

Metabolism of Histones in Functionally Distinct Regions of Chromatin

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Michael J. Hendzel

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METABOLISMS OF HISTONES IN FUNCTIONALLY DISTINCT REGIONS OF CHROMATIN

BY

MICHAEL J. HENDZEL

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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the way that is called The Way is not the eternal Way
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Lao Tzu in *Tao te Ching*

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List of Abbreviations

AUT	acetic acid/urea/Triton X-100
A ₂₆₀	absorbance at 260 nm
bp	base pairs
ddH ₂ O	deionized distilled water
DNase I	deoxyribonuclease I
EDTA	(ethylenedinitrilo) tetraacetic acid
EGTA	[ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EM	electron microscopy
hr	hours
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMG	high mobility group
kDa	kilodaltons
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
Pipes	Piperazine-N, N'-bis (2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
S.E.	standard error
S.E.M.	standard error from the mean
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl)aminomethane

Abstract

In most differentiated eukaryotic cells, only a small proportion of the genome is transcriptionally active. Transcribed genes, like repressed genes, are organized into chromatin by the association with histones. It is known, however, that the chromatin conformation of transcribed genes differs from that of bulk chromatin. This is evidenced by increased sensitivity of transcribed chromatin to digestion by DNase I. We have examined biochemical processes associated with nucleosomal histones that occur actively in chicken erythrocyte chromatin. Suspension cultures of isolated chicken erythrocytes isolated from anemic chickens were incubated with radiolabel to examine ongoing histone methylation, acetylation, and the incorporation of newly synthesized histones into chromatin. We provide evidence that ongoing histone methylation occurs selectively on the small population of chromatin which is undergoing dynamic histone acetylation. In turn dynamic (rapid turnover) histone acetylation is associated with transcribed but not repressed gene chromatin. A small proportion of the repressed gene chromatin participates in a slow turnover class of acetylation. Newly synthesized histones preferentially exchange into transcriptionally competent (i.e. DNase I-sensitive) regions of chromatin but this exchange is not dependent upon ongoing transcription. Thus, the nucleosomes of transcribed chromatin may be considered dynamic with respect to their composition

We also examined the nuclear localization of histone acetyltransferases and histone deacetylases. We provide evidence that these enzymes are associated with the internal nuclear matrix. We propose that dynamic histone acetylation is a solid-state process and that it may be involved in the compartmentalization of the interphase nucleus.

Abstract

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General Introduction

The study of chromatin structure, which attracted early interest by those who wished to understand the molecular mechanisms of gene expression, had seen a diminution of research activity during the mid-80's as many investigators turned to the very popular field of studying sequence-specific transcription factors and the basal transcription machinery. This has led to the development of models in which gene activation is dependent primarily on the availability of specific DNA binding proteins within a cell. The template is often perceived as effectively being naked DNA and readily accessible to these freely diffusible transcription factors. In the past three or four years, however, the study of chromatin structure has found renewed interest amongst those studying the regulation of gene expression. Indeed, with the increased use of reconstituted chromatin templates and genetic studies performed in yeast, novel interactions between the proteins that regulate specific genes and the chromatin template have been observed. The work presented in this thesis deals primarily with the structure and dynamics of transcriptionally active chromatin. The specific biochemical composition of transcriptionally active chromatin, which differs from bulk chromatin, is often overlooked in the design of biochemical reconstitution experiments that examine the role of chromatin structure in gene expression.

Structure of the Eukaryotic Nucleus

The nucleus is the largest organelle in the eukaryotic cell. It is composed of an inner and outer phospholipid bilayer with the outer phospholipid bilayer being continuous with the endoplasmic reticulum. The nuclear envelope contains nuclear pores that allow for the passive diffusion

and receptor-mediated transport of biomolecules into and out of the nucleus. Underneath the phospholipid bilayer is a lamina composed of nuclear lamins, proteins homologous to intermediate-filament proteins of the cytoskeleton. The lamina is composed of helical filaments of lamins and some of the lamins are capable of binding to DNA. The interior of the nucleus contains nucleoplasm and a filamentous nuclear matrix which recent studies indicate may contain proteins homologous to the intermediate-filament family of the cytoskeleton. It is within and around these structures that the eukaryotic genome is organized. The basic features of the eukaryotic nucleus are shown in Fig. 1.

Nucleosomal and Higher Organization of DNA

The primary functions of DNA are to store genetic information such that it can be faithfully inherited by daughter cells and to express genetic information in order to define the specific phenotype of a given cell. Because of the enormous linear length of the eukaryotic genome, it is necessary that a packaging apparatus exist to facilitate the restrictions of nuclear size. The association of the DNA to form chromatin and the organization of chromatin into higher order structures accomplishes this. The major components of chromatin are the DNA template, the "core histones", and the "linker histones". The core histones consist of histones H2A, H2B, H3, and H4. These histones, in the form of two H2A-H2B dimers, and one (H3-H4)₂ tetramer, form the nucleosome in conjunction with 146 bp of DNA. The DNA makes one and three-quarter turns around the histone core of the nucleosome. Nucleosomes form the repeating subunit of eukaryotic chromatin. The intervening DNA, which can vary

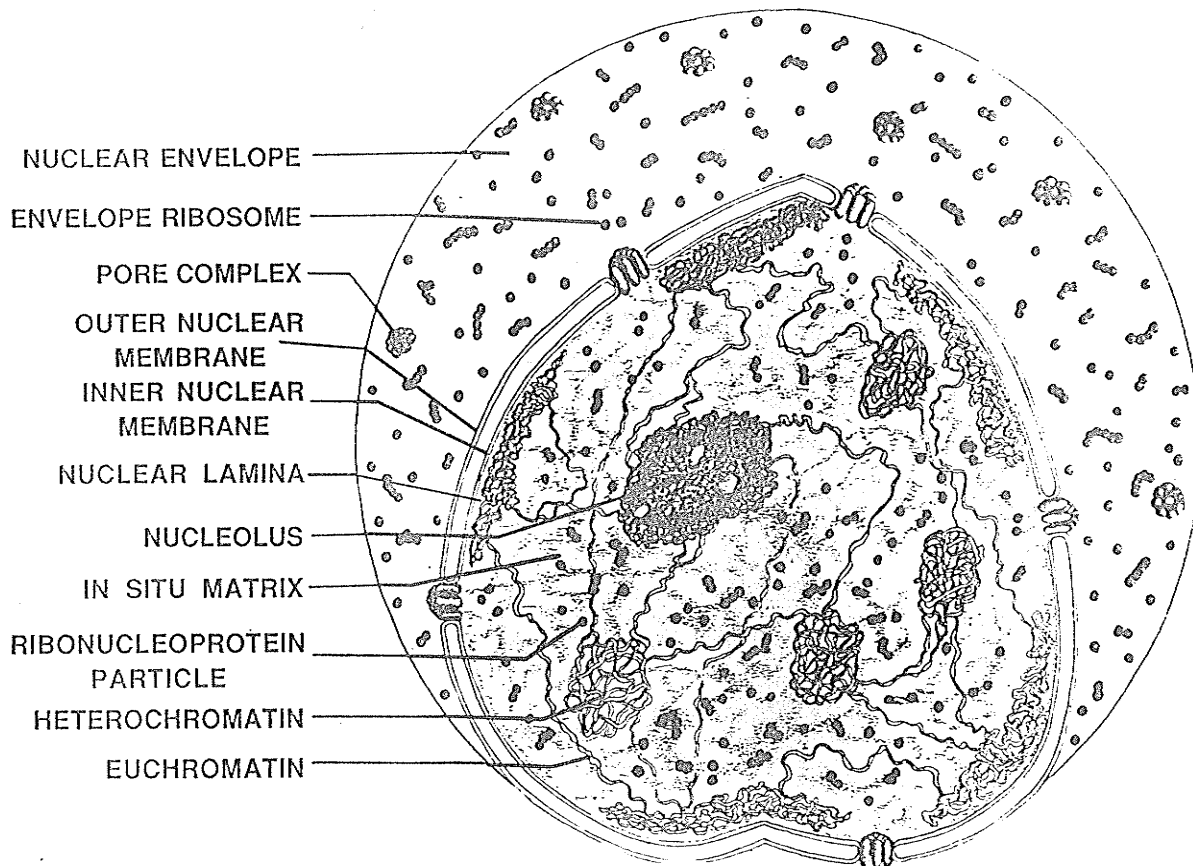


Figure 1. Schematic model of a typical cell nucleus. The nucleus is surrounded by a double-membraned nuclear envelope containing nuclear pore complexes. Ribosome-like structures are found on the surface of the outer nuclear membrane as individual particles and "polysome-like" arrays. The chromatin in the nuclear interior is interpreted as a continuous system of condensed (heterochromatin) and diffuse (euchromatin) regions. The nonchromatin region of the nuclear interior is simplified to contain the nucleolus, RNP (ribonucleoprotein) particles, and an in situ matrix forming a diffuse network which associates with the chromatin and nucleoli in the nuclear interior and the nuclear pore complexes at the periphery. The peripherally localized matrix may correspond to the nuclear lamina often observed in close association with the inner nuclear membrane. Taken from Berezney (1991).

in length from tissue to tissue and at different stages of differentiation of a given cell type, is called linker DNA. The "linker histones" or the H1 histone subtypes bind to the DNA as it exits and enters the nucleosome to seal off two complete turns of DNA about the nucleosome. In doing so, some of the linker DNA is associated with these "linker histones". The linker histones alter a dynamic equilibrium between a folded (30 nm) and unfolded (10 nm) state by stabilizing the 30 nm state. Further compaction is obtained by organizing the 30 nm fiber into domains by association with a proteinaceous scaffold (the nuclear matrix) and coiling of these domains into the metaphase chromosome. In interphase the nuclear matrix is dispersed throughout the nuclear interior and the domains are unfolded from the chromosome scaffold. The organization of DNA into higher order structures and the associated packing ratios are shown in Fig. 2.

Biochemical Characterization of a Model Chromatin Loop

Fundamental to models of chromatin regulated metabolic processes of the genome is the concept of the "chromatin loop". The chromatin loop is the regulated region of chromatin that is fastened to the nuclear matrix at either end. This is fundamental to chromatin models because the establishment of chromatin loops insulates distinct regulated units from being influenced by the state of an adjacent region of chromatin. Thus, chromatin structure can be a means to program regulation of gene expression by altering the condensation state of individual regulated units. There is now considerable evidence to support the chromatin loop as a unit of regulation. The literature on the chicken lysozyme gene is an example.

The chicken lysozyme gene is a biochemically well characterized chromatin loop. Phi-Van and Stratling (1988) established a correlation

between the boundaries of increased sensitivity of the transcribed lysozyme gene to DNase I with restriction enzyme fragments of the lysozyme gene that had a high affinity *in vitro* for nuclear matrix preparations (i.e. are MARs-matrix attachment regions). The MARs for the chicken lysozyme gene have been termed "A" elements. These sequences have a high AT content, characteristic of many MARs/SARs (Phi-Van and Stratling, 1988). Important to a chromatin model of regulation of gene expression, the MARs were shown to confer position-independent and copy number-dependent expression of stably transfected genes in both homologous (chicken HD11/HBC1-promacrophage cells) and heterologous systems (rat fibroblasts) (Stief *et al.*, 1989; Phi-Van *et al.*, 1990). It is important to note that typically stably transfected genes are expressed in position-dependent manners giving a non-copy number-dependent pattern of expression. It is thought that the random expression patterns of stably transfected genes reflects a dominant effect of neighboring chromatin structure on the expression of the gene sequence (reviewed in Bonifer *et al.*, 1991). The results from examination of the chicken lysozyme domain provide strong evidence for the existence of MARs which function to isolate a regulated unit from neighboring chromatin structure. Subsequently, a protein has been purified which bind with high affinity the chicken lysozyme and several other MARs (Phi-Van *et al.*, 1991).

The concept of chromatin loop organization has been substantiated through studies in other systems. In the chicken β -globin locus, locus control regions (LCRs) have been identified. These are characterized by sites which are exceedingly sensitive to DNase I digestion ("superhypersensitive sites") and confer position-independent expression of stably transfected genes (Grosveld *et al.*, 1987). Other SARs/MARs have

also been demonstrated to confer position-independent expression (e.g., Klehr *et al.*, 1992). At the minimum, the data demonstrate that certain sequences function to fragment the genome into independent units of chromatin organization. Whether or not this is accomplished by a structural barrier provided by attachment to the nuclear matrix should be considered more speculative.

Structure of the Nucleosome

Very recently, the structure of the histone octamer was partially solved by X-ray crystallography to a resolution of 3 Angstroms (Arents *et al.*, 1991.). There had previously been some controversy regarding the structure of the nucleosome with the two main groups disagreeing (Burlingame *et al.*, 1985; Richmond *et al.*, 1984). The higher resolution data now produced results in a consistent view of the structure of the histone octamer. The octamer is seen as a tripartite structure consisting of two histone H2A-H2B dimers attached to a central histone H3-H4 tetramer. This is consistent with solution studies on assembly and disassembly of the histone octamer (see van Holde, 1989 for a review). It had previously been thought that each histone occupied a distinct space within the nucleosome by assuming a globular structure. The higher resolution data indicates that the folded histone chains are elongated and histone-histone interactions within both the dimer and tetramer interdigitate extensively with each other (the "handshake motif") resulting in strong intermolecular contacts. Although the dimer-tetramer contact surface is more extensive than contacts between the two H3-H4 half-tetramers, a solvent channel found at the dimer-tetramer interface is present that is likely responsible for the relative lability of the dimer-tetramer contact. Arents *et al.* (1991) suggest

The Formation Of The Radial Loop Chromosome

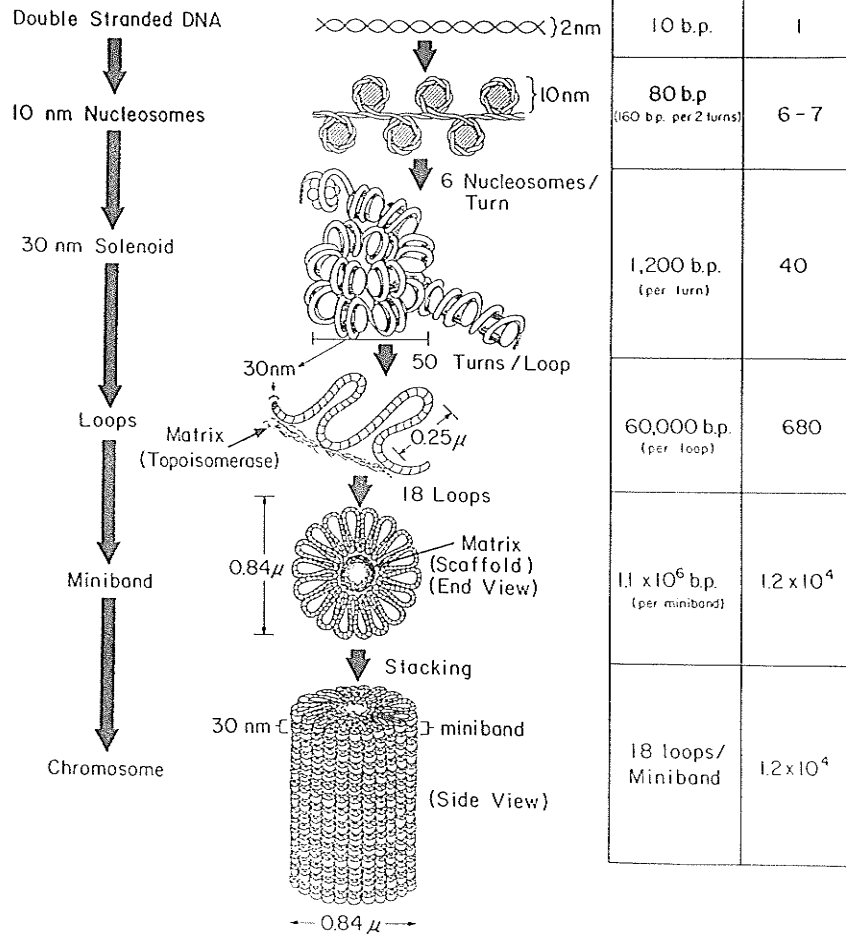


Figure 2. Structural organization of DNA within the eukaryotic nucleus. Approximately 160 bp of 2 nm DNA helix is wound twice around the histone octamers to form the 10 nm nucleosomes. These nucleosomes form a "beads on a string" fiber, which winds in a solenoid fashion with 6 nucleosomes per turn to form the 30 nm chromatin filament. The 30 nm filament forms the 60 kbp DNA loops that are attached at their bases to the nuclear matrix structure. The loops are then wound into the 18 radial loops that form a miniband unit or 1 turn on the chromatid. The minibands are continuously wound and stacked along a central axis to form each chromatid. Variations in intrachromosomal length are achieved by the winding, unwinding, and compacting of the minibands. Topoisomerase II is located at the base of the interphase loop, on the nuclear matrix, and in the central protein scaffold of the metaphase chromosome. Taken from Getzenberg *et al.* (1991).

that this interface may be a site of regulation of chromatin compaction-decompaction. A very interesting feature of the surface of the histone octamer is the presence of a protein superhelix. This forms regularly spaced ridges and valleys that conform to the path of the DNA superhelix in the nucleosome. The protein superhelix is formed by a spiraling assembly of an H2A-H2B dimer on one side of the H3-H4 tetramer and a second dimer on the other side of the H3-H4 tetramer (Fig. 3). Because this structure conforms to the superhelix formed by DNA after local dehydration of the double helix, Arents *et al.* (1991) suggest that this structure evolved to accommodate a preferred structural conformation of DNA and functions by reducing the local water activity upon association with the DNA.

Chromatin Function

Chromatin must serve at least two functions. First, it must serve to package the DNA in such a manner that the long polymers of DNA will fit into a nucleus of relatively small diameter. Second, chromatin must be able to allow the polymerases access to the template. It is this second function that has attracted many researchers to the study of the characteristics of transcriptionally active gene chromatin. The problem is perhaps best illustrated by the title of a recent review by Kornberg: "Irresistible force meets immovable object..." (Kornberg, 1992). That is the DNA template, because of its association with a packaging apparatus, the histone octamer, would seem to be inaccessible to the polymerases. It was assumed then that there would be biochemical differences in transcriptionally active chromatin that would allow this high affinity association between the histone octamer and the nucleosomal DNA to be overcome. Possible

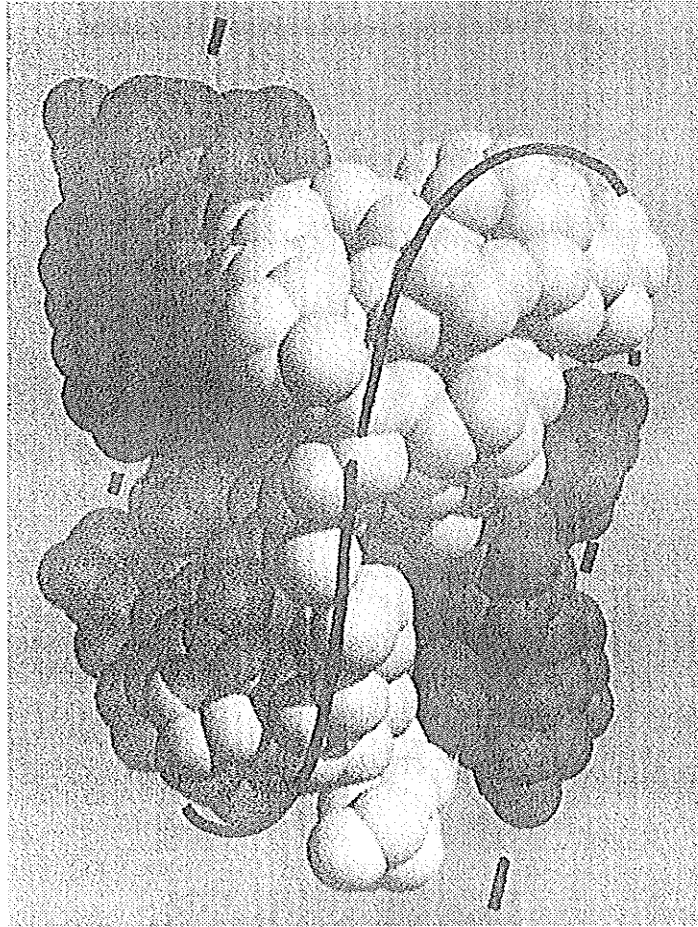


Figure 3. X-ray crystallography structure of the histone octamer. The figure shows the left-handed protein superhelix. The first dimer is at the upper left and sits behind the tetramer, which curves forward at the bottom left, crosses the center, and curves back touching the second dimer at the upper right. The dimers are dark gray, the first half-tetramer is lighter gray, the second half-tetramer is white. In this view, the histone octamer can be perceived as tetrapartite. Black line represents the path of the left-handed protein superhelix. Taken from Arents *et al.* (1991).

mechanisms in which this association is overcome include DNA supercoiling and posttranslational modifications of histones.

Chromatin Structure and Transcription

Since the discovery of the nucleosomal organization of DNA, an obvious philosophical problem that presented itself was how do large macromolecular polymerase complexes transcribe through templates which are tightly associated with the histone octamer. This problem stimulated the original investigations into the composition of transcriptionally active chromatin. It is now apparent that the composition of transcriptionally active gene chromatin differs from that of transcriptionally inactive chromatin. Perhaps the first distinguishing feature of transcriptionally active gene chromatin that was identified was its heightened sensitivity to degradation by nucleases (particularly DNase I). Of all of the biochemical features now associated with transcriptionally active gene chromatin, this is the most consistently observed feature. The increased sensitivity of the template-active chromatin to nucleases has been interpreted to be due to a more "open" chromatin structure. An example of the chromatin structure as assessed by DNase I sensitivity is shown in Fig. 4. The question then arises, what are the features of active chromatin that are responsible for this increased sensitivity and more "open" structure. It is at this point where we reach a dilemma. One can easily directly assess the nuclease sensitivity of specific genes using complementary sequence probes. However, in trying to determine the biochemical composition of transcriptionally active gene chromatin one must first attempt to separate the small proportion of chromatin that is transcriptionally active from the bulk of the chromatin that is inactive. Traditionally, the most commonly used techniques involved

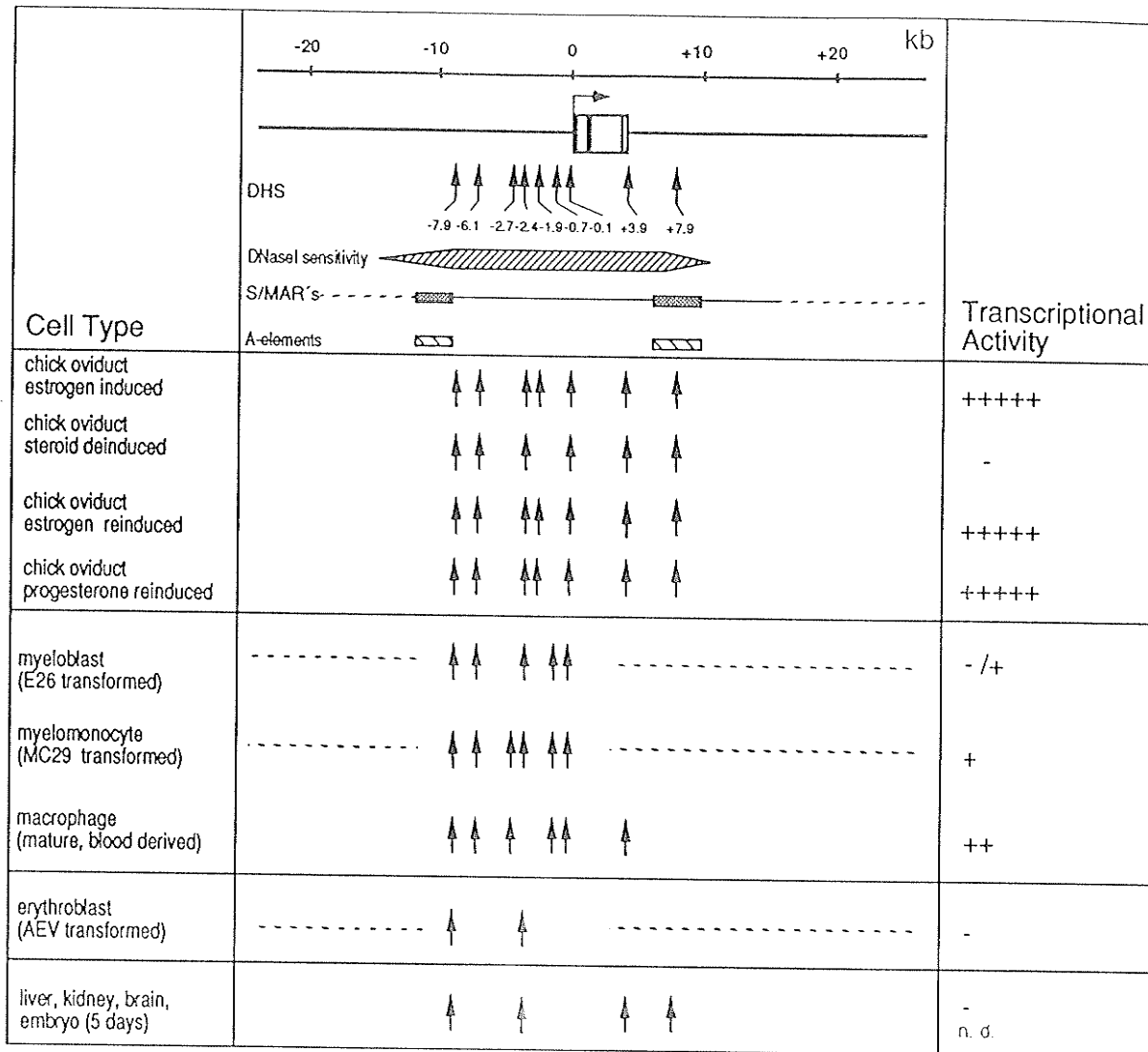


Figure 4. The chromatin domain of the chicken lysozyme gene. The actively transcribed chicken lysozyme gene with its four exons and three introns (top panel; filled and open bars, respectively) resides in a 22kb chromatin domain of elevated DNAase I sensitivity (hatched double arrow). The only scaffold associated or matrix attachment regions (S/MARs; stippled bars) around the gene are found at the edges of the DNAase I sensitive domain and collocate with the A-elements (widely hatched bars). Cell-type specific transcriptional activity of the gene and its mode of regulation (lower panels) correlate with distinct patterns of DNAase I hypersensitive sites (DHS; vertical arrows). The positions of the DHSs relative to the transcriptional start site are given in the top panel of the figure. Dashed lines indicate chromatin regions not analyzed for the presence of DHSs or S/MARs. Taken from Bonifer *et al.* (1991).

fractionation of the chromatin on the basis of differential solubility in buffers of different composition after fragmentation with nucleases (reviewed in van Holde, 1989). The limitations of this technique are determined by the efficiency of the fractionation procedure. These techniques are typically capable of only modest enrichments in transcriptionally active gene chromatin. They are not able to isolate transcriptionally active gene chromatin devoid of contaminants. As such, the results are correlational in nature and the strength of the associations established can only be judged by the degree of coenrichment of various biochemical properties. The lack of the technique's ability to establish direct associations leaves the results open to criticism on this basis (also see van Holde, 1989 for an extensive discussion on this point). Although the techniques are limited, it is possible to establish consistent relationships between chromatin fractions enriched in particular classes of chromatin and specific biochemical features. An example of this is the association between histone acetylation and transcription (see section on histone acetylation for a detailed discussion). Chromatin fractionation procedures were originally used to establish this association and since then more direct immunochemical techniques have confirmed this association.

There is evidence, primarily from using chromatin fractionation procedures that transcribed chromatin is compositionally different from bulk chromatin. Histone H1, regarded as the general repressor of genetic activity is present on transcribed chromatin (Ericsson *et al.*, 1990) but at reduced levels (Kamakaka and Thomas, 1990; Ridsdale and Davie, 1987). Kamakaka and Thomas (1990) demonstrated using antibodies to histone H1 and H5 (an H1 variant expressed in nucleated erythrocytes) and immunoprecipitation techniques that there is an approximately 40%

depletion in histones H1 and H5 in active gene loci confirming previous results from this laboratory using chromatin fractionation techniques (Ridsdale and Davie, 1987; Delcuve and Davie, 1989). Core histone acetylation has been consistently shown to be increased in transcriptionally active gene chromatin (reviewed in Csordas, 1990). Ubiquitination of histone H2A has been variously reported to be associated with transcribed gene chromatin (e.g., Levinger and Varshavsky, 1982) or not (e.g., Huang *et al.*, 1986). The only histone modification clearly associated with the act of transcription, however, is ubiquitination of histone H2B (Davie and Murphy, 1989). It has also been demonstrated that specific nuclear nonhistone chromosomal proteins, in particular HMGs (high mobility group) 14 and 17 are associated with transcribed chromatin (Druckmann *et al.*, 1986; Dorbic and Wittig, 1987; Postnikov *et al.*, 1991).

The nucleosome as a dynamic structure

Although the nucleosome is often regarded as a very stable structure, it is clear that under certain conditions, including those present at least in transcribed chromatin, that the nucleosome is a dynamic structure (reviewed in Hansen and Ausio, 1992). This is particularly relevant to the problem of how the polymerases are able to transcribe through nucleosomal DNA. It has been demonstrated that *in vivo* histone H2A-H2B dimers are labile in the absence of chromatin replication (i.e. interphase) and labeling cells with ³H-lysine to label newly synthesized histones results in the incorporation of primarily labeled histone H2A-H2B dimers into nucleosomes (Louters and Chalkley, 1985, Jackson, 1990). This lability combined with the observation that RNA polymerases bind preferentially to nucleosomes deficient in one H2A-H2B dimer (Baer and Rhodes, 1983; Gonzalez *et al.*, 1987) suggests that the lability of histone H2A-H2B dimers may be an

important first step in allowing the passage of the polymerase through the nucleosome (van Holde *et al.*, 1992). There is evidence that a proportion of the exchange of histone H2A-H2B dimers is directly coupled to the transcription of the template (Jackson, 1990). It has been reported that inhibition of transcription prevents any exchange of histone H3-H4 tetramers (there is a very limited exchange of histone H3-H4 tetramers seen when cells are labeled with ^3H -lysine) (Jackson, 1990). The significance of these observations is that contrary to the impression that the nucleosome is a stable entity, there is a lability to the structure that may be exploited during transcription to allow the passage of the polymerase. The factors that are important in promoting this lability are currently unknown. However, the implications of this process may have functional significance. For example, during G1 and G0, the exchange of histone H2A-H2B dimers favors the replacement of the resident H2A-H2B dimer with one that contains ubiquitinated histones H2A and H2B (Jackson, 1990). Jackson (1990) provided evidence that in interphase the histone H2A/H2B dimer pool is extensively ubiquitinated. The incorporation of ubiquitinated dimers into the nucleosome may facilitate further displacement of H2A-H2B dimers during subsequent rounds of transcription (Morse, 1992) and/or to maintain an unfolded nucleosome conformation (Davie and Murphy, 1990). Additionally, since the complement of histones being synthesized in G1 and G0 differs from that of S-phase (e.g., histone variants H2A.X, H2A.Z, and H3.3, and H1 variants H1^o and H5 are selectively synthesized in G1 phase) (Wu *et al.*, 1986), nucleosomes in transcribed regions of chromatin may be remodeled in a manner which may affect nucleosome structure (Jackson, 1990). These histone variants, at least in some cases, have unique functions essential to cell viability (van Daal and Elgin, 1992).

Histone Acetylation

Acetylation is the most extensively studied of all of the histone posttranslational modifications. Its attractiveness is due to its obvious potential to alter nucleosome structure and histone-DNA interactions. All four core histones may be modified by acetylation. The modification occurs on specific lysine (e.g., 5, 8, 12, and 16 of histone H4) residues in the N-terminal tail of the four core histones (Fig. 5). Histones H2B, H4, and H2A variant H2A.Z can each accept up to four acetyl groups on independent lysine residues. Histone H3 may be pentaacetylated and the major subtypes of histone H2A accept only one acetyl group. Unlike the cotranslational N-terminal acetylation that occurs on many proteins in the cell, the lysine N^ε-acetylation of the nucleosomal histones is reversible.

Acetylated histones are associated with transcribed chromatin

Allfrey originally proposed a role for core histone acetylation in facilitating access to transcription and established correlational evidence to support this (reviewed in Allfrey, 1980). The analysis of transcriptionally active gene-enriched chromatin fractions has consistently revealed increased acetylation in these fractions (e.g., Ridsdale and Davie, 1987; Allegra *et al.*, 1987; Delcuve and Davie, 1989). The development of antibodies recognizing the epitope ϵ -acetyllysine provided direct evidence for this association. Using antibodies specific for highly acetylated histones it was demonstrated in *Tetrahymena* that highly acetylated histones were localized to the transcriptionally active macronuclei but not the transcriptionally inert micronuclei (Lin *et al.*, 1989). Hebbes *et al.* (1988) used antibodies recognizing highly acetylated histone H4 to demonstrate that immunoprecipitated chromatin was 15- to 30-fold enriched in transcribed sequences in 15-day chicken embryo erythrocytes. This

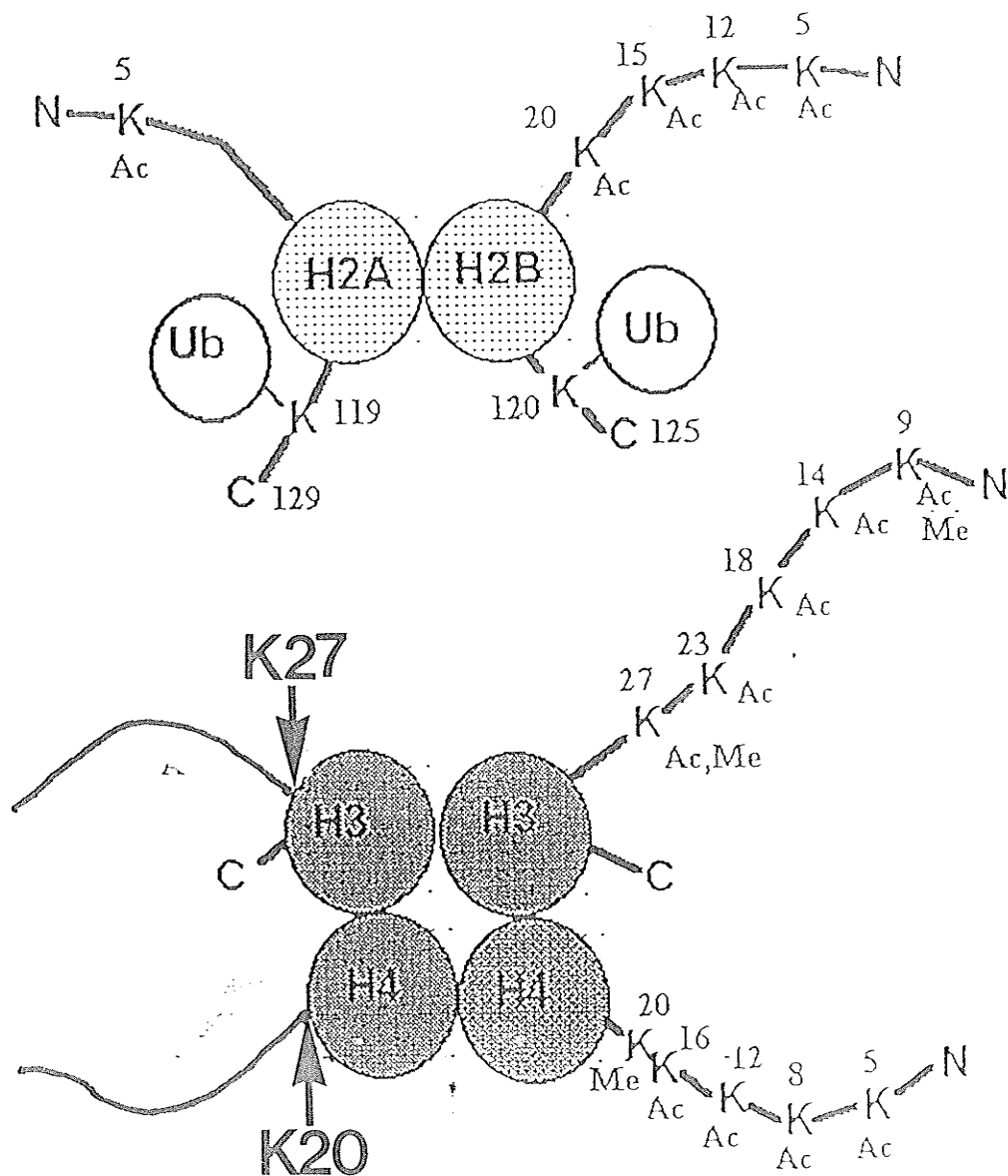


Figure 5. The sites of reversible histone acetylation, ubiquitination, and methylation on the nucleosomal histones. The four nucleosomal histones undergo reversible acetylation at specific lysine residues on their respective N-termini. The sites of acetylation are shown for one H2A/H2B dimer and one of two H3 and H4 histones. Ac, Me, and Ub indicate sites of reversible acetylation, methylation, and ubiquitination, respectively. N indicates the N-terminus. The arrows indicate the sites of cleavage of intact nucleosomes with trypsin.

established a direct link between histone acetylation and transcription. The precipitated chromatin was significantly enriched in acetylated histones with approximately equimolar ratios of the five acetylated isoforms of histone H4. In an extension of this original work Hebbes *et al.* (1992) showed that this antibody-bound highly acetylated chromatin fraction contained transcriptionally competent as well as transcriptionally active gene chromatin, confirming a previous association between transcriptionally competent chromatin and histone acetylation (Ridsdale and Davie, 1987). Furthermore, high levels of histone acetylation were associated with transcriptionally competent erythroid-specific genes prior to developmental expression of these genes (Hebbes *et al.*, 1992). Importantly, using similar antibodies by Pfeffer *et al.* (1989) provided evidence that histone acetylation preceded transcription in newly forming transcriptionally active *Tetrahymena* macronuclei.

The dynamics of histone acetylation

The presence of both histone acetyltransferases and histone deacetylases in eukaryotic cell nuclei provide a mechanism to impart dynamics to the histone acetylation process. Indeed, labeling cells with ^3H -acetate and examining acetylation and deacetylation processes has established that acetyl groups turn over. These studies have demonstrated, however that there are different kinetic classes of histone acetylation. Different regions of chromatin have different acetylation properties. Early studies by Covault and Chalkley (1980) demonstrated in hepatoma tissue culture cells that there existed a small population of rapidly modified histones which reach their maximal acetylation state (e.g., tetraacetylated histone H4) during a short incubation period with sodium butyrate, an inhibitor of histone deacetylase. This kinetic population of acetylated

chromatin is acetylated at a rate of $t_{1/2} = 7$ min and additionally is rapidly deacetylated in the absence of sodium butyrate ($t_{1/2} = 3$ to 7 min). A second class of acetylated histones is less rapidly modified ($t_{1/2} = 200$ to 300 min) and more slowly deacetylated. Covault and Chalkley (1982) originally postulated that the most dynamic class of histone acetylation occurred sequentially throughout the genome and functioned as a general surveillance mechanism for DNA damage and the recognition of different regions of chromatin by regulatory proteins. However, since then, they and others (Ip *et al.*, 1988; Zhang and Nelson, 1988 a,b; Boffa *et al.*, 1991) have demonstrated that this dynamically acetylated chromatin is associated with transcribed sequences in several cell types. Additionally, evidence has been presented that there exists a third class of acetylated species, at least in some cells, that does not actively participate in the acetylation-deacetylation process (Zhang and Nelson, 1986). Whether or not the slower turnover class of acetylation functions as a general surveillance mechanism is unknown. It is worth considering, however, that the slow turnover class of acetylation described by Covault and Chalkley (1980; 1982) may not represent a physiological population. This population had a considerably slow rate of acetylation ($t_{1/2} = 200$ to 300 min). It is detected by incubating cells for extended periods of time (e.g., 18 h) with sodium butyrate. Under these conditions, this second population becomes hyperacetylated (Covault and Chalkley, 1980). The presence of sodium butyrate, however, results in the rapid saturation of the primary substrate of the acetyltransferases, the rapidly acetylated chromatin region. Whether or not this results in the dissociation of histone acetyltransferases from these regions of chromatin and reassociates with regions containing histones with sites not acetylated has not been adequately addressed. If a redistribution

of histone acetyltransferases occurs under these conditions, then this second class of acetylated chromatin may not exist or more likely is overrepresented as a population in studies using extended incubation with sodium butyrate. It can be argued then, one should be particularly cautious in interpreting the physiological relevance of this second, more slowly metabolized, form of histone acetylation.

In contrast, it is well established that transcriptionally active gene chromatin is highly enriched in the higher acetylated species (e.g., tri- and tetraacetylated histone H4) of the four core histones. In turn, these most highly modified histones undergo rapid deacetylation and reacetylation. Thus the acetylation state of transcribed gene chromatin is dynamic (Zhang and Nelson, 1988 a,b; Ip *et al.*, 1988; Boffa *et al.*, 1990). Analysis of steady state levels of acetylation in different chromatin fractions, as is typically done to establish correlations between distinct classes of chromatin and acetylation, fails to appreciate this dynamic nature. Thus, transcribed gene chromatin is often said to be enriched in highly acetylated (i.e. histones with 2, 3, or 4 acetyl groups per histone). However, it has been demonstrated that these highly acetylated histones often exist in a very dynamic state (e.g., Zhang and Nelson, 1988 a,b). The functional significance of the dynamics of the acetylation process is not currently understood. For example, it is not known if dynamic acetylation is associated with transcribed gene chromatin whereas overall high levels of acetylation (dynamic and nondynamic) are characteristic of the transcriptionally competent chromatin configuration. For example Turner *et al.* (1990) found using antibodies specific for particular histone H4 acetylhistone that high acetylation levels are localized to non-coding flanking

sequences of active genes. Additionally, Tazi and Bird (1989) demonstrated high levels of steady-state histone acetylation were associated with non-methylated CpG-rich islands which are enriched in 5'-flanking sequences of transcribed genes. These results suggest that high levels of histone acetylation are associated with flanking but not transcribed regions of chromatin. However, Ip *et al.* (1988) demonstrated that transcriptionally engaged chromatin (i.e. chromatin fractionated on the basis of association with engaged RNA polymerase) was enriched in the highly acetylated rapid-turnover dynamic class of histone acetylation. These results are consistent with the results of Boffa *et al.* (1990) which demonstrate similar associations between rapid turnover dynamic acetylation and transcriptionally active chromatin.

It is not known, whether there are kinetic differences in acetylation turnover between histones associated with flanking sequences and histones associated with transcribed sequences. High steady-state levels of histone acetylation appear to be associated with flanking sequences (Turner *et al.*, 1990; Tazi and Bird, 1991). The high turnover rate of dynamically acetylated histones associated with transcribed regions of chromatin (Ip *et al.*, 1988), may result in lower steady-state levels of histone acetylation in transcribed regions. A distinction, therefore may exist between highly acetylated histones (i.e. histones which are in the highest acetylated state as determined by steady-state analysis) and hyperacetylated histones (histones which obtain the highest acetylated state during incubation with histone deacetylase inhibitors). The discrepancy may be a consequence of differences in deacetylation rates in these regions. Consistent with this hypothesis, if histones are analyzed after a 5 min pulse with [³H]acetate (a

procedure which selectively labels the dynamic class of acetylated histones), label is found predominantly in the lower acetylated species of the core histones. If, however, these cells are subsequently chased for 1 hr in the presence of sodium butyrate, then there is a dramatic redistribution of the label to the most highly acetylated forms of the core histones (Perry and Chalkley, 1982). This suggests that the steady-state level of dynamically acetylated chromatin may be lower due to the dynamic nature of the highest acetylated forms of the core histones.

An important question that remains to be addressed is what is the function of the most dynamic form of acetylation. That is, although we know that this rapid turnover class of acetylated histones is associated with transcribed chromatin, we don't know whether acetylation turnover has a function separate from high levels of acetylation. It is largely assumed that high levels of acetylation reduce the electrostatic charge on the histone "tails" thereby reducing the association with DNA. It is obvious that such a mechanism would have the potential to facilitate transcription of the template. However, for frequently transcribed genes, it would seem to be more efficient for the cell to acetylate the domain once following replication rather than expend energy by establishing a dynamic equilibrium between histone acetyltransferases and histone deacetylases. One possible distinct function of histone acetylation turnover is to modulate levels of supercoiling within the domain. The nucleosome constrains less negatively supercoiled DNA when highly acetylated (Norton *et al.*, 1989). Thus acetylation is capable of releasing negative torsional stress within a domain and storing negative torsional stress by deacetylation (see Norton *et al.*, 1989; 1990). It is also possible that deacetylation serves to maintain nucleosome integrity

following polymerase passage. That is, by facilitating stronger histone-DNA interactions, deacetylation may promote the proper re-establishment of histone-DNA contacts that are altered during polymerase passage. At this point it is unknown if the rapid dynamics of histone acetylation are important for its physiological role during interphase. However, it is the differences in acetylation metabolism that are most striking amongst the differences in histone acetylation between transcribed and nontranscribed regions of chromatin. This largely underinvestigated aspect of histone acetylation deserves further investigation.

The sites of histone acetylation are nonrandomly utilized

An interesting feature of histone acetylation is that the sites of acetylation are nonrandomly utilized (Csordas, 1990; Turner, 1991). This has been consistently observed in all species studied. For example, in calf thymus, lysine 16 is the only acetylated residue in the monoacetylated species (Csordas, 1990). Similar results were obtained in pig liver and HeLa cells (Thorne *et al.*, 1990). Importantly, if HeLa cells were cultured for 24 h in the presence of 7 mM sodium butyrate, the preference for use of specific lysine residues in the acetylated isoforms of the core histones was not significantly altered (Thorne *et al.*, 1990). This indicates that butyrate-induced hyperacetylation of histones reflects the normal highly acetylated state with respect to site-usage. Chicoine *et al.* (1986) demonstrated in *Tetrahymena* that there were differences in preference of usage of histone H4 sites of acetylation between diacetylated newly synthesized histone H4 associated with chromatin replication and diacetylated histone H4 that is transcription-associated.

There is also evidence that the sites of histone acetylation may be important in its biological function. It has been repeatedly demonstrated

that the sites of acetylation on the individual histone molecules are nonrandomly utilized. Grunstein's group has demonstrated that acetylation at lysine 16 of histone H4 is particularly important for the repression of the silent mating type loci in yeast (*S. cerevisiae*) (reviewed in Grunstein, 1990 a,b). These investigators suggest that this region of histone H4 interacts specifically with proteins in yeast nuclei that are important for the regulation of the mating type loci. Whether or not similar interactions exist in higher eukaryotes remains to be determined. These results suggest that different physiological processes distinguish between sites of histone acetylation and imply that the site of acetylation may play specific roles in specific physiological processes.

Function of histone acetylation I- effects on core particle structure

Physicochemical studies on the effects of histone acetylation have been performed on core particles differing in their mean level of acetylation. Core particles are obtained by stripping isolated chromatin fragments of histone H1 and then digesting linker DNA with nucleases to leave a core particle containing the histone octamer and 146 bp of DNA. Typically, acetylated chromatin was isolated from cells treated with the histone deacetylase inhibitor, sodium butyrate. Differences which have been demonstrated on nucleosome core particles have generally been subtle. Bode *et al.* (1983) have demonstrated that acetylated core particles show a retarded migration during nondenaturing polyacrylamide gel electrophoresis. This has subsequently been duplicated in other laboratories (Imai *et al.*, 1986; Ridsdale *et al.*, 1988; Delcuve and Davie, unpublished). Acetylated core particles have been demonstrated to have a slightly altered sedimentation coefficient consistent with a slightly more open conformation (Ausio and van Holde, 1986). Differences in the stability of the

nucleosomes in solutions of high ionic strength (0.7 M) could not be detected on the basis of CD spectral analysis (Ausio and van Holde, 1986). Differences were seen in the sensitivity of core particles to DNase I digestion and thermal denaturation properties which are consistent with weakened histone DNA contacts and include increased susceptibility to digestion with DNase I at a site 60 bp from the ends of the nucleosomal DNA (Ausio and van Holde, 1986). There were, however, only minor differences in micrococcal nuclease sensitivity between control and hyperacetylated core particles (Libertini *et al.*, 1988). No change in nucleosome core particle structure was observed using neutron scatter data (Imai *et al.*, 1986). Together, the results suggest that histone acetylation causes a subtle change in the structure of the nucleosome core particle.

Function of histone acetylation II-effects on higher order chromatin-structure

Although the effects of histone acetylation on the nucleosome core particle are apparently only subtle, there is now evidence that histone acetylation affects the formation of more condensed chromatin structures. It has been shown that hyperacetylation of histones changes the linking number of supercoiled plasmids reconstituted with nucleosomes. That is, hyperacetylated nucleosomes constrain less supercoiled DNA than control nucleosomes containing considerably lower overall levels of acetylation (Norton *et al.*, 1989; Norton *et al.*, 1991). These results have been confirmed *in vivo* by examining the supercoiling of transfected plasmids in cells treated in the presence or absence of sodium butyrate (Thomsen *et al.*, 1991) although the results *in vivo* are controversial (Lutter *et al.*, 1992). Acetylated histones also have reduced binding to DNA on the basis of elution from hydroxylapatite bound-chromatin with increasing concentrations

of NaCl (Hirose, 1988; Nagaraja *et al.*, in preparation). Possibly related to this ability to release torsional tension into the domain, Bode *et al.* (1992) observed that A+T-rich matrix attachment sequences (MARs) would become stably unwound (single-stranded) in the presence of torsional tension and that this single-stranded property rather than specific sequence was required for its function as a MAR. More importantly, it was found that the histone deacetylase inhibitor sodium butyrate as well as the histone deacetylase-specific inhibitor Trichostatin A stimulated transcription of stably transfected genes (Klehr *et al.*, 1992). These results suggest that increased levels of histone acetylation cause topological alterations in the chromatin facilitating the unwinding of the MAR element and its subsequent binding to the nuclear matrix (thereby isolating the transfected domain from neighboring repressed chromatin structures).

Studies which more specifically analyzed the effects of histone acetylation on higher-order chromatin structures and chromatin folding have clearly demonstrated important differences between control and hyperacetylated chromatin. In the presence of monovalent cations near physiological concentrations, polynucleosome fibers will adopt a more condensed structure and in the presence of histone H1, these polynucleosome fibers will aggregate and precipitate out of solution. Ridsdale *et al.* (1988) used this precipitation assay to examine the formation of H1-dependent higher-order structures in solution amongst functionally distinct regions of the chicken erythrocyte genome. It was found that sequences enriched in transcriptionally competent (i.e., not transcribed but DNase I sensitive) and transcriptionally active gene chromatin would resist precipitation at physiological ionic strength following reconstitution with H1 histones. In contrast, under these conditions, the majority of the

reconstituted chromatin was insoluble. Because these polynucleosomes had been treated in a manner which removed virtually all non-histone proteins, it was apparent that these differences reflected alterations at the nucleosome level. The most apparent difference in the biochemical composition of the nucleosomes of the precipitation-resistant chromatin was that it was enriched in acetylated species of the core histones (Ridsdale and Davie, 1987). Subsequently, Ridsdale *et al.* (1990) utilized H1 reconstitution onto polynucleosomes prepared from chicken erythrocytes in which the level of histone acetylation had been altered by incubating the cells in the presence or in the absence of sodium butyrate. These experiments directly demonstrated that high levels of histone acetylation prevented H1-induced precipitation of the polynucleosome fibers at physiological ionic strength. These results were recently confirmed (Perry and Annunziato, 1991). Garcia-Ramirez *et al.* (1992) have performed studies comparing the folding properties of 12-mer oligonucleosomes with 12-mer trypsinized oligonucleosomes (trypsinized nucleosomes, in which the highly basic N-termini of the nucleosomal histones are cleaved, is often used as a model for histone acetylation) and found that the N-termini are absolutely required for folding of oligonucleosomes in the absence of histone H1. The data is consistent with an alteration in the path of the DNA within the nucleosome resulting in the loss or alteration of the H1 histone binding sites (histone H1 stabilizes the 30 nm conformation of chromatin). Importantly, electron spectroscopic imaging microscopy demonstrates that the low salt-soluble polynucleosome fraction from chicken erythrocyte (a highly acetylated fraction) contains nucleosomes with altered DNA paths (Locklear *et al.*, 1990). It can be concluded that histone acetylation plays a very important

role in the modulation of the condensation state of a chromatin domain. The data relevant to gene expression are summarized in Fig. 6.

Histone Deacetylases

Association of histone deacetylase with the nuclear matrix

Despite the wealth of information correlating histone acetylation with transcription, there has been very little investigation of the enzymes responsible for this reversible and dynamic posttranslational modification of histones. Histone deacetylases have been studied in mammalian cells, plant cells, and fungi. In both HeLa cells and Chinese Hamster Ovary cells, the enzyme was found to be associated primarily with high molecular weight material. In Chinese hamster ovary cells, approximately 75% of the total nuclear histone deacetylase activity was soluble in EDTA following digestion with micrococcal nuclease. Of this 75%, 50% of the total nuclear activity was associated with very high molecular weight material, 4.6% was found as free enzyme, 9.7 % was associated with a unique class of mononucleosomes (resolved from total mononucleosomes by nondenaturing polyacrylamide gel electrophoresis), and the remaining 11.3 % was associated with di- and polynucleosomes (Mold and McCarty, 1987). In HeLa cells, all of the detectable histone deacetylase activity was associated with very high molecular weight material. The histone deacetylase remained associated with this high molecular weight complex even in the presence of 2 M NaCl but the complex was partially dissociated in the presence of 2-mercaptoethanol (Hay and Candido, 1983 a,b). The authors of this study suggested that this may reflect histone deacetylase association with the nuclear matrix. The nuclear matrix is the residual nuclear material following extensive nuclease digestion and extraction with high-salt buffers (see page 74).

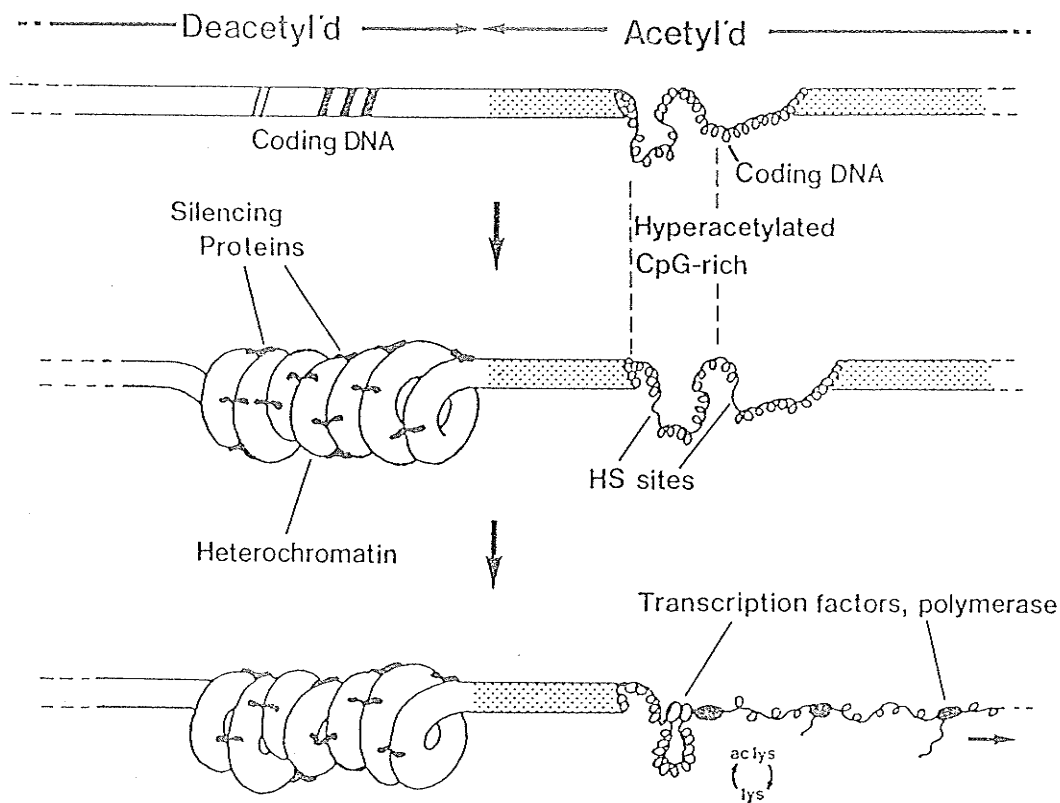


Figure 6. A diagram showing some of the changes in higher-order chromatin structure that accompany gene activation and inactivation and in which histone acetylation may play a role. Chromatin is shown as the 30 nm solenoid or, in the case of the potentially active gene, the DNase I-sensitive, 10 nm nucleosomal fibre. The labels 'deacetyl'd' and 'acetyl'd' refer either to the overall level of acetylation, or to acetylation at one, or a small number of sites. A hyperacetylated CpG-rich island is shown incorporating the upstream control elements and part of the coding region of the potentially active gene. Heterochromatin formation is shown diagrammatically in the middle and lower parts of the figure as further coiling of the 30 nm fibre, though the actual mechanism is unknown, as is the distribution of the silencing proteins responsible for mediating the condensation process. The model proposes that the spread of heterochromatin stops at the boundary between acetylated and deacetylated chromatin. Activation of the gene on the right of the figure involves the generation of DNase I-hypersensitive sites (HS sites) by loss of selected nucleosomes, possibly through displacement by transcription factors or other proteins. The fully active gene is shown in the lower part of the figure. Upstream chromatin has looped out to allow interaction between proteins bound to upstream control elements and the promoter region. The transcribed DNA contains nucleosomes, possibly structurally modified, which may undergo rapid turnover of their histone acetate groups. Taken from Turner (1991).

In contrast, Brosch *et al.* (1992) found that when nuclei from *Zea mays* were isolated in a buffer containing 10 mM 2-mercaptoethanol (the significance of the use of 2-mercaptoethanol during isolation will become apparent later-see fig. 57), histone deacetylase was not (i.e. 3% remained) associated with 0.4 M/2.0 M KCl nuclear matrices. Similar results were obtained with nuclear matrices from *Physarum polycephalum* (Brosch *et al.*, 1992).

Evidence for a soluble/chromatin bound histone deacetylase

Although there is evidence that histone deacetylase is associated with the nuclear matrix, earlier studies characterized histone deacetylases from mammalian cells which were extracted with buffers of moderate ionic strength from isolated nuclei. Fujimoto's group is responsible for the majority of work on solubilized histone deacetylase(s). Histone deacetylase activity could be extracted from isolated calf thymus nuclei with 0.2 M phosphate buffer (pH 7.0) (Kaneta and Fujimoto, 1974), or 0.05 M phosphate buffer in the presence (but not in the absence) of 0.4 M NaCl (Inoue and Fujimoto, 1972). This group provided evidence for at least two histone deacetylases in calf thymus. Their respective molecular weights, as determined by gel exclusion chromatography, are approximately 550,000 and 300,000 (Kikuchi and Fujimoto, 1973). Interestingly, while both enzymes were capable of deacetylating free histones in solution, only the high molecular weight form was capable of deacetylating chromatin bound histones (Kaneta and Fujimoto, 1974). Additionally, if unlabelled free histones were included in an incubation with labeled chromatin, the low molecular weight form was then capable of deacetylating the chromatin bound histone. In this case, the distribution of enzyme activity between the high and low molecular weight peaks was similar to that seen with free

histones as a substrate. This indicates that in the presence of free histones, the low molecular weight form is nearly as efficient at deacetylating chromatin-bound histones as the high molecular weight peak. The significance of the requirement for free histones to deacetylate chromatin remains obscure. In apparent contrast to the results of Fujimoto's group, a single histone deacetylase of apparent molecular weight of 150,000 (determined by gel exclusion chromatography) was extracted from calf thymus nuclei by extraction with an isotonic buffer following isolation of nuclei either in a nonaqueous buffer or in buffer containing 0.25 M sucrose (Vidali *et al.*, 1973). The authors provided evidence that this histone deacetylase bound to chromatin and determined that its maximal activity was at 150 mM NaCl and pH 8.0. Its apparent pI was 4.5 (determined by isoelectric focusing chromatography) and thus the protein is acidic. Amino acid analysis suggested that this protein may be phosphorylated

In plant cells (*Zea mays*), histone deacetylases have been described which are solubilized from whole cell extracts with buffers containing 0.5 M ammonium chloride and 15 mM 2-mercaptoethanol (Lopez-Rodas *et al.*, 1991, Georgieva *et al.*, 1991). During nuclear isolation, only 15% of the total histone deacetylase activity was retained in isolated nuclei (the remainder was released into the soluble cellular lysate). It is important to note however that the isolation procedure used by these authors leaves a minority of the nuclei intact (Lopez-Rodas *et al.*, 1991). By chromatographic behavior, the authors of this study demonstrated the presence of two distinct forms of histone deacetylase which were differentially expressed during plant cell development. Thus, there is evidence for both soluble and nuclear matrix-associated forms of the histone deacetylase.

Phosphorylation regulates histone deacetylase substrate specificity in *Zea mays*

Loidl's group has extended their original studies of histone deacetylase activities in *Zea mays*. They have recently used Mono Q anion exchange chromatography to demonstrate the presence of multiple forms of histone deacetylase 1 (this is the major histone deacetylase found post-germination in *Zea mays*). They observed that when cellular extracts were chromatographed on a Mono Q column, a broad peak of histone deacetylase activity with an evident shoulder was observed. Importantly, when the cellular extract was treated with alkaline phosphatase, the histone deacetylase activity could then be resolved into two distinct peaks on a Mono Q column (Brosch *et al.*, 1992). Using soluble histones as a substrate, they presented evidence that phosphorylation alters the substrate specificity of the histone deacetylase (phosphorylation increases the utilization of H2A as a substrate by more than 2-fold and decreases the use of H3 as a substrate by approximately 60 %). These results suggest that histone deacetylase may be a target of signal transduction mechanisms and/or cell cycle regulation by cell-cycle dependent protein kinases. The suggestion of signal transduction-mediated alterations in histone deacetylase activity implicates histone deacetylase as a mechanism to modulate chromatin structure during alterations of gene expression in response to these signals.

Evidence for an important *in vivo* function of histone deacetylase

The *in vivo* importance of histone deacetylase in normal cell physiology is exemplified by the treatment of cells with the specific histone deacetylase inhibitor Trichostatin A and supported by experiments that

involve treatment of cells with the histone deacetylase inhibitor sodium butyrate. Trichostatin A, originally identified as an antifungal agent, was found to induce differentiation of murine erythroleukemia cells (Yoshida *et al.*, 1987) and cause growth arrest in both G1 and G0 phases in rat fibroblasts (Yoshida and Beppu, 1988). Because sodium butyrate had similar effects on murine erythroleukemia cell differentiation and cell growth, these authors assessed the effect of this drug on histone deacetylase activity. They found that Trichostatin A is a potent ($K_i=3.4$ nM) and specific noncompetitive inhibitor of histone deacetylase *in vivo* and *in vitro* (Yoshida *et al.*, 1990). Caution must be exercised when interpreting the results of studies with sodium butyrate because of its pleiotropic effects on cell physiology (e.g., Boffa *et al.*, 1980). However, where the results of these studies parallel those of the Trichostatin A experiments, it is likely that the inhibition of histone deacetylase activity within the cell is responsible for the perturbation of normal cell function. A second example which indicates important *in vivo* functions for histone deacetylase is the *Suvar(2)1* mutation in *Drosophila*. *Suvar* mutants are *Drosophila* mutants which suppress position effect variegation. Position-effect variegation is a phenomenon of mosaic expression of a particular gene in cells in which the gene would normally be expressed. It occurs, when by some chromosomal rearrangement, a particular gene is placed next to a region of heterochromatin. The neighboring heterochromatin spreads to encompass the expressed gene in some cells and thereby suppresses its expression. Because the heterochromatin does not spread into the expressed gene in all cells, a mosaic expression of the gene can be observed phenotypically. One particular *Suvar* mutant in *Drosophila*, *Suvar(2)1*, which is characterized by being sensitive to sodium butyrate, shows significantly reduced levels of

histone H4 deacetylation in hetero- and homozygous flies (reviewed in Reuter and Spierer, 1992). These results suggest that this mutant is either a histone deacetylase mutant or a mutant in a gene which modulates histone deacetylase activity. More importantly, the results indicate that histone deacetylase activity is involved in the regulation of heterochromatin, a highly condensed chromatin structure found in the interphase cell.

Thus, alterations in histone acetylation levels appear necessary for normal chromosomal changes during the cell cycle. The patterns of histone acetylation change throughout the cell cycle implying that different acetylation states for each of the four core histones is necessary for different functions during the cell cycle (reviewed in Bradbury, 1992). The Trichostatin A and sodium butyrate-induced block to the cell cycle suggest that the core histones must be acetylated and deacetylated during chromosomal changes throughout the cell cycle in order to function normally. Elevated levels of histone acetylation may result in the activation of specific genes responsible for initiating erythroleukemia cell differentiation.

Histone Acetyltransferases

Histone acetyltransferases have been more extensively studied. There have been several reports of extensive purification of histone acetyltransferases. A nomenclature has been developed to distinguish amongst the types of acetyltransferases that have been identified. Histone acetyltransferase A refers to the nuclear form of the enzyme which acetylates histone H1 and the four core histones *in vitro* and the four nucleosomal histones *in vivo*. Histone acetyltransferase B refers to a cytosolic form of the enzyme which has a strong preference for histone H4

in vitro. This acetyltransferase is responsible for the deposition-associated acetylation of histone H4 *in vivo*. Histone H4 is diacetylated (at the sites of reversible acetylation) after synthesis in the cytoplasm and is deposited onto DNA in the diacetylated form. Histone acetyltransferase A is responsible for the nuclear acetylation of histones in their chromatin context. There is considerable evidence for at least two nuclear histone acetyltransferases. Two nuclear histone acetyltransferases have been identified in both rat and pig liver (Kelner and McCarty, 1984; Yukioka *et al.*, 1984; Attisano and Lewis, 1990). Attisano and Lewis (1990) have purified to apparent homogeneity two nuclear histone acetyltransferases from porcine liver. One enzyme is 105 KDa while the other is composed of two subunits of 50 KDa and 40 KDa. The nuclear enzymes can be typically extracted with intermediate salt concentrations (i.e. ≈ 0.5 M) in the presence of reducing agents. The nuclear histone acetyltransferases are of low abundance in the cell. Estimates include a few thousand per cell (Belikoff *et al.*, 1980) and one acetyltransferase per 500 nucleosomes (Attisano and Lewis, 1990). The latter estimate predicts that there is approximately 1 molecule of histone acetyltransferase per chromatin loop. An additional nuclear histone acetyltransferase has been identified and named acetyltransferase DB (for DNA-binding) (Bohm *et al.*, 1980). This acetyltransferase differs from the acetyltransferase A in that it binds to a DNA cellulose column whereas the acetyltransferase designated type A in this study did not. Whether or not this is a distinct form of histone acetyltransferase that deserves a separate classification remains controversial.

Chan *et al.* (1988) demonstrated that the nuclear histone acetyltransferases are not randomly distributed within the eukaryotic genome. These investigators showed that histone acetyltransferase is

preferentially associated with transcriptionally active gene-enriched chromatin in chicken erythrocyte. There was an approximately 4.8-fold enrichment in 0.15 M NaCl-soluble chromatin versus 0.15 M NaCl-insoluble chromatin in chicken erythrocyte chromatin fractions. When the extent of micrococcal nuclease digest is minimized, much of this acetyltransferase activity is found in the transcriptionally active gene-enriched low salt soluble polynucleosomes. These authors also presented evidence that the enzyme was bound to linker DNA (the amount of free enzyme liberated was very sensitive to digestion by nucleases suggesting that the enzyme was associated with nuclease sensitive linker regions of DNA).

Histone-Lysine Methylation

Histones H3 and H4 may be methylated at specific lysine residues of the basic N-terminus. The primary sites of methylation in histone H3 are lysine 9 and lysine 27 and in histone H4 is lysine 20 (Wu *et al.*, 1986; see Fig. 5). Although some reports indicate that histone methylation begins during late S-phase and proceeds through G2 (e.g., Tidwell *et al.*, 1968; Shepherd *et al.*, 1971), a careful kinetic study demonstrated that histone methylation begins very soon after histone biosynthesis and proceeds throughout the cell cycle (Thomas *et al.*, 1975). Histone methylation appears to be a stable modification with at most a very slow turnover (Duerre and Lee, 1974; Wu *et al.*, 1986; van Holde, 1989). The role of histone methylation is unknown. Because of the timing of histone methylation relative to the cell cycle and as a result of the large proportion of histones methylated, methylation has been proposed to play a role in chromatin condensation during mitosis (Honda *et al.*, 1975).

The sites of histone methylation occur at structural boundaries within the nucleosome

The most striking feature of histone methylation is that the methylation sites lysine 27 of histone H3 and lysine 20 of histone H4 occur at structural boundaries within the nucleosome. If chromatin is digested with trypsin, which indicates the accessibility of trypsin cleavage sites when the histones are presented in the form of a nucleosome, the limit digests of histone H3 and H4 coincide with the sites of methylation. That is, the limit peptide of histone H3 is from residue 27 to residue 129 and the limit peptide of histone H4 is from residue 20 to residue 102 (Bohm *et al.*, 1981; see Fig. 5). These results suggest that these sites of histone methylation occur at the structural boundary between the structureless N-terminal basic tails and the globular hydrophobic core of these histones. By inference, it is possible that histone methylation can modulate the structure of the nucleosome at this boundary. Additionally, the site of histone H4 methylation occurs within a region of the H4 protein which is in direct contact with DNA. Utilizing crosslinking techniques, Mirzabekov's group demonstrated that lysine 15 and histidine 18 are in direct contact with DNA at a site where the nucleosome mediates a sharp bend in the DNA path (Ebraldise *et al.*, 1988). The crosslinking of histidine 18 to DNA has been shown to be reduced in HSP 70 when this gene is induced (Nacheva *et al.*, 1989) indicating that histone-DNA contacts may be altered during transcription. This suggests the possibility that postsynthetic modifications, of which there are at least two potential sites (lysine 16-acetylation and lysine 20-methylation), may modulate histone-DNA contacts within this region of the nucleosome and the path of the DNA within the nucleosome. Implicitly, nucleosome

structure may be altered at this site by postsynthetic modification of histone H4 within this region of the histone H4 N-terminus.

Histone methylation progresses to completion during the cell cycle

Despite the potential functional role of histone methylation in the modulation of nucleosome structure, this histone modification remains largely underinvestigated. Perhaps the most informative study on histone methylation was that performed by Thomas *et al.* (1975). These investigators labeled cells (Ehrlich ascites tumour cells) with ^3H -lysine and studied the incorporation of label into mono-, di-, and trimethyllysine over a period of time sufficient for all cells in culture to complete one cell cycle. They found that histone methylation proceeded at a slower rate than histone biosynthesis but that over the course of a cell cycle histone H3 and H4 methylation reached its theoretical limit with H3 attaining methylation levels of 1:3:1 for mono-, di-, and trimethyllysine derivatives whereas all of the histone H4 was found in the dimethylated state. This data suggest that unlike other histone modifications such as histone acetylation, the entire histone population is modified. The authors determined theoretical limits by assuming that in histone H3 only lysines 9 and 27 were methylated and in histone H4 only lysine 20 was methylated. This assumption is reasonable because although there are other sites of methylation in histone H3 (e.g., lysine 4 and 36), these are minor and have not been found in all tissues studied (Wu *et al.*, 1986; van Holde, 1989). van Holde (1989) has suggested that methylation of histones H3 and H4 is involved in the maturation of nucleosomes following incorporation into chromatin.

In contrast to the studies involving determination of methyllysine derivatives which suggest that histone methylation occurs throughout the genome, other studies have demonstrated a nonrandom methylation of

histones with specific regions of chromatin undergoing methylation (Reneker and Brotherton, 1991). Additionally, there is evidence that methylation of histones is related to the conformation of the chromatin template. These experiments involve labeling cells with ^3H -methionine in the presence of an inhibitor of protein synthesis and examining fluorographically the incorporation of labeled new methyl groups into chromatin utilizing either chromatin fractionation techniques or treatment of cells by heat shock or with inhibitors of transcription or topoisomerase I. Arrigo (1983) and Tanguay's group (Camoto and Tanguay, 1982; Desrosiers and Tanguay, 1985; Desrosiers and Tanguay, 1988; Tanguay and Desrosiers, 1990) have demonstrated that heat shock of *Drosophila* cells, which results in the transcriptional repression of the bulk of the genome, is associated with decreased histone H3 and H4 methylation. Desrosiers and Tanguay, 1985) suggested that this decrease in methylation was associated with the repression of gene expression. This infers that ongoing histone methylation may be associated with transcribed chromatin. Using chromatin fractionation procedures, Reneker and Brotherton (1991) found that incorporation of label into newly methylated histones was not randomly distributed amongst chromatin fractions. Specifically, they observed an association of newly methylated histones with acetylated species of histone H4 and a depletion of newly methylated histones amongst chromatin insoluble in physiological ionic strength buffers. This fraction is likely to represent chromatin that contains low levels of acetylation and is in a transcriptionally repressed chromatin structure (i.e., will form condensed structures under these ionic conditions). Support for this hypothesis is presented in this thesis. It is exceedingly difficult to resolve the disparity between these two types of experiments. One possible explanation is that

different regions of the genome are methylated at different points in the cell cycle.

Histone Ubiquitination

Ubiquitination occurs on the ϵ -amino group of C-terminal lysine residues of histones H2A (Lys 119) and H2B (Lys 120) by covalent attachment of the ubiquitin protein via an isopeptide bond (see Fig. 5). In most cell types studied the ubiquitinated species of histone H2A are far more abundant (generally approximately 10-fold) than those of histone H2B (van Holde, 1989). Ubiquitination of histones H2A and H2B is dynamic and there is a nuclear isopeptidase activity which cleaves the ubiquitin moiety from the parent histone (van Holde, 1989). In the cytoplasm, ubiquitination of proteins is associated with a ubiquitin-mediated proteolytic pathway (Herschko, 1988). More specifically, cytoplasmic proteins that are targeted for degradation are polyubiquitinated and ubiquitin plays an important role in proteolytic degradation of proteins during heat shock. In the nucleus, polyubiquitination of both H2A and H2B may occur but the parent monoubiquitinated species is more abundant.

Ubiquitin is cleaved from the parent H2A and H2B proteins prior to metaphase such that metaphase chromosomes lack ubiquitinated histones (Wu et al., 1981). Additionally, ubiquitin is cleaved from uH2A to yield H2A during erythropoiesis (Goldknopf et al., 1980). These observations have led to the suggestion that ubiquitination of histones interferes with the higher-order condensation of chromatin (i.e., ubiquitination may play a role in maintaining decondensed chromatin structures such as in transcriptionally active chromatin) (van Holde, 1989). Because histone H2A is much more readily detected by electrophoretic techniques and conventional protein

staining, much more attention has been paid to uH2A than uH2B in the literature. uH2A has been shown to be enriched in transcribed gene chromatin (Levinger and Varshavsky, 1982) but the extent of this association is controversial (e.g., Dawson *et al.*, 1991). Using chromatin fractionation techniques, our laboratory has found only modest enrichments of uH2A in transcriptionally active gene chromatin-enriched fractions (Nickel *et al.*, 1987; Nickel *et al.*, 1989; Ridsdale and Davie, 1987; Delcuve and Davie, 1989). In contrast, the association of ubiquitinated H2B with transcribed gene chromatin is better established. Ubiquitinated H2B species were enriched in transcriptionally active/competent gene-enriched chromatin fractions (Nickel *et al.*, 1987; Nickel *et al.*, 1989; Ridsdale and Davie, 1987; Delcuve and Davie, 1989). Most importantly, when T47D-5 cells, a human breast cancer cell line, were heat shocked, a process which results in repression of a large number of cellular genes, the levels of ubiquitinated H2A and H2B dropped to very low levels, whereas polyubiquitination of histone H2A was induced. In contrast, when cells were treated with inhibitors of transcription (DRB and/or actinomycin D), levels of uH2B but not uH2A dropped to virtually undetectable levels using immunochemical staining of acid-soluble nuclear proteins (Davie and Murphy, 1990). Upon release of DRB-induced transcription block, the levels of uH2B returned to pretreatment levels within 30 min. This important study demonstrated that ubiquitination of histone H2B but not H2A was directly coupled to transcription. This is the first and only histone modification to date which has been demonstrated to be transcription dependent. These results have been confirmed in other cell types (J. Davie and L.C. Murphy, unpublished results). Davie and Murphy (1990) suggest that because the carboxy-terminal site of ubiquitination of histone H2B is normally buried within the

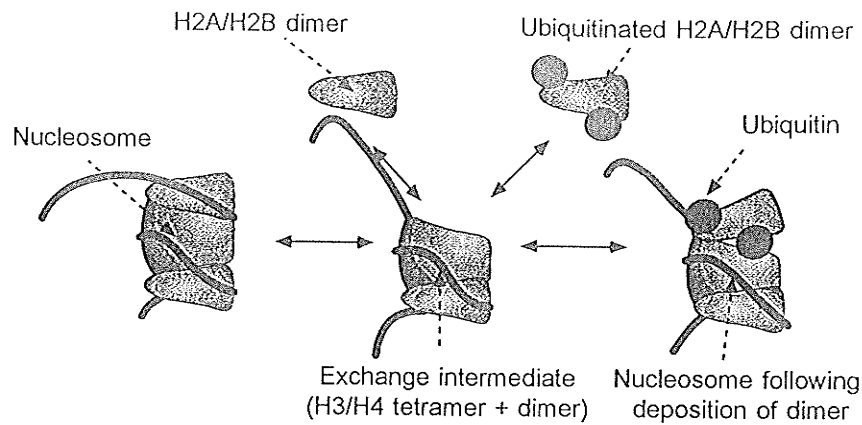
nucleosome, this site would be unavailable for ubiquitination. In the presence of transcription, the nucleosome structure is disturbed by the passage of the polymerase in such a manner as to make available the site of ubiquitination in histone H2B. Once ubiquitinated, the ubiquitin moiety may prevent normal refolding of the nucleosome and thus result in a more open nucleosome structure.

A second interesting feature of histone ubiquitination is that in the absence of chromatin replication, the histone pool is altered in a manner such that free H2A-H2B dimers, which are available for exchange into chromatin, are extensively ubiquitinated (Jackson, 1990). This results in a remodeling of chromatin via an exchange process between a soluble histone pool and preexisting nucleosomes. This exchange process is associated primarily with transcriptionally active gene chromatin and has been suggested to be responsible for the enrichment of these proteins in transcriptionally active chromatin fractions (Jackson, 1990). It is clear from the results of Davie and Murphy (1990) that this process cannot account for the association of ubiquitinated histones with transcribed chromatin but the remodeling of active gene chromatin by replacing nonubiquitinated histone H2A-H2B dimers with ubiquitinated dimers may be of functional importance in maintaining the transcriptionally competent chromatin structure. The mechanisms of histone ubiquitination are summarized in Fig. 7.

As noted above, the levels of ubiquitination are cell cycle regulated. In *Physarum polycephalum*, which can be grown easily in synchronous cultures, uH2A and uH2B are found to be present through S phase and G2 phase up to prophase. The removal of the ubiquitin group is then catalyzed at this stage and the histones remain deubiquitinated until anaphase where reubiquitination occurs (reviewed in Bradbury, 1992). There is an

interesting observation found in some cell cycle mutants in which mitosis-related phosphorylation of H1 and H3 is disrupted at nonpermissive temperatures. Two temperature-sensitive cell cycle mutants have been isolated independently that are defective in histone phosphorylation and the ubiquitination or deubiquitination of H2A. In one instance the defective gene *RCC1* (for regulator of chromosome condensation) has been isolated and codes for a 45 KDa DNA binding protein of unknown function (reviewed in Bradbury, 1992). Thus, there may be a relationship between the cell cycle regulation of histone phosphorylation and the cell cycle regulation of ubiquitination/deubiquitination.

A Incorporation of uH2B into the Nucleosome by H2A/H2B Dimer Exchange



B Ubiquitination of Histone H2B during Transcriptional Elongation

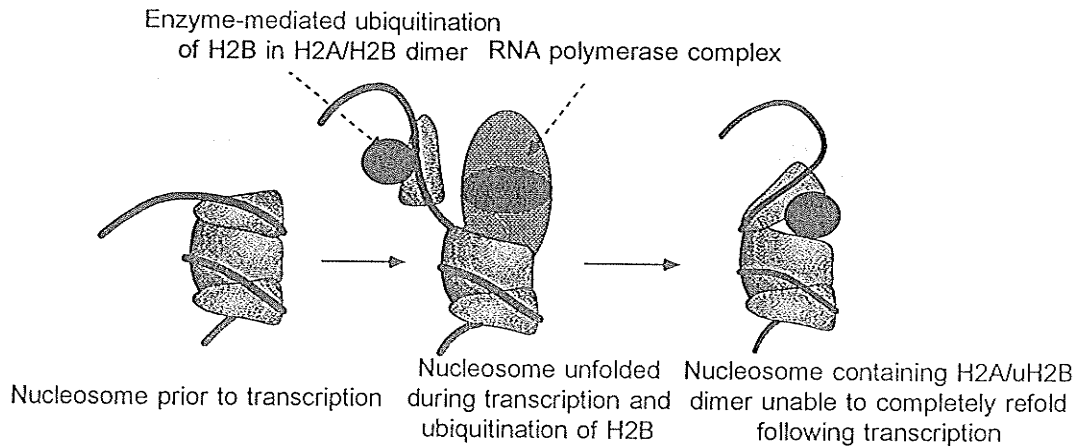


Figure 7. Mechanisms of histone H2B ubiquitination. Panel A shows incorporation of ubiquitinated histone H2B into nucleosomes through an exchange process with a nuclear histone pool. In the absence of DNA synthesis, the nuclear histone pool is enriched in ubiquitinated species of histone H2A and H2B (Jackson, 1990). Ubiquitinated histone H2B can be incorporated into nucleosomes via an exchange process. H2A/H2B dimer exchange does occur outside of S-phase. Panel B shows incorporation of ubiquitinated histone H2B into nucleosomes by an enzyme-mediated reaction that is coupled to transcriptional elongation. The structure of the nucleosome is disrupted as the polymerase elongates through it. This briefly makes accessible the C-terminal tail of H2B. At this point the an enzyme-mediated transfer of ubiquitin to H2B occurs. As the polymerase passes, the nucleosome refolds. Ubiquitin may alter the refolded nucleosome structure.

Histone Phosphorylation

All four core histones may be posttranslationally modified by phosphorylation (Wu *et al.*, 1986). More extensively studied however has been phosphorylation of the H1 histones. Phosphorylation of histone H1 has been implicated in both chromatin condensation (reviewed in Bradbury, 1992) and transcription (reviewed in Roth and Allis, 1992). During mitosis, histone H1 becomes hyperphosphorylated (presumably the cell-cycle dependent histone H1 kinase *cdc 2* is involved). This lead to models which suggested that hyperphosphorylation was necessary for chromosome condensation. In contrast, Allis's group found that histone H1 dephosphorylation was associated with chromatin condensation in *Tetrahymena* (Lin *et al.*, 1991) and suggested that histone H1 phosphorylation plays a role in decondensation of chromatin necessary for transcription. In support of his model, he cites the work of Sung *et al.* (1977) who demonstrated that histone H5 was dephosphorylated during chicken erythrocyte maturation, a process that leads to transcriptional repression. These results would be consistent with dephosphorylation being required for condensation of the chromatin fibril. The resolution of this apparent conflict may reside in the specific sites of phosphorylation and the extent of phosphorylation.

Phosphorylation of core histones has been less extensively studied. A recent report however deserves special attention. Mahadevan *et al.* (1991) attempted to identify proteins phosphorylated during response to growth factors and phorbol esters that correlate with the activation of early response genes such as *c-fos* and *c-jun*. These authors identified two proteins which were rapidly phosphorylated in response to stimulation with growth factors, phorbol esters, okadaic acid (a phosphatase 1 and 2A

inhibitor), and cycloheximide. One of these two proteins was identified by N-terminal amino acid sequencing as histone H3. The authors found a strong correlation between the rapid induction of histone H3 phosphorylation (phosphorylation is markedly increased within 5 min of stimulation with epidermal growth factor or tissue plasminogen activator) and the induction of the early response genes. The significance of this observation is that it indicates that signal transduction mechanisms may directly affect chromatin structure by inducing the posttranslational modification of histone H3 by phosphorylation.

Histone Poly ADP-ribosylation

Poly ADP-ribosylation occurs on all histones accepting many ADP-ribose moieties per histone. There appears to be an important role for poly ADP-ribosylation in DNA repair (van Holde, 1989). It may also be involved in DNA replication and perhaps transcription (Boulikas, 1990). There is an association between histone acetylation and poly ADP-ribosylation that suggests an involvement in transcription (Boulikas, 1990). ADP-ribose groups attached to histones will significantly alter charge by adding negatively charged groups to the histone. Additionally, it appears that poly ADP-ribose polymerase is associated with the nuclear matrix (Kauffman *et al.*, 1991).

Genetic Dissection of Histone Function *In Vivo*

A mechanism to directly assess the function of a protein *in vivo* is to utilize deletion or expression of mutant proteins in living cells. The function of histones may be assessed *in vivo* using the yeast system. *S. cerevisiae* contains two genes for each of the four core histones. Both H2A and H2B have two distinct subtypes (the two H2A isoforms differ by two amino acids

whereas the two H2B isoforms differ by four amino acids) while the two copies of H3 are identical as are the two copies of H4 (Grunstein, 1990). Mutants can be constructed from these cells in which their sole source of a specific histone encoding gene (e.g., H3) is that of a transfected plasmid whose sequence may be mutated at specific sites or otherwise altered to test the function of the entire histone *in vivo* or specific regions of a histone. These studies have provided many novel insights into histone function within the yeast cell.

Function of histones H2A and H2B in yeast deletion mutants

All four core histones have highly basic hydrophilic N-terminal tails and globular central hydrophobic cores. Because of the very basic nature of these N-termini, they may be anticipated to be involved in important electrostatic interactions between the histone core octamer and the nucleosomal DNA. The functional significance of the N-termini have attracted considerable attention in the genetic studies. Grunstein's group has demonstrated that deletion of either but not both N-termini of histones H2A and H2B does not alter the viability of the cells. Double mutants are not viable (reviewed in Grunstein, 1990a,b). These studies indicated that the N-termini of H2A and H2B serve functionally redundant roles in chromatin structure. Only one functional N-terminal tail per H2A-H2B dimer is necessary for viability.

Genetic evidence for a functional role of histones H3 and H4 N-terminal tails in gene regulation

Because of their high degree of evolutionary conservation, histones H3 and H4 and their N-terminal tails have been the focus of most of the genetic studies of histone function in yeast. Deletion studies indicated that, much the same as histones H2A and H2B, deletion of either H3 or H4 N-

terminal is not lethal (Grunstein, 1990a). Deletions of only small portions of the central hydrophobic core, however, is lethal (Grunstein, 1990a). Wolffe's group has demonstrated that *in vitro*, it is the central hydrophobic core of histones H3 and H4 which are primarily responsible for the organization of the DNA within the nucleosomes (Hayes *et al.*, 1991). Thus, the primary organizational function of the repeating nucleosome structure may be preserved in mutants with large N-terminal deletions resulting in viability. Further investigations of the roles of histones H3 and H4 N-terminal tails have, however, revealed important functional roles for these N-terminal tail regions in activation or repression of specific genes in yeast.

The sites of reversible histone modification by acetylation of N-terminal lysines attracted interest for study by yeast genetics because in histones H3 and H4 these modifications are conserved in both site and number and because reversible acetylation of N-terminal lysine residues has long been implicated in gene activation. Two basic types of experiments were performed to assess the function of histone acetylation. One involved substitution of lysine with arginine (which is not able to be acetylated). The second type of experiment involved substitution of these same lysines by uncharged residues to simulate the acetylated state of the lysine residue. These studies revealed novel functions for the histone H4 N-terminus in cell function. Arginine substitutions of individual lysine residues result in viability. Importantly, substitution of lysine 16 with an arginine or substitution of histidine 18 (which may be phosphorylated) with tyrosine resulted in a significant reduction in mating efficiency (Megee *et al.*, 1990). Substitution of all four reversibly acetylated lysines with either arginine or asparagine was lethal (Megee *et al.*, 1990) or resulted in a substantial

reduction in growth rate (Grunstein, 1990b; Durrin *et al.*, 1991). The results of Megee *et al.* (1990) demonstrate that reversible histone acetylation plays an essential role in chromosome dynamics throughout the cell cycle. Interestingly, these results are supported in higher eukaryotes by the previously discussed use of an inhibitor specific for histone deacetylase which causes cells to stop cycling in G1 and G2 and at higher concentrations is lethal (Yoshida *et al.*, 1990).

Grunstein's group performed similar genetic studies in yeast and found that the yeast H4 N-terminus contains a repressor domain containing amino acids 16 through 19 (Johnson *et al.*, 1990). Further to this, a genetic interaction with *SIR3* was detected. Mutations in this gene could suppress mutations in this repressor domain of histone H4. The SIR3 protein is involved in the repression of the silent mating loci (*HML α* and *HMR α*). An additional aspect of this story is that Roth *et al.*, (1990) have demonstrated that the yeast $\alpha 2$ repressor specifically positions nucleosomes. Furthermore, this stable positioning of the repressor (which is involved in the suppression of a-specific mating type genes) is disrupted by mutations in the N-terminal region of histone H4 (Roth *et al.*, 1992). This interaction was found to involve residues 4-23 and individual residues lysine-16 and histidine-18.

A more recent study by Grunstein's group found that the N-terminus of histone H4 was additionally involved in the activation/repression of several inducible genes in yeast (Durrin *et al.*, 1991). The region of the histone H4 N-terminus containing residues 4-23 was found to be essential for the proper induction of the galactose inducible genes *GAL1*, *GAL7*, and *GAL 10*. All of these genes are induced by the acidic activator protein GAL4. Interestingly, substitution of the four reversibly acetylated lysine

residues with arginine also resulted in a 50-fold decrease in induction of *GAL1*. Additionally, this same substitution resulted in a 100-fold decrease in the induction of *PHO5* and a 10-fold decrease in the induction of *CUP1*. It is important to note that these inducible genes normally contain nucleosomes positioned specifically within the promoter or upstream activating sequences. These results indicate that this region of the histone H4 N-terminal tail is involved in modulating histone-DNA contacts or in direct interaction with activator proteins during the displacement of nucleosomes from the promoter or activator regions during induction. In addition, the neutralization of charges on the reversibly acetylated lysine residues is implicated as being involved in this process. These results are summarized in Fig. 8

Novel functions of the histone H3 N-terminus were also detected in genetic studies utilizing yeast (Mann and Grunstein, 1992). Deletion of the N-terminal residues 4-40 allowed cell viability. However, novel functions for the N-terminal domain of histone H3 were found to be involved in the induction of the *GAL1*, *GAL2*, *GAL7*, and *GAL10* genes. Deletions of residues 4-15 as well as acetylation site substitutions 9, 14, and 18 with either glutamine or arginine allow for hyperactivation of these genes. Unlike histone H4, these deletions or substitutions had little effect on mating efficiency or in the repression of other inducible genes such as *PHO5*. These results indicate that this novel function of the N-terminus of histone H3 is different from that of H4. Specifically, the histone H3 N-terminus is involved in the repression of transcription of these GAL4 regulated genes in a manner which is affected similarly by mutations of reversibly acetylated lysine residues that will conserve charge (replacement by arginine) or those that alter charge and mimic acetylation (replacement

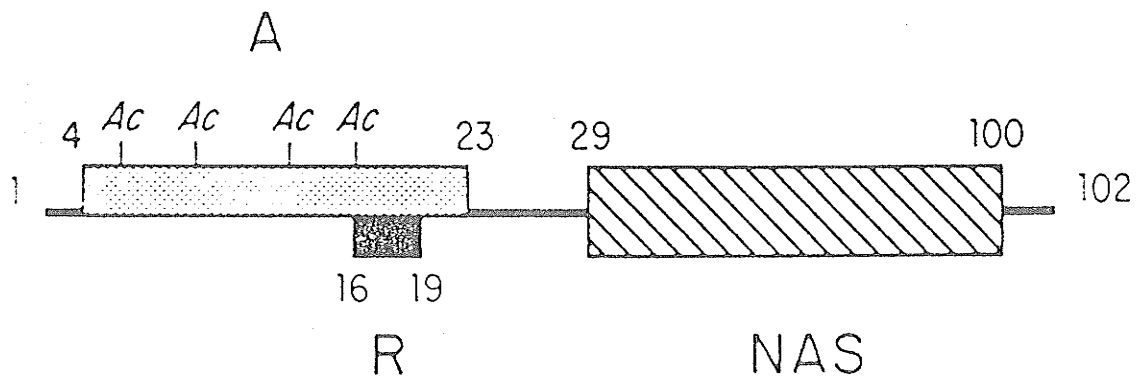


Figure 8. A map of the functional domains of histone H4 identified by genetic studies in yeast. Domain A is the promoter activation domain located within amino acids 4-23 and containing the four reversibly acetylated lysine residues (Ac). Domain R is the repressor domain (residues 16-19) that interact genetically with the repressor protein Sir3 to silence *HML* and *HMR*. NAS is the hydrophobic, nucleosome assembly and stability domain that includes the C-terminal amino acids from 29-100. Taken from Durrin *et al.* *Cell* 65, 1023 (1991).

by glutamine). Additionally, the histone H3 N-terminus appears to be involved in a specific interaction with regulatory proteins of the galactose-inducible genes as this function was not observed on the *PHO5* gene which is also an inducible gene. This contrasts with the histone H4 N-terminus which influenced repression of several types of inducible genes regulated by different mechanisms. The data suggest that there may be a specific interaction between a regulatory protein involved in the expression of these genes (e.g., GAL4) and the N-terminus of histone H3.

Effect of nucleosome depletion on chromatin structure and function in yeast

Often repression of inducible gene expression (e.g., hormone responsive, heat shock responsive, or nutrient responsive) involve the specific positioning of nucleosomes over regulatory regions of inducible genes (Grunstein, 1990a). In turn, activation of these genes upon induction involves specific displacement of this nucleosome (Grunstein, 1990a). It may be expected that nucleosome loss, accomplished by the downregulated expression of a transfected histone gene replacing the native copies, may be expected to facilitate activation of these inducible genes. Grunstein's group performed these studies using both histone H2B and histone H4 regulated by a galactose-inducible promoter. These cells would arrest in G2 phase and showed evidence of nucleosome depletion. In these cells, there was little effect on the expression of constitutively expressed sequences but inducible genes showed significant induction (reviewed in Grunstein, 1990a). These results demonstrate that nucleosomes play a dominant role in the repression of inducible-gene expression in yeast.

Summary, implications, and limitations of the yeast genetic studies

As a collection, the genetic studies in yeast demonstrate novel functions of individual histone domains and the lysines involved in reversible histone acetylation and an important role for nucleosomes in gene repression. Of particular interest is the identification of specific protein-protein interactions between regulatory proteins and specific histone domains. These investigations promise to yield the discovery of more novel interactions between histones and other nuclear proteins as well as dissect the mechanisms involved. An important consideration when reflecting on this data is its relevance to multicellular eukaryotes. It seems possible, if not probable, that specific interactions between regulatory proteins and specific histone domains are involved in the regulation of inducible gene expression in higher eukaryotes. It is clear, however, that the regulation of gene expression in multicellular eukaryotes is more complex than in yeast. Multicellular eukaryotes, in which individual cells once terminally differentiated often express only a small proportion of their genome have additional mechanisms of gene regulation. The most relevant of these is the presence of histone H1 and the compaction of the chromatin fibre into a 30 nm filament in an H1-dependent mechanism. An established function of histone acetylation in multicellular eukaryotes is to prevent the H1-mediated condensation of chromatin (Ridsdale *et al.*, 1991). Histone acetylation may function by regulating higher-order chromatin structure in higher eukaryotes but this function may not be amenable to analysis in the yeast system in the absence of H1-like proteins. This is not to suggest that histone acetylation may not favor a more decondensed structure in yeast but rather that in the absence of a histone H1-like protein to stabilize the condensed state, mutations of the reversibly acetylated lysines in the N-termini of the core

histones may not be as detrimental to viability as in a multicellular eukaryote. Note that H1 histones have not yet been identified and may not exist in yeast (van Holde, 1989). This would predict that in multicellular eukaryotes, single lysine mutations may have more dramatic effects on cell viability.

An additional limitation of the yeast studies to date is the manner in which they have assessed the function of reversible histone acetylation. To date, the yeast genetic studies have examined the function of histone acetylation by mutating individual histone components of the histone octamer. This may not have a dramatic effect on the nucleosome if other histone N-terminal tails can compensate for this change. For example, there is genetic evidence that the N-terminal tails of histones H3 and H4 as well as H2A and H2B are functionally redundant (reviewed in Grunstein, 1990b). Thus in yeast cells containing mutant constructs of histone H4 containing substitutions with arginine or glycine effects in chromatin structure that are mediated by histone acetylation may be compensated by changes in acetylation states of histone H3 relative to the wild-type strain. Because the acetylation states of the other core histones was not assessed in these studies, it is not known if such changes have taken place in the mutant cells. If histone acetylation is able to cause local changes in chromatin through a charge neutralization process in which the net charge of the histone octamer is more important than the net charge of individual histones in the octamer, then this function of histone acetylation cannot be assessed by the studies to date. The yeast studies to date have assessed the function of individual histone N-terminal tails and the sites of reversible acetylation within these tails. To specifically assess the function of acetylation of the histone octamer, mutants will have to be established

which contain point mutations of sites of reversible acetylation in two, three, and all four core histones in combination.

An additional complexity in higher eukaryotes is the presence of sequence-divergent histone variants. Of particular interest in this respect is an H2A variant, H2A.Z. The *Drosophila* homologue of this H2A variant was found to be essential for cell viability (van Daal and Elgin, 1992). There is no corresponding yeast variant. Another recently discovered histone H2A variant is particularly novel and apparently not present in yeast. This histone, macro (m)H2A, contains an N-terminus containing a full length H2A protein and a C-terminal extension bringing the size of the protein to 42 KDa and containing a leucine-zipper motif often involved in protein dimerization (Pehrson and Fried, 1992). This suggests novel interactions between this histone and other nuclear proteins. This form of H2A is present at approximately 1 copy per 30 nucleosomes in mouse liver (Pehrson and Fried, 1992).

Regulation of Gene Expression

The study of regulation of gene expression is perhaps the most popular pursuit in molecular biology today. Many of the studies have used techniques such as transient transfection assays to detect sequences of functional significance (e.g., enhancers, silencers), *in vitro* gel mobility shift assays and DNase I footprinting assays to detect sequence specific DNA-protein interactions and the sequences involved. The current evidence indicates that genes are regulated through the interaction of sequence specific DNA binding proteins with specific regions of sequences which typically flank the gene. These sequences are called enhancers, silencers, or promoters depending upon their biological function. When these

sequences are functional in a cell, they are demarked by DNase I hypersensitivity. That is, the binding of sequence specific DNA binding proteins to their target sequence results in a nucleosome free region of the DNA which has increased sensitivity to digestion by DNase I. It is believed that all regions of DNA showing DNase I hypersensitivity are of functional significance (Elgin, 1988; Gross and Garrard, 1988).

In addition to enhancer sequences, silencer sequences, and promoters, there exist regions of DNA called locus control regions or LCRs. These sequences are responsible for organizing the gene into a transcriptionally competent state and will confer position-independent and copy-number dependent expression of a stably transfected sequence containing LCRs. These sequences may represent matrix attachment sequences (Jarman and Higgs, 1988) and may function by organizing the transfected gene into an independent domain via attachment to the nuclear matrix (reviewed in Bonifer *et al.*, 1991). This isolates the gene from the effects of the local chromatin conformation of adjacent sequences such that it is not repressed by a repressed chromatin state of an adjacent gene.

Function of Nucleosomes-Results from Reconstitution Experiments

Amongst the most informative studies done in the field of chromatin over the past few years have been those involving reconstitution of nucleosomal templates for the study of transcription in a chromatin context. Many fundamental questions about the role and fate of nucleosomes in *in vivo* transcription are difficult to determine due to the vast majority of the genome being nontranscribed and associated with histones. To establish the function of nucleosomes, reconstitution experiments with purified components have yielded more definitive results without the complexity of

the *in vivo* situation. There are several important aspects of how the transcriptionally active chromatin state is established and maintained that can be more easily answered using defined components in reconstituted systems. The most fundamental of these questions are: 1) does the deposition of nucleosomes on promoter sequences prevent the assembly of active transcription complexes on a DNA template? 2) what is the fate of nucleosomes during the elongation phase of transcription? and 3) does the presence of nucleosomes on the transcribed template decrease the efficiency of elongation by the RNA polymerase?

Does nucleosome assembly on the promoter prevent the assembly of active transcription complexes on a DNA template?

Lorch *et al.* (1987) and Losa and Brown (1987) demonstrated that reconstitution of nucleosomes on promoter sequences eliminated initiation from small linear templates by SP6 polymerase and RNA polymerase II. Since this report, results using different methods of reconstitution have consistently reported that preassembly of the promoter sequences into nucleosomes effectively eliminates assembly of an active transcription complex being formed on the promoter (Almouzni *et al.*, 1990; Becker and Wu, 1992; Workman *et al.*, 1988; Laybourn and Kadonaga, 1992). These results imply that the establishment of a transcriptionally competent chromatin structure involves a dynamic competition between transcription factors and nucleosomes during chromatin replication and this has been demonstrated to occur under specific conditions using chromatin assembly extracts from *Xenopus* oocytes (Almouzni *et al.*, 1990). The requirements for the establishment of a transcriptionally competent chromatin structure has also been investigated *in vitro*. For example, when TFIID (a chromatographic fraction that binds to TATA sequences in the promoters of

RNA polymerase II-transcribed genes) is included in a chromatin assembly extract and adenovirus major late promoter is assembled into chromatin *in vitro*, the gene is assembled into a repressed chromatin structure and no transcription occurs from the template. In contrast, if the immediate early protein of the pseudorabies virus, a protein capable of transactivating the template, is included in the assembly reaction, a template which is transcribed is produced (Workman *et al.*, 1988). These results suggest that transcriptional activators can stabilize the TFIID-TATA complex such that it can effectively compete with nucleosomes for the assembly of the template into nucleoprotein structures. The pseudorabies virus protein was incapable of reversing repression by preassembly into chromatin prior to addition of transcription factors (Workman *et al.*, 1988). Similar results were obtained with the 5S rRNA gene of *S. cerevisiae*. In this instance, TFIIA (which binds to the internal control region of the gene) alone was incapable of competing with nucleosomes, but in the presence of TFIIB and C, a transcriptionally competent chromatin structure was assembled (Felts *et al.*, 1990). The hsp70 promoter can be assembled into a potentiated chromatin structure if cloned yeast TFIID is preincubated with the template or is present during the assembly of chromatin. This template is then capable of responding to heat shock factor present in a heat shocked transcription extract subsequent to assembly (Becker *et al.*, 1991). It seems likely that cellular genes, whether to be transcribed or inducible, must have at least a subset of the necessary transcription factors assembled onto the promoter, enhancer, or locus control region, during replication. These results are summarized in Fig. 9.

What is the fate of nucleosomes during transcriptional elongation?

This is a particularly difficult question to assess *in vivo*. It is clear that transcribed genes are associated with histones but whether individual nucleosomes are displaced by polymerases and reform after passage, or whether they unfold and remain bound but in an altered conformation is unclear. To date, *in vitro* experiments have not yet yielded definitive answers to this problem. Earlier studies using short linear DNA templates indicated that polymerases could transcribe through nucleosomes but in some cases nucleosome loss occurred (Lorch *et al.*, 1987) while in other instances nucleosomes were retained on the template (Losa and Brown, 1987). This difference may have been due to underlying DNA sequence. Lorch *et al.* (1988) provided evidence that the strong nucleosome positioning sequence found in the 5S rRNA gene used in the studies of Losa and Brown (1987) were more likely to retain nucleosomes following passage of the polymerase than were random sequences from rat liver. An important deficiency of these early studies, however, was that in both cases linear DNA templates containing only single nucleosomes were transcribed. The *in vivo* context differs in two obvious manners. First, *in vivo*, polymerases must often transverse polynucleosome templates to transcribe a gene. Second, the chromatin template is thought to exist as a topologically constrained system and supercoiling of the template is thought to occur during transcription elongation (Lui and Wang, 1987). The effects of supercoiling could not be assessed in these constructs. Pfaffle *et al.* (1990) and Clark and Felsenfeld (1992) provided evidence that nucleosomes are displaced under conditions where topological changes would be expected to occur on polynucleosome templates. In the studies of Pfaffle *et al.*, (1990) nucleosome loss was assessed by topological changes of a closed-circular DNA template following

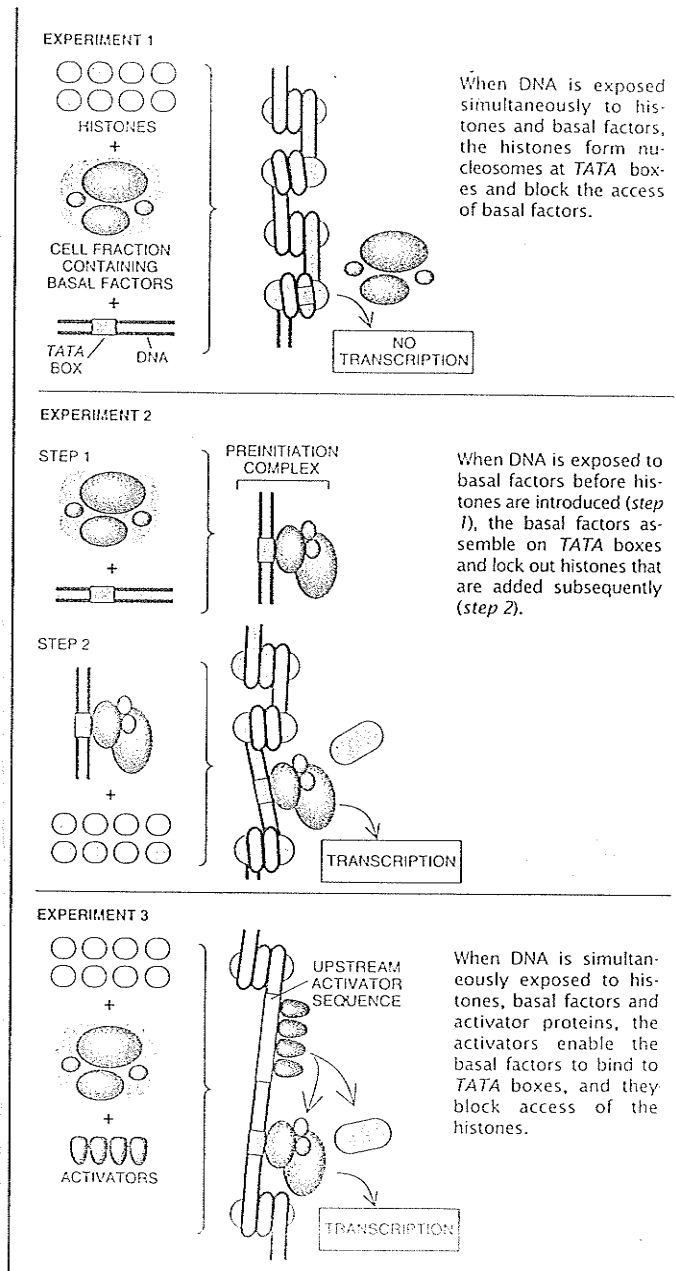


Figure 9. Summary and diagrammatic representation of the results of reconstitution experiments. In the late 1980s, studies of extracts from human cells demonstrated that histones and basal factors compete for access to TATA boxes and that histones generally win (*experiment 1*), except under special circumstances (*experiments 2 and 3*). These findings suggested that activator proteins or proteins they influence might be the agents that free TATA boxes from nucleosomes in intact cells, allowing transcription to occur. Taken from Grunstein (1992).

transcription reactions. Because the nucleosome constrains one negative supercoil of DNA, a loss of negative supercoiling upon relaxation of the template with topoisomerase I was interpreted to reflect nucleosome loss. However, it is not known whether an altered nucleosome conformation would yield similar results.

Does the presence of nucleosomes on the transcribed DNA template affect the rate of transcriptional elongation?

Early studies using short linear templates indicated that nucleosomes did not reduce the efficiency of the elongation phase of transcription (Lorch *et al.*, 1987; Losa and Brown, 1987). This was an unexpected result. However, subsequent studies using longer arrays of nucleosomes have consistently demonstrated that the presence of nucleosomes in a polynucleosome template reduces the rate of transcription apparently by increasing the frequency of pausing by the polymerase on the DNA template (Izban and Luse, 1991; O'Neill *et al.*, 1992). Inhibition of elongation is also affected by conditions that lead to chromatin folding. In the absence of histone H1 when transcription is performed under ionic conditions which favor chromatin compaction, inhibition of elongation is increased significantly on chromatin templates (Hansen and Wolffe, 1992). In this respect, it is important to note that H1-independent chromatin compaction requires the presence of the highly basic N-termini of the nucleosomal histones (Garcia-Ramirez *et al.*, 1992). Alterations in the charge of the N-termini by reversible histone acetylation may prevent the formation of a more compacted chromatin state (Ridsdale *et al.*, 1991; Garcia-Ramirez *et al.*, 1992) and as such histone acetylation may function *in vivo* by alleviating compaction-induced inhibition of elongation.

Novel interactions between transcription factors and chromatin

The use of chromatin templates in the analysis of *in vitro* transcription reactions and the function of sequence-specific DNA binding transcription activator complexes has led to the recognition of novel mechanisms of function in chromatin templates. During a study of the function GAGA, a sequence-specific transcription factor involved in the regulation of the *Drosophila Kruppel* gene, they observed an "antirepression" mechanism of action for the GAGA protein. Specifically, the results obtained in *in vitro* transcription reactions varied with the method of preparation and were most consistent with the activator acting not by a true activation mechanism but rather by counteracting the repression of basal transcription by a general repressor activity present in some extracts (they defined this mechanism as antirepression) (Kerrigan *et al.*, 1991). Purification of this repressor from a nuclear extract prepared by the method of Dignam *et al.* (1983) led to its identification as histone H1 (Croston *et al.*, 1991).

Following the identification of the general repressor activity as histone H1 in transcription extracts, the mechanism and dynamics of the "antirepression" activity of transcription factors was investigated. In the initial study Croston *et al.* (1991) examined the interaction between the basal transcription complex, histone H1, the DNA template, and one of GAGA, Sp1, or a GAL4-VP16 fusion protein. In the absence of histone H1 and in the presence of the GAGA protein an approximately 2-fold decrease in transcription was seen. In contrast Sp1 and the GAL4-VP16 fusion protein both increased transcription over that of the basal transcription rate. The remarkable result however, was that in the presence of histone H1, GAGA, Sp1, and GAL4-VP16 functioned primarily by counteracting H1-mediated repression. That is increasing H1 content in the reaction led to

reduction and elimination of transcription from the basal transcription machinery present in the nuclear extract but the added transcription factors prevented this repression of transcription. This led to a relative activation of the template in levels comparable to that seen in expression in cells. Furthermore, preinitiation complexes, if preassembled onto the template, resisted H1-mediated repression for a single round of transcription but preinitiation complexes could not form on templates preincubated with histone H1. Activator proteins, when present, would allow multiple rounds of transcription to take place in the presence of histone H1. The results indicated that histone H1 would out compete the preinitiation complex for assembly onto the template in the absence but not in the presence of a transcriptional activator. Moreover the results indicated that in an environment in which histone H1 is present (such as the physiological environment of the nucleus) that transcriptional activators function primarily by preventing repression of the template (i.e. "antirepression") rather than true activation. A separate group found that CTF/NF-I functioned by an "antirepression" mechanism also and furthermore that there were CTF/NF-I associated cofactors (coactivators) that were required for this function (Dusserre and Mermod, 1992).

When the function of the transcription factors Sp1 and the GAL4-VP16 fusion protein was examined in chromatin templates in the presence of histone H1, a similar "antirepression" effect was seen at approximately physiological nucleosome density (Laybourn and Kadonaga, 1991). At an H1 histone density of 0.5-1.0, GAL4-VP16 and Sp1 prevented H1-mediated repression of the chromatin template that was observed in their absence. This resulted in a relative activation of 200-fold in the case of GAL4-VP16 at an H1-histone stoichiometry of 0.5 H1 molecules per nucleosome

(Laybourn and Kadonaga, 1991). At an H1 histone density of 1.0-1.5 H1 molecules/nucleosome a sharp nonlinear decrease in transcription factor-activated transcription was observed. This presumably reflects the cooperative nature of H1 assembly onto (and hence repression of) chromatin. These investigators also found that long-distance activation, which is observed *in vivo* with transcription activator protein but which has not been observed in *in vitro* transcription reactions with naked DNA templates, could occur on reconstituted H1-containing chromatin templates in an H1-dependent manner (Laybourn and Kadonaga, 1992). This effect was also dependent upon assembly of the template into a nucleosomal array. A second unique feature of the function of transcriptional activators in H1-containing reconstituted chromatin templates was a non-linear response of a template containing a 5-mer of GAL4 binding sequence to activation ("antirepression") by increasing concentrations of a GAL4-VP16 fusion protein (Laybourn and Kadonaga, 1992). This may mimic the response of genes to transcriptional activation by concentrations of diffusible transcription factors in the establishment of differentiation patterns during development (e.g., *Drosophila* embryo segmentation).

The Replication-Expression Hypothesis

Because once the transcriptionally repressed state is established, accessibility of functional sequences within the 30 nm chromatin fibre is thought to be limited, a hypothesis has emerged for the reprogramming of cell type-specific gene expression. The model makes the logical assumption that replication, during which the chromatin is systematically disassembled and the template is momentarily accessible, allows the reprogramming of cell type-specific gene expression. This model applies most appropriately to

differentiation-specific gene expression where upon terminal differentiation a cell type will express a specific and characteristic set of genes which will determine its phenotype. This model has been extensively elaborated in a recent review by Wolffe (1991). As discussed earlier, *in vitro* reconstitution experiments have consistently shown that preassembly of a DNA template into chromatin results in an essentially irreversible repression of transcription if the template is subsequently challenged with sequence-specific or basal transcription factors. However stable assembly of TFIID onto polymerase II promoters allows for subsequent expression of the template upon reconstitution into chromatin. Similar conditions may arise during replication where for brief moments a short linear array of DNA template is naked due to a minimal amount of DNA required for the initial assembly of the H3-H4 tetramer. Wolffe (1991) suggests that the H3-H4 tetramer as well as the naked DNA template is programmable and the programmability of the underlying sequence is reduced as the H2A-H2B dimers assemble (approximately 20 min following replication of the DNA template) and refractory to reprogramming following the H1 assembly onto the newly assembled nucleosomes. This correlates with differences in DNase I sensitivity. The template remains nuclease sensitive (relative to mature chromatin) in the presence of only the H3-H4 tetramer and upon the assembly of the H2A-H2B dimer this nuclease sensitivity is lost (Fig. 10). Sequence-specific DNA binding proteins, which have much smaller recognition sequences than the amount of DNA that is sequestered into the nucleosome may be able to access the template at a point prior to its assembly into nucleosomes. Wolffe (1991) suggests that during chromatin replication, there exists a dynamic balance between the concentration of transcription factors and the stability of the transcription complex

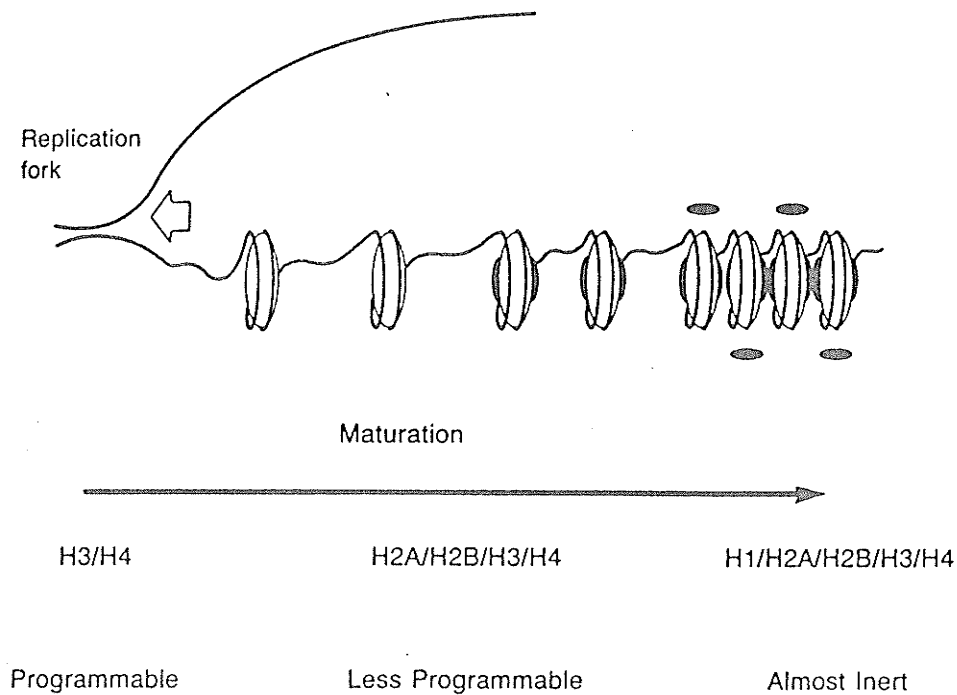


Figure 10. The maturation of chromatin following the replication fork. The sequential deposition of histones H3 and H4 (open ellipsoids) onto DNA followed by histones H2A and H2B (filled ellipsoids) and histone H1 (filled ellipsoids above and below chromatin fiber) is shown after the replication fork (open arrow). The addition of histone H1 causes the chromatin fiber to become more compacted. The accessibility of chromatin to transcription factors as it matures following replication is also shown. The progressive maturation of chromatin following replication fork passage is indicated by the horizontal arrows going from early (left) to late (right). Taken from Wolffe (1991).

on the specific sequence with the concentration of histones and the stability the nucleosome on the underlying DNA sequence. The relative affinities and stabilities of these two competing processes will determine which nucleoprotein complex will form on the DNA. A second aspect of the replication-expression hypothesis is that the availability of sequence-specific DNA binding proteins and the basal transcription machinery will differ at different time points during S-phase. Specifically, it is believed that the concentration of sequence-specific DNA binding proteins and basal transcription factors will be greatest early in S-phase when relatively few genes have replicated. As a consequence of this, it has been proposed genes will have a better chance of being assembled into a transcriptionally active state if they are replicated early in S-phase when the concentration (and therefore effectiveness of competition with assembling nucleosomes) is greatest. Consistent with this hypothesis, it has been demonstrated that cell type-specific genes tend to be replicated early in S-phase if they are to be expressed during the next cell cycle (Hatton *et al.*, 1988; Dhar *et al.*, 1988). This is not to suggest that replication is essential for the reprogramming of all genes. Well established exceptions to this rule are inducible genes such as steroid-responsive genes which are expressed in response to the presence of steroids in the absence of any template replication. It has been demonstrated for the glucocorticoid response element in the mouse mammary tumour virus promoter that the glucocorticoid receptor can displace nucleosomes specifically positioned on its recognition sequence (reviewed in Hayes and Wolffe, 1992).

A further extension of the replication-expression hypothesis was recently proposed by Travers (1992). Travers suggests that some of the

conditions of DNA replication (i.e. topological changes generating an increase in superhelical tension, discussed in more detail later in this introduction) can be generated in the absence of DNA replication. Specifically, a helicase either located at the end of the domain or one which is mobile and associated with a specific domain can generate sufficient superhelical tension to displace nucleosomes or transcription factors allowing a window of time where transcription factors and nucleosomes could compete for assembly onto the underlying DNA in the absence of replication. Genes have been isolated which have homology to ATP-dependent DNA, DNA-RNA, and RNA helicases (e.g., *SWI2/SNF2* from *S. cerevisiae*) and these genes have been implicated in transcriptional activation of specific subsets of genes in both yeast and *Drosophila* (Travers, 1992). The presence of such genes provides tentative support for this model.

The replication-expression hypothesis offers a simple mechanism to establish new regimes of gene expression in differentiating cells and to maintain programs of gene expression in established cell lines. It may be that these conditions can be duplicated in the absence of DNA replication for specific gene loci in a controlled manner during interphase.

The Looped-Domain Model of Chromatin Organization

Another parameter which may influence the ability of polymerases to transverse the nucleosome is DNA topology. It is now generally accepted that chromatin fibers are further organized into topologically constrained loops. One or several related genes are found in each loop and the loop size is somewhat variable. On average the loops are approximately 85 kbp in length (Jackson *et al.*, 1990; note that estimates of loop size vary

depending upon the methods used to determine loop size). The chromatin loops are constrained by attachment to an RNA/protein matrix in the nucleus (see page 74 for more details). The chromatin loop is thought to represent the unit of regulation with respect to gene expression. An important feature of the chromatin loop is that topological changes can influence the chromatin structure of the domain either locally or globally. According to the model of Lui and Wang (1987), transcription may be a major source of torsional strain (Fig. 11). This model is based on the assumption that during transcription, as the RNA polymer lengthens, the polymerase is unable to rotate around the DNA template. In instances where the DNA is topologically constrained, such as in a chromatin loop, this generates positive supercoiling ahead of the elongating polymerase and negative supercoiling behind the elongating polymerase. These topological changes may have important implications for nucleosome integrity on the template. For example, Clark and Felsenfeld (1991) have demonstrated that although nucleosomes form readily on negatively supercoiled DNA, they form very slowly on positively supercoiled DNA. By measuring changes in linking number that accompany nucleosome assembly onto DNA (the nucleosome stores one negative supercoil of DNA and as a result will change the linking number by one), Pfaffle *et al.* (1990) have presented evidence that the positive supercoiling generated ahead of the elongating polymerase displaces nucleosomes from the template. The negative supercoils formed behind the elongating polymerase serve as a deposition site for the displaced nucleosomes. Clark and Felsenfeld (1992) have subsequently demonstrated that a nucleosome of known position is displaced during transcription *in vitro*. Thus, Pfaffle *et al.* (1990) and Clark

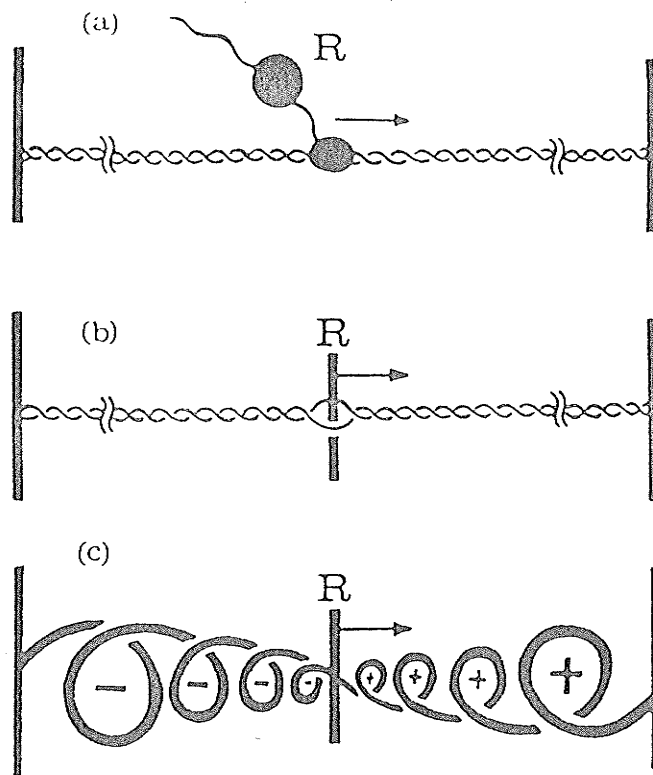


Figure 11. A graphical illustration of the mechanics of transcription. (a) A transcription ensemble R including the polymerase, the nascent RNA, and proteins bound to the RNA is moving in the direction of the arrow along a DNA segment: the ends of the DNA segment are anchored on a large structure represented by the solid bars. (b) The transcription ensemble can be viewed as a divide separating the helical DNA into two parts. (c) If R is moving from left to right without turning around the DNA, the DNA in front of the polymerase becomes overwound, or positively supercoiled: the DNA behind the polymerase becomes underwound, or negatively supercoiled.

and Felsenfeld (1991) have proposed a model whereby nucleosomes are displaced ahead of the elongating polymerase and rapidly reform behind the elongating polymerase. This model provides a solution to the philosophical problem of how the polymerases are able to access a template that is tightly bound in the nucleosome. As will be discussed in detail later, however, other data suggests that nucleosomes are not displaced during transcription but rather unfold or only partially dissociate from the template.

Evidence for the Importance of DNA Topology in Transcription

The increasingly popular model of Lui and Wang (1987) has been validated in recent years by experimental data. Lee and Garrard (1991a) observed half-nucleosome cleavage by DNase I in the presence of transcription on the *HSP82* gene of yeast, where the nucleosomes are phased (i.e. specifically positioned with respect to underlying DNA sequence). These investigators suggested that this half-nucleosome cleavage was the consequence of nucleosome "splitting" in response to positive supercoiling ahead of the transcribing polymerase. In a subsequent study, Lee and Garrard (1991b) utilized a yeast strain, *top1 top2-ts*, which allowed the supercoiled state of DNA to be manipulated *in vivo* by growing the yeast at either 24°C (permissive temperature) or 37°C (nonpermissive temperature). At 37°C, the topoisomerase II is inactivated and positive supercoils accumulate due to the preferential relaxation of negative supercoils by topo I. At 24°C, minichromosomes examined showed relative resistance to DNase I but at 37°C, minichromosomes showed high DNase I sensitivity. Although the DNase I sensitivity did not correlate with transcription rates of the *REP2* gene contained on the minichromosomes, the authors suggested that positive supercoiling was responsible for

establishing the DNase I sensitivity and that a similar mechanism may establish the DNase I sensitive structure of transcribed genes. Two additional important observations were made in this study: 1) there was no evidence for a significant displacement of histones due to positive supercoiling and 2) relaxation of positive supercoiling did not reverse the DNase I sensitivity of the *REP2* locus. The lack of displacement of histones from DNA by positive supercoiling indicates that topological changes alone are insufficient to remove nucleosomes ahead of the transcribing polymerase. The observation that relaxation of positive supercoils does not affect the DNase I sensitivity of the domain in turn suggests that once established, memory mechanisms exist which will propagate the DNase I sensitive structure (a likely candidate is posttranslational modifications of histones) and that a domain need not be transcribed to be maintained in a DNase I sensitive conformation. This data is consistent with the observed DNase I sensitivity of non-transcribed but transcriptionally competent (i.e. DNase I sensitive) chromatin.

A second implication of topological changes coupled to transcription is with respect to DNA conformation. DNA is typically found in the B-form conformation under physiological conditions. However, other conformations are possible under certain conditions. Relative to DNA topology, Z-form DNA can be induced under physiological conditions in the presence of negative supercoiling. In an extensive computer search of genomic DNA sequences with the potential to form Z-form DNA, Schroth *et al.* (1992) observed a striking nonrandom distribution of potential Z-forming sequences. Specifically, there was a strong tendency for potential Z-forming DNA sequences to be found in the 5' portions of genes near the transcription start site and a lack of potential Z-forming DNA sequences in

the 3' regions of genes (Fig. 12). Furthermore Wittig *et al.* (1989, 1991) demonstrated that torsional strain regulates the level of Z-DNA and utilized anti-Z DNA antibodies to demonstrate that transcription and to a lesser extent replication is associated with substantially increased Z-DNA formation in metabolically active permeabilized mammalian cell nuclei. Furthermore, negative supercoiling as a consequence of transcription has been demonstrated *in vivo* (Giaever and Wang, 1988; Brill and Sternglanz, 1988; Wu *et al.*, 1988). The functional significance of Z-DNA formation is less clear.

It is important to note that transcription of prokaryotic genes is facilitated by DNA supercoiling mediated by a gyrase activity. The role of supercoiling in eukaryotic gene transcription has, until recently, been largely ignored. However, there is evidence that eukaryotic genes may be similarly torsionally strained (e.g., Leonard and Patient, 1991; reviewed in Saavedra, 1990). Additionally, there is evidence that supercoiling is responsible for generating the DNase I sensitive conformation which is characteristic of transcribed gene chromatin (reviewed in van Holde, 1989; Lee and Garrard, 1991b). Most importantly, Ljungman and Hanawalt (1992) demonstrated that torsional tension is nonrandomly distributed within specific genes. These authors used psoralen crosslinking and UV irradiation to demonstrate that negative torsional tension is localized in the 5' region of genes and low levels of positive torsional tension is found near the 3' region of genes. This was under conditions in which no net torsional tension was detected in the bulk of the genome. Psoralen preferentially intercalates DNA that is underwound by negative torsional tension, forming interstrand DNA crosslinks upon UV irradiation. Crosslinked DNA can then be separated from

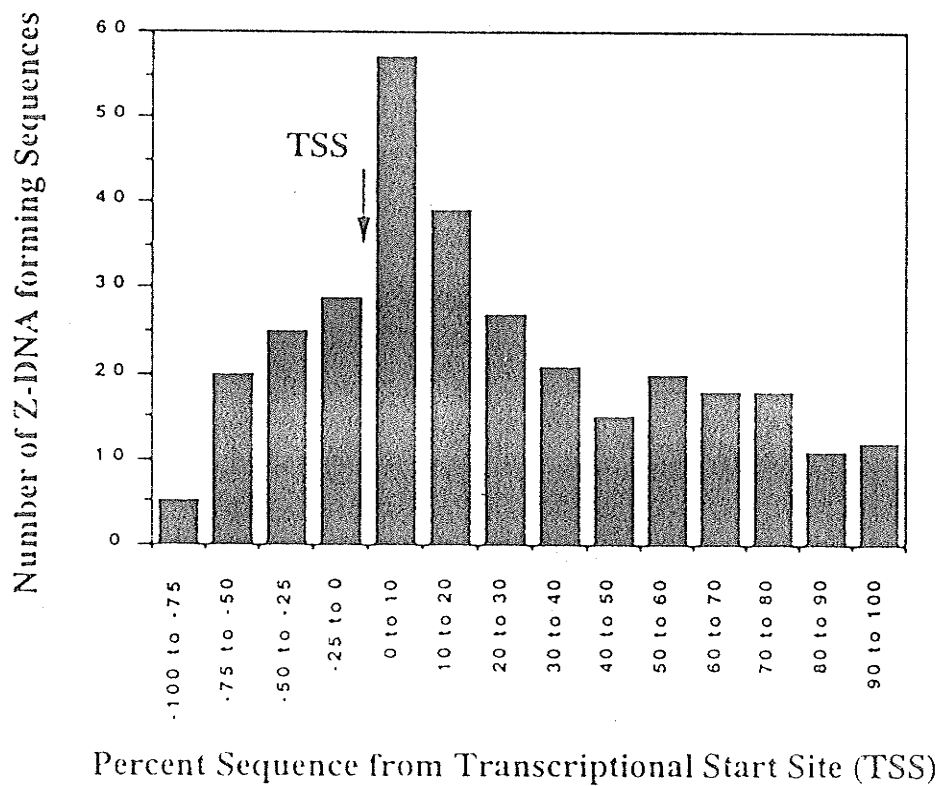


Figure 12. Distribution of potential Z-DNA-forming sequences across 137 human gene sequences. A graph of the number of potential Z-DNA-forming sequences *versus* the percentage of base pairs in each gene that are upstream (5') and downstream (3') to the TSS of the gene. The potential Z-DNA-forming sequences have been grouped into larger percentages in the 5' end to illustrate that this region represents a smaller proportion (16%) of the total data set, relative to the amount of sequence in the data set which is 3' to the TSS (84%). Taken from Schroth *et al.* (1992).

non-crosslinked DNA by hydroxylapatite chromatography following a denaturation/renaturation cycle (the crosslinked DNA will renature and elute as ds DNA whereas the noncrosslinked DNA will have a low probability of renaturing, dependent upon gene copy number, and will elute as single stranded DNA). The crosslinking efficiency for a particular sequence is compared between samples previously exposed to x-irradiation and those exposed to x-irradiation after crosslinking. X-irradiation will create nicks in the DNA which will allow the relaxation of supercoiling. The authors found that prior x-irradiation decreased crosslinks in the 5' region of the dihydrofolate reductase gene and the 5' region of the 45S rRNA gene and an increase in the crosslinking of the 3' region of the DHFR gene. These results provide strong support for the twin-domain model of Lui and Wang (1987) by demonstrating elevated negative torsional tension in the 5' region of a transcribed gene and elevated positive torsional tension in the 3' region of a transcribed gene but no evidence for torsional stress generally throughout the genome. It thus seems likely that torsional strain is a very important component in generating a readily transcribed polynucleosomal template.

The Nuclear Matrix

There is now considerable evidence that the eukaryotic nucleus contains a network of protein and RNA that is responsible for structural organization. Electron microscopic investigations reveal an underlying network which resembles the intermediate filament network of the cytoskeleton (Jackson and Cook, 1988; He *et al.*, 1990). Indeed, proteins homologous to the intermediate filament protein family which are localized exclusively in the nucleus have been cloned and sequenced and shown to

be essential for cell viability (Mirzayan *et al.*, 1992; Yang *et al.*, 1992). Nuclear matrices are prepared by digesting nuclei extensively with DNase I and extracting with high salt concentrations. The residual nuclear material is defined as the nuclear matrix. It contains residual nucleoli, the nuclear pore-lamina complex, and an internal nuclear matrix. The internal nuclear matrix contains both protein and heterogeneous nuclear RNA. It is believed that this structure reflects an underlying nuclear scaffolding structure present in intact cells which serves to structurally organize the nuclear contents. An *in vitro*-prepared nuclear matrix is shown in Fig. 13. It is the attachment to the nuclear matrix which divides the genome into topologically constrained loops. These loops are the regulated unit of expression and replication. Although molecular biology has developed models for replication and gene expression in which soluble and freely diffusible factors interact with the DNA to perform their appropriate functions, there is an emerging accumulation of evidence which suggests that much of the nuclear machinery responsible for transcription, replication, RNA splicing, and transport of RNAs is physically associated with the nuclear matrix and not freely diffusible.

If we accept these studies as reflecting associations that exist *in vivo*, then we are required to think of these processes as not occurring in solution as the molecular biology models suggest but rather as solid state processes. There are many inherent advantages of such solid state processes. For example, by localizing relevant enzymes at specific sites in the nucleus, locally high concentrations of the necessary machinery can be placed at the appropriate sites in the nucleus without the necessity for large quantities of these factors to be synthesized in order to efficiently perform their function. Additionally, spatial organization of the appropriate enzymes on the nuclear

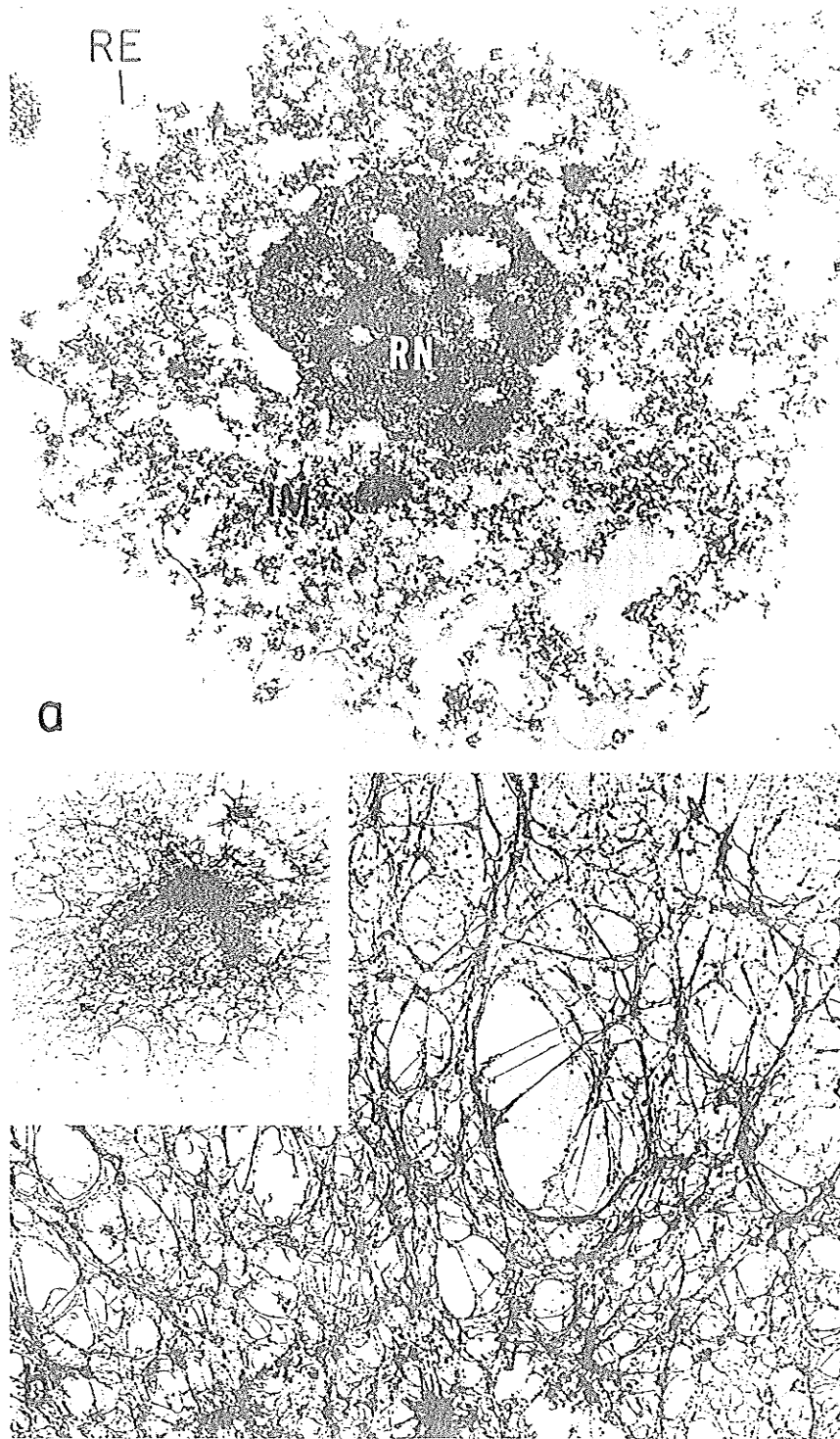


Figure 13. Study of nuclear matrix structure by thin sectioning and whole mount electron microscopy. a: Thin sectioning of an isolated rat liver nuclear matrix reveals the basic tripartite structure of the nuclear matrix. IM, internal matrix; RE, residual nuclear envelope or nuclear lamina; RN, residual nucleoli. b: Whole mount electron microscopy of an isolated nuclear matrix spread on an aqueous surface reveals an overall fibrous network structure (inset). At higher magnification details of the elaborate fibrogranular network are seen. The specimen was critically point dried and rotary shadowed with platinum-palladium. The delicate matrix lacework is considerably disrupted in the absence of critically point drying. Taken from Berezney (1991).

matrix can facilitate the organized processing of biomolecules such as RNA after synthesis. RNA can be properly spliced and transported in such a manner where the product of one reaction serves as the substrate for the next reaction. Because the orderly processing of these molecules is not diffusion mediated, these processes can be very efficient.

Solid-state RNA processing: a case in point

The rapid kinetics of RNA splicing and transport from the nucleus suggest that a vectorial energy dependent mechanism is involved (Carter and Lawrence, 1991). This is further suggested by the specific non random localization of specific mRNAs within the cytoplasm (Lawrence and Singer, 1986) and the association of mRNAs with the cytoskeleton (Ornelles *et al.*, 1986; Biegel and Pachter, 1992). Taneja *et al.*, (1992), using fluorescent *in situ* hybridization to poly(A) RNA and immunofluorescent probes, demonstrated that most of this RNA colocalizes with the actin filament system of the cell. Furthermore, Ornelles *et al.* (1986) demonstrated that the association of mRNAs with the cytoskeleton is necessary for their translation. Early studies have indicated that mRNA precursor RNAs are associated with the nuclear matrix (e.g., Ciejek *et al.*, 1982). In 1987, Zeitlin *et al.* (1987) provided the first experimental evidence to suggest that RNA processing was a solid-state process. These investigators demonstrated that a construct containing introns produced transcripts that were associated with isolated nuclear matrices from transfected cells. More importantly, they noted that if they complimented a soluble micrococcal nuclease treated nuclear splicing extract with nuclear matrices, the normally observed lag time before splicing began did not occur. The authors concluded that nuclear matrices contain preassembled splicing complexes

that must otherwise assemble in solution in the soluble splicing extract. This was the first indication of a solid-state processing of nuclear RNA into mature mRNA. The significance of this observation, however, may not have been fully appreciated until the publication of a fluorescent microscopy study of specific transcripts (Lawrence *et al.*, 1989). These authors used cells containing integrated copies of the Epstein-Barr Virus genome to visualize specific viral transcripts in intact nuclei and whole cells. The transcripts were detected by hybridization to a fluorescently labeled probe. The transcripts were then visualized by fluorescent microscopy. The authors found that curvilinear tracks containing several hundred kb of specific transcripts could be observed. These tracks extended from the nuclear interior to the nuclear periphery and contained sharp boundaries (see Fig. 14). These results strongly argued against diffusion-mediated RNA processing and were subsequently confirmed for *c-fos* transcripts (Huang and Spector, 1991). Since the publication of this report, the use of fluorescent microscopy and immunofluorescent microscopy techniques have provided overwhelming evidence in support of a solid-state vectorial transport and processing mechanism. Important additions to our understanding of RNA processing include the following. 1) These RNA tracks are preserved morphologically during and remain associated with nuclear matrix preparations (Xing *et al.*, 1991). 2) Many components of the splicing machinery are found in discrete clusters within the interphase nucleus. These components include several essential small nuclear RNAs (Carmo-Fonseca *et al.*, 1991a,b; Huang and Spector, 1992) and protein components of the splicing machinery (Carmo-Fonseca *et al.*, 1991a; Spector *et al.*, 1991; Zamore and Green, 1991; Elliot *et al.*, 1992). The visual pattern produced by fluorescent detection of any one of these splicing

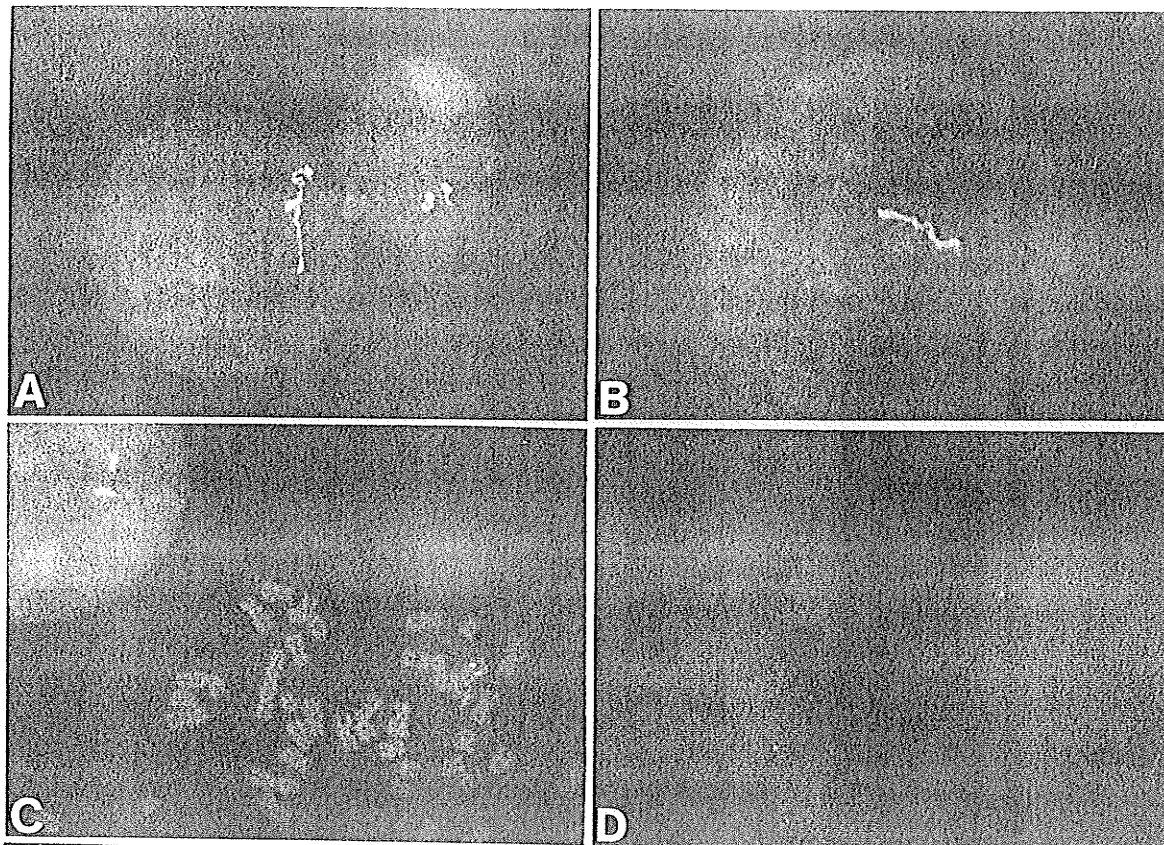


Figure 14. Fluorescent detection of specific RNA and DNA sequences within nuclei stained simultaneously for total DNA. Biotinated probes were hybridized *in situ*, and specific hybridization was detected with fluorescein-avidin. Total DNA was stained with either propidium iodide or DAPI. (A)-(D) show hybridization to cytogenetic preparations of Namalwa cells. (A) Track of EBV BamHI fragment W (Bam W) RNA within nondenatured interphase nuclei of Namalwa cells. Total magnification 1000x. (B) Same as in (A), in DAPI-stained nuclei. 1400x. (C) Simultaneous hybridization to both viral RNA and DNA in denatured samples. Spots of yellow fluorescence on each sister chromatid of chromosome 1 indicate the localization of integrated EBV genomes (see (D) below). Interphase nuclei show larger fluorescent signals, indicative of Bam W RNA. 1000x. (D) Hybridization to BamW DNA in denatured and RNAase H-treated samples. Signal in G1 interphase nuclei appears as two closely spaced fluorescent spots indicative of the two EBV genomes closely integrated on one homologue of chromosome 1. Due to increased chromatin condensation, on metaphase chromosomes the two fluorescent spots coalesce into one signal on each of the two sister chromatids. 1000x. Taken from Lawrence *et al.*, 1989.

components shows discrete foci where these components are present in high concentrations and these foci generally have very distinct boundaries. Because these distinct boundaries represent very steep concentration gradients between the foci and the surrounding nucleoplasm, these results imply that the splicing machinery is not freely diffusible within the nucleus. Transcription is also compartmentalized within the nucleus. Carter *et al.* (1991) demonstrated that poly (A) RNA was located in discrete foci which they called "transcript domains". Together these results indicate that RNA is transcribed in discrete nuclear domains which are intimately associated with nuclear matrix-associated splicing and RNA transport machinery. The results are consistent with a solid-state model but not a diffusion-mediated model of RNA synthesis, transport, and processing.

Identification of targeting sequences that direct processing machinery to discrete nuclear compartments-a mechanism to impart nuclear compartmentalization

If specific nuclear machinery, such as that involved in RNA processing and transport, is compartmentalized to the extent that immunofluorescent microscopy and fluorescent *in situ* hybridization experiments indicate, then a mechanism is needed to impart such nuclear organization. In this respect, three recent publications deserve special attention. Li and Bingman (1991) investigated the function of related 120 amino acid arginine/serine (RS)-domains found in two unrelated pre-mRNA splicing regulators-*suppressor-of-white-apricot* (*su(w^a)*) and *transformer* (*tra*). These domains were found to be interchangeable and functioned to localize both the native proteins and a β -galactosidase fusion protein to discrete nuclear sites which colocalized with the Sm antigen (a general splicing factor associated antigen) (Fig. 15).

A. Colocalization of RS-targeted reporter and splicing proteins

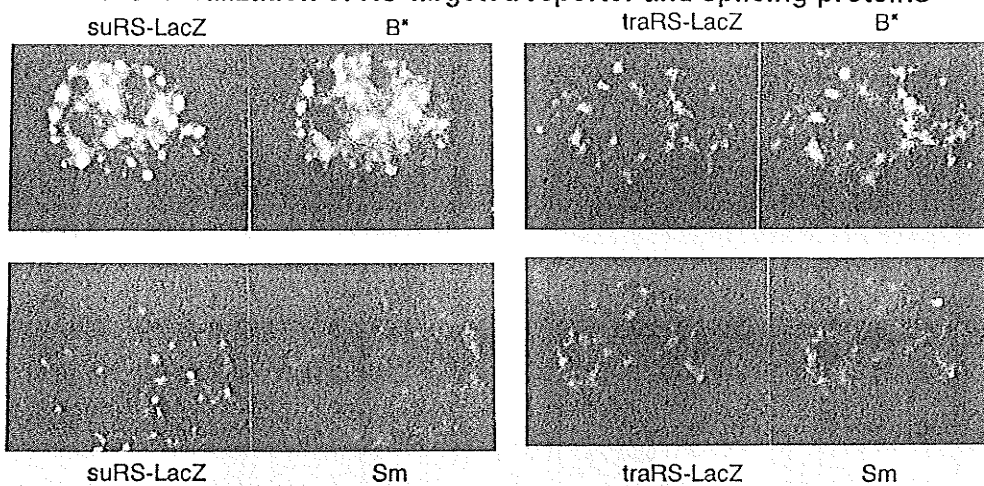


Figure 15. Immunolocalization of RS domain-targeted β -Galactosidase in COS-1 cells. The figure shows double-label immunolocalization of the suRS-LacZ and traRS-LacZ proteins with the B'' and Sm epitopes associated with constitutive splicing proteins. These RS-LacZ proteins colocalize with the punctate portions of the distributions of these splicing proteins. Taken from Li and Bingham (1991).

In this respect, these domains resemble similar domains on proteins targeted to subcellular organelles such as the mitochondrion. RS-domains have also been identified in *transformer-2*, (*Drosophila*) and SF-2/ASF (vertebrate) proteins which are also involved in RNA splicing. Removal of the domain was found to eliminate function. Importantly, if the RS-domain of *su(w^a)* was replaced with the 10 amino acid nuclear localization signal of the SV40 T antigen, a diffuse nuclear distribution of this protein was seen. However, the biological activity of the protein was significantly reduced. These results support the hypothesis that nuclear compartmentalization results in a more efficient metabolic process.

The other studies of significance are that of Leonhardt *et al.* (1992) and Elliot *et al.* (1992). Leonhardt *et al.* (1992) investigated the nuclear localization of DNA methyltransferase and the function of a C-terminal domain not required for enzyme activity *in vitro*. Using indirect immunofluorescent microscopy, these authors found that DNA methyltransferase was diffusely distributed throughout the nucleus until S-phase at which time it redistributed to discrete nuclear domains that corresponded with sites of DNA replication (as determined by incorporation of bromodeoxyuridine). A region of the C-terminal domain from amino acids 207-455 which is moderately hydrophobic was found to be capable of targeting a β -galactosidase fusion protein to replication foci during S-phase. Elliot *et al.* (1992) demonstrated in yeast that a splicing protein PRP6p localized to discrete subnuclear domains that were the sites of RNA splicing but more importantly, they provided evidence that there were nuclear receptors for PRP6p. Overexpression of this protein in yeast resulted in a much greater staining of the nucleoplasm as well as a punctate nuclear

staining. These results are suggestive of a saturable and discrete number of nuclear receptors that are responsible for localizing proteins to discrete nuclear sites.

The demonstration of specific protein domains capable of directing the localization of a protein to discrete nuclear domains imparts a mechanism to functionally compartmentalize the nucleus. The functional organization of sequentially required enzymes responsible for specific nuclear metabolic processes may be important for efficient processing of biomolecules as indicated by the study of Li and Bingham (1991). It is reassuring to note that targeting sequences are established receptor-mediated mechanisms of obtaining subcellular localization of proteins. For example, such sequences and their receptors have been identified for mitochondrial proteins and proteins retained in the endoplasmic reticulum. It is important to note that both DNA replication (Berezney, 1991) and RNA processing (Xing and Lawrence, 1991) have been demonstrated to be associated with the *in vitro* nuclear matrix. This implicates the nuclear matrix as the site of the receptors for these targeting sequences and as the structural component around which these processes are organized.

Evidence for an *in vivo* homologue to the *in vitro* nuclear matrix

The nuclear matrix has traditionally been a very controversial structure. It has been argued that due to the very high concentration of protein and nucleic acid in the nucleus that any alteration of ionic strength may result in precipitation (see Cook, 1988 for a discussion). This argument views residual nuclear structure as a precipitate formed during the extraction procedure. Thus, there has been reluctance to accept the existence of such a structure *in vivo*. It is only within the past two years

that very compelling evidence for a homologous structure existing *in vivo* has appeared. The new data has utilized fluorescent probes to demonstrate fibrogranular networks *in situ* and has provided evidence for spatial organization of specific genes within the nucleus as well as vectorial transport of specific RNAs. There is now solid evidence that splicing occurs in association with the nuclear matrix, that RNA transport is both vectorial and solid state, that transcribed sequences are specifically localized within the nucleus, and that replication occurs via fixed polymerases (Zeitlin *et al.*, 1987; Jackson, 1991; Nelson *et al.*, 1986; Lawrence *et al.*, 1989; Carter *et al.*, 1991; Xing and Lawrence, 1991; Nakayasu and Berezney, 1989). Additionally, there is an emerging evidence that at least some sequence-specific DNA binding proteins may be associated with the nuclear matrix (Dworetzky *et al.*, 1992; Stein *et al.*, 1991; Sun and Davie, unpublished). Additionally, it is well established that steroid hormone receptors which are involved in the activation of steroid-inducible genes in steroid-responsive tissues by binding to steroid response elements are established components of the nuclear matrices of responsive tissues upon steroid treatment ((Getzenberg *et al.*, 1991; van Driel *et al.*, 1991).

In summary, there is now evidence for the existence of a skeletal framework in the *in vivo* nucleus. This skeletal framework, most typically called the nuclear matrix, is likely to serve several important functions in the eukaryotic nucleus. First, it both functionally and topologically isolates individual domains containing one or several related genes (e.g., the α - and β -globin loci) from linearly adjacent sequences. The significance of topological isolation is discussed in more detail below. Second, it serves as a structure around which the nuclear DNA can be functionally organized and compartmentalized. Third, it serves as a site for many important nuclear

metabolic processes. Important nuclear enzymes such as DNA and RNA polymerases and RNA splicing and transport machinery are associated with this structure. Fourth, it may serve as a nucleation site for the assembly of transcription factors onto regulatory sequences during the programming of transcriptional competence.

The Role of the Nuclear Matrix in the Organization of Transcriptionally Active Chromatin

Transcriptionally active gene chromatin is known to have an insoluble nature during nuclear fractionation (Gross and Garrard, 1987) and maintain dynamic associations with the nuclear matrix (Bodnar, 1988). The insoluble nature of transcriptionally active gene chromatin is thus likely to reflect association with the nuclear matrix. Discussions on how this association is maintained and its functional significance can only be speculative at this point. However, emerging data on the structure and biochemical composition of the nuclear matrix suggest interesting possibilities. There is evidence to suggest that transcriptionally-engaged RNA polymerases are associated with the internal nuclear matrix (reviewed in Cook, 1989). The challenge to this hypothesis in the literature (Roberge *et al.*, 1988) can be resolved by the study of nuclear matrix architecture of He *et al.* (1990). Roberge *et al.* (1988) found that the reported association of RNA polymerases with the high-salt nuclear matrix were not found when nuclei were extracted sequentially with 0.4 M KCl and 2.0 M KCl. In this case, most of the total polymerases were extracted with 0.4 M KCl but almost all of the actively engaged RNA polymerase II was associated with the 0.4 M KCl matrix. Because virtually all of the RNA polymerases were extracted with 2.0 M KCl, the authors concluded that RNA polymerases were not

nuclear matrix associated but rather the association of the RNA polymerases with high salt nuclear matrices in the literature reflected a high salt-induced precipitation of the polymerases on the matrix. The study of He *et al.* (1990) examined the architecture of internal nuclear matrices prepared by either high salt extraction or intermediate followed by high salt extraction. They found that the nuclear matrices prepared by high salt extraction resembled those prepared by intermediate salt extraction and consisted of a thick fiber network interspersed within the nucleus. In contrast, using the sequential extraction procedure, they found that the overlying thick fibres could be removed to reveal an underlying intermediate-filament like network. Because the 2.0 M NaCl matrix prepared under these conditions contains mostly structural components, it is likely that functional components such as polymerases would be components of the thicker filaments. The association that Roberge *et al.* (1988) observed between the 0.4 M KCl matrices and engaged RNA polymerases can now be considered to support this model when interpreted in the context of the data of He *et al.* (1990). Thus, RNA polymerases may be one mechanism of establishing dynamic associations between transcriptionally active gene chromatin and the internal nuclear matrix. A second possibility is that nuclear matrix-associated chromatin modification machinery establish dynamic associations between chromatin and the nuclear matrix. A third possibility is that transcribed gene chromatin is dynamically associated with the internal nuclear matrix as a consequence of nuclear matrix association of elongating transcripts. Because the splicing machinery is associated with the internal nuclear matrix (Xing and Lawrence, 1991; Zeitlen *et al.* 1987), an association may be maintained by the elongating RNA transcripts associating with the splicing machinery. In all likelihood, all three

mechanisms contribute to the insolubility of transcribed gene chromatin during nuclear fractionation and the dynamic association of transcriptionally active chromatin maintains with the nuclear matrix.

A second mechanism whereby the internal nuclear matrix plays an important role in the organization of transcriptionally active gene chromatin is via stable nuclear matrix attachment sites. Locus control regions (LCRs) are important for the establishment of position-independent gene expression. It is likely that LCRs possess some capacity for nuclear matrix attachment (Grosveld *et al.*, 1987). It has further been established that nuclear matrix attachment sequences flanking a construct can also establish position-independent expression of a stably transfected gene (Stief *et al.*, 1989; Phi-Van *et al.*, 1990). These regions correspond to the limits of biochemical nuclease sensitivity for native genes (Phi-Van and Stratling, 1988). It appears that these stable nuclear matrix attachment sites insulate the loci from the chromatin state of the neighboring domain. Thus, stable nuclear matrix attachment sites are essential for the establishment of a transcriptionally active domain architecture. The association of LCRs with the nuclear matrix implies that the transcription complex may be nucleated on the internal nuclear matrix. Additionally, these stable sites of nuclear matrix attachment which bind to the attachment region binding protein (ARBP), Lamin B₁, and the tissue specific SATB1 (von Kries *et al.*, 1991; Luderus *et al.*, 1992; Dickenson *et al.*, 1992) and often to topoisomerase II (Gasser and Laemmli, 1987) are probable sites for the introduction of torsional stress into the domain by topoisomerases or gyrase-like activities. This may aid in the initial establishment of the DNase I-sensitive architecture (Lee and Garrard, 1991a,b).

Is Transcription a Solid-State Process?

It is broadly assumed that soluble, freely diffusible nuclear transcription factors regulate the initiation of transcription by soluble freely diffusible RNA polymerases. In turn, these RNA polymerases transcribe the template in a manner analogous to a train on a train track, that is they are mobile and elongate down the DNA track. However, there is little evidence that either transcription factors or polymerases are freely diffusible when functionally active. For example, *in vitro* transcription extracts and extracts containing nuclear transcription factors are generally prepared by extracting isolated nuclei with a buffer containing a moderate salt concentration (typically 0.4 M monovalent ions). The ability to extract these transcription factors by using moderate ionic strength buffers would seem to support the idea of soluble nuclear factors. However, invariably the efficiency of extraction of these components is not quantitated in such procedures. In turn, because we do not know if there exists a population which is not extracted, we cannot distinguish between this extract representing the entire pool of a particular transcription factor or whether this represents a subpopulation that is inactive at the time of extraction. Indeed, there is some evidence that the second possibility may be the case. For example, Roberge *et al.* (1988) found that although RNA polymerases I and II could be extracted from nuclease digested nuclei with 0.4 M KCl, if only the transcriptionally active pool of RNA polymerases I and II was examined (using a photoaffinity label to label the active RNA polymerases), essentially all of the active polymerase pool remained associated with the insoluble material (nuclear matrix) following 0.4 M KCl extraction. In the instance of transcription factors, steroid hormone receptors represent an example in which soluble and insoluble fractions have been analyzed. Because steroid

hormone receptors not only activate transcription by binding to DNA in a sequence-specific manner but also can bind specific ligands with high affinity, it is possible to quantitate the amount of receptor which remains associated with insoluble nuclear material or nuclear matrix by using a radiolabeled ligand. Barrack and Coffey (1980) demonstrated that the majority of high affinity nuclear receptors for both estradiol and dihydrotestosterone were associated with the nuclear matrix. Interestingly, they found that this association was dependent upon the presence of ligand. If rats were castrated, the binding of dihydrotestosterone to isolated nuclear matrices was eliminated within 24 hrs. However, when rats were subsequently injected with dihydrotestosterone, the binding of dihydrotestosterone to the nuclear matrix returned to precastration levels within 30 min of the injection. These results suggest that for at least some steroid hormone receptors, the functionally active receptor is bound to nuclear matrix whereas the soluble receptor is transcriptionally inactive. In the absence of antibodies to specific transcription factors, the lack of a procedure which is effective at solubilizing nuclear matrix proteins while maintaining activity makes it difficult to assess the association of specific transcription factors with the nuclear matrix. The results from studies of steroid hormone receptors and the identification of NF-1 (Sun and Davie, in preparation) and an ATF-like factor (Dworetzky *et al.*, 1992) suggest that at least some sequence-specific transcription factors which are important in the regulation of gene expression are nuclear matrix-associated. A fixed-polymerase model for gene transcription is shown in Fig. 16.

Thus, there is evidence that RNA polymerase II and at least some transcription factors are nuclear matrix associated. We also know that there is a transcription-dependent association of transcribed genes with the

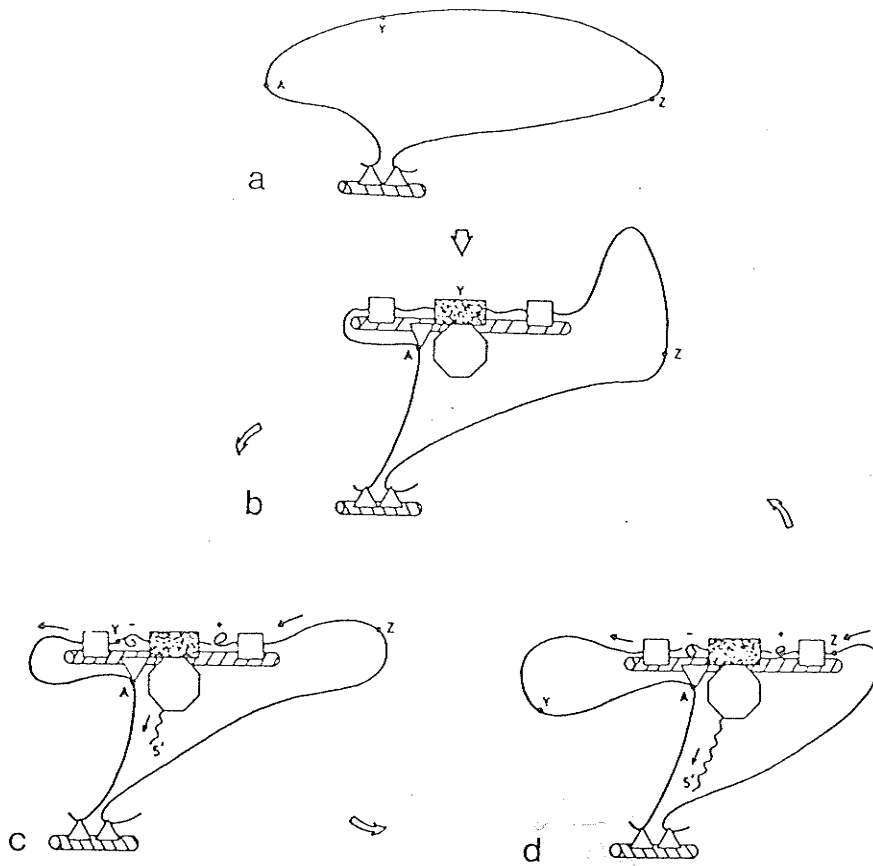


Figure 16. A schematic model for transcription. (a) A loop of DNA is shown attached to the skeleton (rod) at two sites (Δ). These attachments probably persist whether or not the loop is transcribed or replicated. The gene (Y-Z) out in the loop cannot be transcribed as it is remote from any attached polymerase. A marks an upstream activating sequence. (b) During development, the gene is activated by binding to the skeleton and assembly into an attached transcription complex containing polymerase (stippled rectangle), upstream binding sites (∇), topoisomerases (small circles) and RNA processing site (octagon). For the sake of simplicity, the complex is assembled on an additional skeletal element; transcription factors and another loop formed by an enhancer are also excluded. The upstream binding site now permanently tethers the gene to the complex and abuts the polymerase so that they can inter-communicate through physical contact or indirectly through variations in supercoiling of the connecting loop. (c,d) After initiation, DNA moves (arrows) through the complex and RNA (wavy line) is synthesized and processed. Probably the 5' RNA end is attached and a loop of RNA is extruded, rather than as shown. Positive and negative supercoils appear transiently as shown but are removed by topoisomerases. After the transcript is completed, A remains attached to the gene can easily return to its position in (b) and reinitiate synthesis.

nuclear matrix (e.g., Delcuve and Davie, 1989; Andreeva *et al.*, 1992). Thus, some dynamic associations exist between the nuclear matrix and transcribed gene chromatin (Bodnar, 1988). There may additionally be an association between the nuclear matrix and chromatin modification machinery (Hay and Candido, 1983a,b; Kaufmann *et al.*, 1991). It has also clearly been demonstrated that RNA processing and transport are nuclear matrix associated (reviewed in Carter and Lawrence, 1991) and that transcription occurs in nuclear compartments called "transcript domains" (Carter *et al.*, 1991; Carter and Lawrence, 1992).

Combined, these results suggest an alternative to the "text book" model of the regulation of gene expression. This model, perhaps best elaborated by Jackson (1990), views the nuclear matrix as the principal organizational structure and the site of much of the nuclear metabolic processes. In establishing chromatin domains, the nuclear matrix is essential for defining the units of regulation within the genome. Additionally, in the establishment of tissue-specific patterns of gene expression, this model implicates the assembly of a particular nuclear matrix configuration (i.e. a site which contains transcription and processing machinery) during replication of the nuclear matrix as the key regulatory feature in the establishment of a transcriptionally active chromatin structure. This could be considered an extension of the replication-expression hypothesis. The principal argument for this model is that diffusion-mediated processes involving large biomolecules are likely to be considerably more inefficient inside a nucleus (where very high protein and nucleic acid concentrations are likely to make the solution phase of the nucleus very viscous) than in a dilute solution such as a soluble nuclear extract. A solid-

state process, in contrast, would be very efficient by virtue of a compartmentalization of reaction components. This is analogous to a multi-enzyme complex. The elegance of the model is that it provides an additional mechanism of transmission of information during developmental programming. That is, spatial information (e.g., localization of specific mRNAs to specific regions within the cell) can be an additional mechanism that the cell can draw upon in making developmental decisions. Unequal distribution of specific RNAs between daughter cells as a consequence of nonrandom localization within the cell provides a mechanism for the determination of developmental fate.

There is a large body of literature which has isolated and characterized important functional components of the transcription process from soluble nuclear extracts. In contrast, there is a relatively small body of literature characterizing the insoluble functional components of the transcription process. In part this reflects the ease of working with soluble components and the difficulty in working with insoluble material. Awareness of the alternative solid-state model for transcription is important because it demands that experiments be designed differently to assess its validity. Work with soluble nuclear extracts has led to a dangerously naive view of gene expression in which important physiological mechanisms of regulation such as chromatin folding and domain organization are ignored. Recent results on the functional compartmentalization of the nucleus seriously challenge our current perspective on the transcriptional process.

Materials and Methods

Tissues

Adult white leghorn chickens were made anemic by injections of 0.25% 1-acetyl 2-phenylhydrazine dissolved in 95 % ethanol using the following injection schedule. day 1-0.7 ml, day 2-0.7 ml, day 3-0.6 ml, day 4-0.4 ml, day 5-0.7 ml, day 6-0.8 ml. On the seventh day, immature erythrocytes were harvested by decapitation or severing the carotid arteries and draining the blood into an approximately equal volume of 10 mM Tris-Cl pH 7.5, 75 mM NaCl, 25 mM EDTA. In some instances 30 mM sodium butyrate was included in the collection buffer. Erythrocytes were washed twice in the collection buffer and a Pasteur pipette was used to remove the white cells from the top of the packed erythrocytes following centrifugation at approximately 500 *g* for approximately 4 min. Erythrocytes were then either frozen at -80° C or washed in an isotonic buffer (130 mM NaCl, 5.2 mM KCl, 7.5 mM MgCl₂, 10 mM Hepes pH 7.2) and then in incubation media when cells were to be used for labeling. Mature erythrocytes were obtained from a local slaughter house and treated in a similar manner to the immature cells. Trout livers were excised from freshly killed rainbow trout and frozen immediately in liquid nitrogen and stored at -80° C until use. In some instances, erythrocytes were flushed from the livers by perfusing the livers with RSB buffer. Chicken livers were collected in an identical manner.

Labeling of Newly Methylated Histones in Adult Chicken Immature Erythrocytes

Isolated immature chicken erythrocytes were preincubated in DMEM-deficient media (Sigma) pH 7.5 with 2×10^{-4} M cycloheximide (Sigma) for 30 min. L-[methyl-³H] methionine (70-85 Ci/mmol; Amersham Corp.) was

added to the cell suspension (final concentration between 50 and 75 $\mu\text{Ci/ml}$) which was incubated for 60 min at 37°C. For labeling in the presence or absence of sodium butyrate, cells were preincubated in DMEM-deficient media in the presence of cycloheximide, label was added and mixed into the cell suspension, cells were split into two equal volumes, and sodium butyrate was added to a concentration of 10 mM to the plus butyrate cells. For determining rates of acetylation of labeled methylated histones, cells were labeled as above but then resuspended in Swim's S-77 (Sigma) pH 7.5, which contains unlabeled methionine, and chased for 2 hr in this media. Cells were then resuspended in Swim's S-77 media pH 7.5 containing 10 mM sodium butyrate and samples collected at time points between 0 min and 2 h.

Labeling of Acetylated Histones

Immature or mature erythroid cells resuspended at one-third volume packed erythrocytes and two-thirds volume Swim's S-77 media pH 7.5 were preincubated for 30 min with 20 μM cycloheximide. Cells were then labeled with [^3H] acetic acid, sodium salt (27 Ci/mmol; ICN Radiochemicals) at a final concentration of 0.1 mCi/ml. Labeling proceeded for the period indicated (15 min to 2 hr) as indicated. Cells were collected by centrifugation and washed with Swim's S-77 media pH 7.5 containing 0.1 mM sodium acetate and 10 mM sodium butyrate. Subsequently, cells resuspended in the same media with sodium acetate and sodium butyrate were incubated a further 60 min essentially as described by Zhang and Nelson (1988a).

Inhibition of Ongoing Methylation with Adenosine Dialdehyde

Cells were suspended in DMEM-deficient medium pH 7.5 with (methyl group labeling) or without (acetyl group labeling) 10 mM sodium butyrate. L-methyl [³H] methionine (85 Ci/mmol, New England Nuclear) or [³H] acetic acid was then added to a final concentration of 0.1 mCi/ml, and the cells were incubated for 1 h (methionine labeling) with adenosine dialdehyde present at a concentration of 10 μM. For acetic acid labeling, cells were collected by centrifugation and washed once in Swim's S-77 media containing 0.1 mM sodium acetate and 10 mM sodium butyrate. Subsequently, cells were resuspended in the same media with sodium acetate and sodium butyrate and incubated a further 60 min in the presence or absence of 10 μM adenosine dialdehyde. Cells were then collected by centrifugation and stored at -70°C until use.

Labeling of Newly Synthesized Histones with ³H-Lysine

Cells were resuspended to 2.5×10^8 cells/ml in Dulbecco's modified Earle's Medium (pH 7.5) deficient in lysine and supplemented with 10 mM sodium butyrate. Cells were preincubated for 30 min with 5×10^{-6} M aphidicolin to inhibit DNA synthesis (Affolter *et al.*, 1987). Aphidicolin (Sigma) and sodium butyrate were found not to influence labeling of total acid-soluble nuclear proteins. Aphidicolin was included, however, to prevent labeling of the few replicating cells (e.g., erythroblasts) present in the peripheral red blood cells of anemic birds (Williams, 1972). Sodium butyrate, an inhibitor of histone deacetylase, was included to maintain both the high level of acetylated histones that are complexed to active/competent DNA and the solubility of active/competent gene polynucleosomes in 0.15 M NaCl (Ridsdale *et al.*, 1990). For some incubations 15 μg/ml actinomycin D (Sigma) was added during the preincubation period to inhibit transcription.

L-[4,5-³H] Lysine monohydrochloride (85 Ci/mmol; Amersham) was added to the cell suspension to a final concentration of 100 μ Ci/ml and cells were further incubated for 60 or 90 min at 37°C. Incubations of 90 min were typically used because the greater amount of labeled histones incorporated into chromatin after this labeling period facilitated their detection among the chromatin fractions. The duration of labeling did not affect either the spectrum of labeled histones incorporated into chromatin or the distribution of the labeled histones amongst the various chromatin fractions. Cells were collected by centrifugation and stored at -70°C.

Nuclei Isolation

Nuclei were isolated by lysing erythrocytes in RSB pH 7.5 (10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 10 mM sodium butyrate). NP-40 (0.25 % v/v) was included in the first two of four washes and nuclei were collected by centrifugation at 4500 rpm in an SS34 rotor. Trout liver, trout hepatocellular carcinoma, and chicken liver nuclei were prepared by homogenization in 10 mM Pipes/1.0 M hexylene glycol/1% v/v thiodiglycol/2 mM MgCl₂/30 mM sodium butyrate pH 7.0 (Buffer A) with 0.25% NP40 present during the first two of three washes. After the first homogenization, the homogenate was filtered through cheese-cloth to remove connective tissue. The nuclei were collected by centrifugation at 3500 rpm in an SS34 rotor for 10 min. After the third wash, nuclei were resuspended in a small volume of Buffer A and layered onto 50 mM Tris-HCl pH 7.5/150 mM KCl/5 mM MgCl₂/0.7 M sucrose. The nuclei were collected by centrifugation at 4000 rpm in an SS34 rotor for 10 min.

Isolation of Nuclei under Isotonic Conditions-Trout Liver

Trout livers were scissor-minced and then homogenized in 100 mM KCl, 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 mM sodium butyrate, 0.25% NP-40 with 1 mM phenylmethanesulfonylfluoride added fresh from a 100 mM stock. The nuclei were pelleted by centrifugation at 2500 rpm for 10 min in an SS34 rotor. The nuclear pellet was then resuspended in a small volume of 0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 150 mM KCl, 10 mM sodium butyrate, and 5 mM MgCl₂ and the suspension was layered onto 30 ml of the same buffer containing 0.7 M sucrose after homogenization by three passages through a 22-gauge needle. The nuclei were then collected by centrifugation at 4500 rpm for 10 min in an SS34 rotor.

Quantitation of DNA

DNA was quantitated by absorbance at 260 nm in a spectrophotometer. One A₂₆₀ unit is equal to 50 µg of pure DNA. For samples of nuclei or soluble and insoluble chromatin fractions, a 1/10 or 1/100 dilution was made for each sample with 5 M urea/ 2 M NaCl. The spectrophotometer was adjusted to zero against a solution of 5 M urea/ 2 M NaCl. For quantitation by the diphenylamine assay, to the DNA containing sample in 10 % perchloric acid was added an equal volume of 4% diphenylamine in glacial acetic acid, followed by 1/40 volume 1.6 % acetaldehyde. This was incubated overnight at 30°C overnight while shaking. The optical density difference at 595-700 nm was determined. A concentration standard curve was made with fraction S_E where it was found that the absorbance at 260 nm was an accurate measurement of the DNA content. A blank containing all solutions but minus the DNA was also

prepared to zero the spectrophotometer. The method is that described by Giles and Myers (1965).

Quantitation of Protein

Histones were most commonly quantitated using a TCA turbidity assay. 10 μ l or 100 μ l aliquots of the sample were made to 0.8 ml with H₂O. To the diluted sample, 0.4 ml of 50 % TCA was added and the sample was mixed vigorously. The fine precipitate was allowed to develop for about 10 min and light scattering of the sample was measured by reading its absorbance at 400 nm. The spectrophotometer was adjusted to zero against 16.6 % TCA. The protein concentration of the sample was calculated according to the formula:

10 μ l sample: A_{400} reading \times 12900 = x μ g/ml protein

100 μ l sample: A_{400} reading \times 1290 = x μ g/ml protein

Other proteins were quantitated using the Bio-Rad protein microassay (Bio-Rad). The manufacturer's instructions were followed. Samples were diluted to 0.8 ml using H₂O. This sample was then mixed with 0.2 ml of a dye reagent and mixed vigorously. The samples were then measured for absorbance at 595 nm. Protein was quantitated by using a standard curve generated by a known range of bovine serum albumin standard.

Polyacrylamide Gel Electrophoresis and Fluorography

Preparation of samples for polyacrylamide gel electrophoresis

Protein samples were either dialyzed against ddH₂O and lyophilized to concentrate them or they were precipitated by addition of TCA to 20 % final concentration and incubating them on ice for 10 min. The TCA precipitates were then collected by centrifugation at 12000 *g* for 10 min and washed with acetone. The acetone washed material was recentrifuged

and the pellet was vacuum dried. Samples were either resuspended in ddH₂O or directly in the appropriate sample buffer for electrophoresis. For SDS PAGE, the sample was combined with an equal volume of 2x SDS sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). The sample was boiled for approximately 1 min and then loaded onto the gel.

SDS polyacrylamide gel electrophoresis

A discontinuous two part system was used. The appropriate concentrations of acrylamide for stacking gels (typically 4 or 6 %) or separating gels (typically 8, 10, or 15%) was made by dilution of a 30 % acrylamide, 0.8 % bis acrylamide stock solution. For 2 ml stacking gels, 1.4 ml of acrylamide + H₂O was added to 0.5 ml of 0.5 M Tris-Cl pH 6.8. 20 µl of 10 % w/v SDS, 20 µl 10 % w/v ammonium persulfate, and 2 µl TEMED were added. For 7 ml separating gels, the appropriate amount of acrylamide stock solution was added to 1.75 ml 1.5 M Tris-Cl pH 8.8, 70 µl 10 % w/v SDS, 40 µl 10 % w/v ammonium persulfate, 3.5 µl TEMED, and made to the final 7 ml volume with H₂O. The gels were 8 cm high (about 6 cm of separating gel). The gels were run in a minislab apparatus (Idea Scientific) at a continuous 170 V for 1.5 hr. The electrophoresis tray buffer consisted of 200 mM Tris, 1.52 M glycine and 0.4 % SDS.

Coomassie Brilliant Blue staining of proteins and fluorography

All gels were fixed and stained with 0.04 % Serva Blue (Coomassie Brilliant Blue G250 equivalent) in 45 % methanol, 9 % acetic acid. They were destained first in 25 % methanol, 12.5 % acetic acid for 0.5 to 2 hr depending on the gel thickness and then transferred into 5 % methanol and 7.5 % acetic acid where they were stored. Fluorography was performed by placing a destained gel in Autofluor (National Diagnostics) and incubating

the gel in this solution for 1-2 hr at room temperature. The gel was then vacuum dried under heat. The gel was then placed under preflashed Kodak X-OMAT X-ray film (preflash was typically at 30 inches with a flash unit identical to that described by Laskey and Mills (1985)).

Acetic acid-urea-Triton X-100 polyacrylamide gel electrophoresis

The 5.4 % acetic acid-6.6 M urea-0.375 % Triton X-100 discontinuous electrophoresis system provides good resolution amongst the highly basic histone proteins. The 6 % stacking gel and the 15 % separating gel portions were made in the following proportions

	Stock Solution	Stacking Gel (100 ml)	Separating Gel (160 ml)
1	30 % acrylamide/0.8 %bisacrylamide	25.0 ml	80.0 ml
2	4 % TEMED/43.1 % acetic acid		20.0 ml
3	Urea	40.0 g	64.0 g
4	TEMED	1.0 ml	nil
5	0.004 % riboflavin	10.0 ml	16.0 ml
6	3 M K acetate pH 4.0	12.5 ml	nil
7	0.3 M Triton X- 100	2.0 ml	3.2 ml
8	thiodiglycol	1.0 ml	1.6 ml

The gels were polymerized by placing them in front of a white light source. The gels were electrophoresed for 3.5 hours at 200 V for a 8 cm gel. The electrophoresis buffer was 0.9 N acetic acid.

Nuclease Digestion and Fractionation of Nuclei

Nuclei were resuspended at 50 A_{260} units per ml in Buffer A and digested with 25 u/ml of micrococcal nuclease for 20 min. Nuclease digestion was terminated by the addition of EGTA to 10 mM and nuclei were collected by centrifugation at 4000 rpm in an SS34 rotor. Nuclei were resuspended and lysed in 10 mM EDTA pH 7.5 for 30 min on ice. Soluble chromatin was separated from insoluble nuclear material by centrifugation at 10,000 rpm in an SS34 rotor for 10 min. The EDTA soluble chromatin (S_E) was then made 150 mM NaCl by the addition of 4 M NaCl dropwise with agitation. This material was immediately centrifuged at 10,000 rpm in an SS34 rotor for 10 min to yield two fractions (S_{150} and P_{150}). Fraction S_{150} was concentrated by placing the sample into a hydrated 3.5 K MWCO 9.3 ml/cm dialysis bag (Spectra-P) and placing the dialysis bag in a generous amount of dry polyethylene glycol 8000 (Fisher) overnight at 4°C. The 150 mM NaCl-soluble chromatin fragments of fraction S_{150} were subsequently size-fractionated on a Bio-Gel A-5m (2.5 cm x 100 cm or 2.5 cm x 30 cm). The smaller column was typically run at a flow rate of 1.2 ml/min at 4°C. A flow rate of approximately 25 ml/hr was used for the larger column and the fractionation was carried out in a 4°C room. This yielded fractions F_I , F_{II} , F_{III} , and F_{IV} (Delcuve and Davie, 1989). Fractions F_I and F_{II} contain the salt-soluble polynucleosomes while fraction F_{IV} has the mononucleosomes. The percentage of DNA in each fraction was determined by diphenylamine assay (Giles and Myers, 1965). Fraction P_E was extracted with 0.6 M

NaCl. The suspension was then kept on ice for 10 min and then centrifuged at 10,000 rpm for 10 min in an SS34 rotor to obtain a soluble ($S_{0.6}$) and an insoluble fraction ($P_{0.6}$).

Preparation of Nuclear Matrices

Isolation of the 2 M NaCl (high salt) nuclear matrix

2 M NaCl matrices from chicken erythrocytes were prepared according to the procedure of Cockerill and Garrard (1986). Nuclei were resuspended to a final concentration of 20 A_{260} units/ml in RSB-0.25 M sucrose. The nuclei were digested for 1 hr at 23°C with 100 μ g/ml DNase I (Sigma Type IV). All subsequent steps were performed on ice with buffers pre-chilled to 4°C. 1 mM PMSF was added immediately following resuspension of nuclear pellets for each extraction. The nuclei were collected by centrifugation at 3500 rpm for 10 min in an SS34 rotor. Nuclear pellets were resuspended in one-half of the nuclease digestion volume in RSB-0.25 M sucrose and an equal volume of 4 M NaCl, 20 mM EDTA, and 20 mM Tris-HCl (pH 7.4) was added. After incubation on ice for 15 min, residual nuclear material was collected by centrifugation at 3500 rpm for 10 min in an SS34 rotor. The pellets were reextracted twice by resuspension in buffer containing 2 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.4) followed by centrifugation at 6500 rpm in an SS34 rotor. The residual nuclear pellet constituted the 2 M NaCl nuclear matrix. The 2 M NaCl-soluble material was saved and it was dialyzed against 10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl (TEN) pH 8.0 at 4°C. The nuclear matrix was washed once in RSB (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM sodium butyrate) pH 7.5 containing 1 mM PMSF and resuspended in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1 mM PMSF.

Intermediate-/ high-salt nuclear matrix isolation

Nuclear matrices from chicken erythrocytes were prepared as described by Roberge *et al.* (1988). Nuclei were resuspended at a concentration of 40 A₂₆₀/ml in RSB-0.25 M sucrose pH 7.5 and digested for one hour at room temperature with 200 µg/ml DNase I. Nuclei were collected by centrifugation at 3000-4000 rpm for 10 min in an SS34 rotor. The nuclear pellet was resuspended in 0.4 M KCl, 0.2 mM MgCl₂, 1 mM PMSF, 0.25 M sucrose, and 10 mM Tris-HCl pH 7.4. Following a 15 min incubation on ice, the matrices were collected by centrifugation and both the pellet and supernatant were saved. The pellet was resuspended in the same buffer with 2.0 M KCl and treated as before. Thus, we obtained a digestion supernatant, a 0.4 M KCl soluble fraction, a 0.4 M KCl nuclear matrix, a 2.0-0.4 M KCl soluble fraction, and a 0.4/2.0 M KCl nuclear matrix.

Preparation of 0.2 M (NH₄)₂SO₄ nuclear matrices

Nuclei were resuspended at 1 mg/ml DNA in RSB-0.25 M sucrose and incubated with 250 µg/ml DNase I for one hour at 23°C. The nuclei were collected by centrifugation and resuspended in 10 mM Tris-Cl pH 7.5, 0.2 mM MgCl₂ an equal volume of the same buffer containing 0.4 M NaCl was then added while mixing. The nuclei were left on ice for 15 min and the 0.2 M (NH₄)₂SO₄ nuclear matrices were collected by centrifugation.

Preparation of nuclear matrix core filaments

Nuclear matrices and nuclear filaments were prepared according to the method of He *et al.* (1990). Briefly, purified nuclei were resuspended in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, and 0.5% v/v Triton X-100) at a concentration of 1 mg/ml, DNase I was added to a final concentration of 100 µg/ml and the

nuclei were digested for approximately 30 min at 23°C. Ammonium sulfate was added dropwise from a 4 M stock to a final concentration of 0.25 M and the nuclei were pelleted by centrifugation at 2500 rpm for 10 min in an SS34 rotor. The pelleted nuclei (Ammonium sulfate matrix) were resuspended in digestion buffer and reextracted by slowly adding NaCl to a final concentration of 2.0 M from a 4.0 M stock solution while mixing. This was centrifuged at 6500 rpm in (or 2500 rpm for samples prepared for electron microscopy) for 10 min in an SS34 rotor to produce a soluble (matrix) and an insoluble fraction (nuclear filaments plus nuclear pore-lamina complex).

Nuclear pore-lamina complex isolation

Nuclear pore-lamina complexes were prepared from chicken erythrocytes as described by Kaufmann *et al.* (1983). Nuclei were resuspended at a concentration of 40 A₂₆₀/ml in RSB-0.25 M sucrose pH 7.5 and digested for 1 h on ice with 500 µg/ml DNase I and 500 µg/ml RNase A (boiled). The nuclear pellet and supernatant fractions were saved following centrifugation. The nuclear pellet was resuspended in a low-salt buffer (10 mM Tris-HCl pH 7.4 and 0.2 mM MgSO₄). High-salt buffer (10 mM Tris-HCl pH 7.4, 0.2 mM MgSO₄, 2 M NaCl) was then added to a final NaCl concentration of 1.6 M. 2-mercaptoethanol was subsequently added with gentle agitation to a final concentration of 1% v/v and the suspension was incubated on ice for 15 min. The nuclear pore-lamina complexes (pellet) and supernatant were collected by centrifugation at 6500 rpm for 10 min in an SS34 rotor. The pellet was resuspended in the same manner in the absence of 2-mercaptoethanol. After 15 min incubation on ice, the pellet and supernatant were collected. This procedure resulted in the production of a soluble fraction which contained the internal nuclear matrix (1.6 M NaCl/1 % v/v 2-mercaptoethanol S) and an insoluble fraction which contained nuclear pore-lamina complexes.

Nuclear Chromatography

Nuclei were extracted with a linear gradient of ammonium sulfate/2-mercaptoethanol to resolve nuclear histone deacetylase activities. Chromatography was performed using nuclei which had been digested under the conditions described above for the isolation of nuclear pore-lamina complexes. The digested nuclei were pelleted by centrifugation at 3500 rpm in an SS34 rotor and resuspended in a small volume of RSB-0.25 M

sucrose/1 mM PMSF. To this was added 1 g of dry Sephadex G-25 superfine (Pharmacia) per 5 ml volume. This was allowed to swell for about 10 min on ice. A 2.3 cm diameter column was prepared by adding a small volume (2- 3 ml) of pre swollen Sephadex G-25 to the bottom of the column. The nuclei/Sephadex mixture was then poured on top of the Sephadex beads and the excess buffer was allowed to flow through the column (sometimes a peristaltic pump was used to accomplish this-the flow rate was 0.3-0.4 ml/min). A flow adapter was then placed into the column and the column was developed by pumping a linear gradient (typically 200 to 300 ml) from 50 mM Tris-Cl pH 7.0 to 50 mM Tris-Cl pH 7.0/0.25 M ammonium sulfate/1% 2-mercaptoethanol against gravity through the column. 7 ml fractions were collected. Chromatography was performed at a flow rate of 0.3-0.4 ml/min at 4°C. During some preparations, the column would cease to flow. This usually occurred only at the beginning of the gradient. If this happened, the bed was agitated using a glass stir rod and the gradient was then continued following restoration of flow.

Assay for Histone Deacetylase Activity

Isolated soluble fractions were prepared for histone deacetylase assay by dialysis against deionized distilled H₂O or TEN (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 150 mM NaCl) buffer. When a precipitate formed during dialysis, these samples were assayed as suspensions. The insoluble pellet fractions were resuspended in deionized distilled H₂O or in assay buffer. Aliquots of 290 µl or less from each fraction were assayed. To assay chromatin fractions, each chromatin fraction was adjusted to equivalent A₂₆₀ concentrations (typically 4 absorbance units in a final volume of 300 µl) in TEN buffer. Each fraction was assayed in a final volume of 0.30 ml in

TEN buffer and incubated at 37°C for 60 min in the presence of 100 µg of [³H] acetate-labeled histones. Background activity was determined from the amount of dpm released into the ethyl acetate phase using a boiled matrix fraction as an enzyme source and subtracted from the measured activity of each assayed sample. Substrate for histone deacetylase activity was prepared by labeling chicken immature erythrocytes with [³H] acetic acid as described in the "labeling of acetylated histones" section except that labeling proceeded for one hour and 10 mM sodium butyrate was present during the entire incubation period.

Assay for Histone Acetyltransferase Activity

Isolated nuclear fractions were dialyzed overnight at 4°C against 50 mM Tris-Cl pH 7.5 using 6-8 K molecular weight cut off dialysis tubing (Spectra-Por) prior to analysis for histone acetyltransferase activity. Histone acetyltransferase activity was assayed by incubating an aliquot of each fraction in a final volume of 150 µl containing 50 mM Tris-Cl pH 7.5, 50 mM sodium butyrate, 15 mM 2-mercaptoethanol, 100 µg calf thymus histones, and 0.5 µCi Acetyl CoA [Acetyl-³H] (New England Nuclear, 4.7 Ci/mmol). The reaction was incubated for 30 min at 37°C and then spotted onto 5 cm x 5 cm squares of P81 phosphocellulose paper (Whatman). The unincorporated label was removed by washing the phosphocellulose squares in 50 mM sodium carbonate, pH 9.1 (pH adjusted by addition of sodium bicarbonate) for typically 2 x 10 min and 2 x 1 hr washes of approximately 200 ml per 10 squares. The incorporated label was then assayed by scintillation counting of the washed phosphocellulose squares.

Preparation and Analysis of Protein Samples

Histones were isolated from the various chromatin fractions by extraction with 0.4 N H₂SO₄. Briefly, samples were made 0.4 N H₂SO₄ and incubated on ice for at least 30 min. The sample was centrifuged for 10 min at 10,000 rpm in an SS34 rotor and the supernatant containing the histones was retained. The sample was then dialyzed overnight against 1 N acetic acid and then twice for at least 4 h each against ddH₂O. Dialysis was carried out at 4°C using 6-8 K molecular weight cut off dialysis tubing (Spectra-Por). For some experiments chromatin fractions S_E, P₁₅₀, S₁₅₀, and pooled Bio-Gel A-5m column fractions were prepared by elution from a Bio-Gel HTP hydroxylapatite column. Fraction P₁₅₀ was resuspended in

H₂O and loaded directly onto the column. Fractions S_E, S₁₅₀, and pooled Bio-Gel A-5m fractions were loaded directly onto the column. All fractions were washed with 0.45 M NaCl/0.1 M potassium phosphate (pH 6.7) before elution of the histones with 2.0 M NaCl/0.1 M potassium phosphate (pH 6.7). Treatment of chromatin with 0.45 M NaCl before eluting the histones removes any non-nucleosomal histones that may be bound to the surface of chromatin (Seale, 1981; Jackson, 1990). Additionally, this procedure removes the majority of nonhistone proteins as well as the H1 histones and some of the histone H5.

Determination of chromatin distribution of newly synthesized histone H2A by densitometry

Enrichments of H2A were determined by comparing peak heights of densitometer readings of the fluorograms for each fraction on equivalently loaded AUT polyacrylamide gels. Hydroxylapatite prepared histones were used for determination of labeled H2A enrichments in the various chromatin fractions. H2A was chosen because it is well resolved from other labeled species. The enrichment of newly synthesized H2A in the 0.15 M NaCl-soluble polynucleosomes represents a mean of enrichments of fractions F_I and F_{II}.

Quantification of Enrichments of Methylated Histones H3 and H4 in 150 mM NaCl-Soluble Chromatin Fractions by Densitometry

Equivalent amounts of acid-soluble proteins isolated from fraction S and total nuclei were subjected to SDS 15% polyacrylamide gel electrophoresis. Fluorograms were prepared from Coomassie Blue-stained gels and the fluorograms were scanned using a densitometer. Using the peak height of the exposure of histones H3 and H4 on the fluorogram, the

enrichments of each histone in fraction S was determined by dividing the peak height of each histone in fraction S by the peak height of each histone from total nuclei.

Analysis of Rates of Acetylation

The rates of acetylation of the newly methylated histone H4 species was determined according to the procedure of Covault and Chalkley (1980). Cells were collected at various time points during incubation with sodium butyrate and histones were isolated. The acetylated species of histone H4 were resolved on AUT 15% polyacrylamide gels. Fluorograms were prepared and scanned with a densitometer. The peak heights of the unacetylated and each acetylated species of histone H4 were totaled for each time point. The percentage of this total that comprised the unacetylated species of histone H4 at 0 min incubation with sodium butyrate was defined as 100%. The percentage of unacetylated relative to total H4 for each time point was also determined and this was in turn compared to the time 0 figure and expressed as a percentage of this number. The percentage of unacetylated species relative to total for each time point in comparison to that of time 0 was then plotted on logarithmic paper and the rates of acetylation were determined from this plot.

Part 1: Metabolic Properties of Histone Acetylation in Chicken Erythrocytes

Introduction

The chicken erythrocyte as a model system

Throughout much of the original experimental work presented herein, chicken mature and immature erythrocyte cells are used as model systems to characterize specific aspects of chromatin structure and their association with functional nuclear processes. These cell populations have been extensively characterized by Williams (1972). Blood isolated from anemic chickens contain 2.0 % nonerythroid cells, 0.1 % erythroblasts, 3.0 % mature erythroid cells, and 95 % immature erythroid cells (early, mid, and late polychromatic cells). Normal chicken blood contains 99.3 % mature erythroid cells and 0.7 % nonerythroid cells (Williams, 1972). Because cells isolated from both anemic blood and normal blood do not incorporate radiolabel into DNA when incubated with appropriate labeled precursors, it is clear that this cell population contains very little replicating chromatin. This is important because many of the features of transcribing chromatin (e.g. DNase I-sensitivity, elevated levels of acetylated histones) are also common to replicating chromatin. Cell populations containing replicating chromatin can, as a result, complicate the establishment of correlations between biochemical characteristics of chromatin and transcriptional activity of the underlying sequence. Both immature and mature erythroid cells synthesize RNA but the synthesis of RNA is significantly reduced in mature erythroid levels (which has led to reports of an absence of RNA synthesis in these cells (reviewed in Gasaryan, 1982; see also Affolter *et al.*, 1987). 90

% of the total RNA synthesized in immature erythroid cells belongs to the globin family (Gasaryan, 1982). Thus, these cells are highly differentiated and express only a small fraction of their genome. The developmental expression of the erythroid-specific histone H1 variant, histone H5, contributes to the global repression of the erythroid genome (Affolter *et al.*, 1987). Biochemical correlations between chromatin structure and chromatin function can be established in these cells using a chromatin fractionation procedure developed for these cells that partially resolves transcriptionally active, competent, and repressed chromatin fragments from each other (Ridsdale and Davie, 1987; Delcuve and Davie, 1989; described in detail below). It is important to note that this procedure results in the isolation of fractions with unusually high enrichments in transcriptionally competent and transcriptionally active chromatin fragments. The combination of a highly efficient chromatin fractionation procedure and the absence of replicating cells are the major advantages to using chicken erythrocyte cells as a model system for examining the association between chromatin structure and chromatin function.

The fractionation of chicken erythrocyte chromatin

Traditionally, chromatin fractionation techniques have been used in an attempt to study the biochemical composition of specific functional classes of chromatin (reviewed in van Holde, 1989). This is the technique used to ascribe characteristics of transcribed and repressed chromatin in this thesis. In this regard, it is of interest to understand how chromatin fractionation procedures in general and the fractionation procedure used herein work.

The ability to separate functionally distinct classes of chromatin from each other depends upon differences in the physical properties of functionally different regions of chromatin (e.g., transcribed *versus*

transcriptionally repressed sequences). For example, transcribed chromatin can be partially separated from repressed sequences by exploiting density differences between chromatin associated with polymerases and RNPs and those which are not (Ip *et al.*, 1988). The procedure used here exploits differences in the folding properties of different regions of chromatin and insolubility during nuclear lysis to produce fractions which are modestly enriched to greatly enriched in transcriptionally active and transcriptionally competent sequences.

A schematic representation of the chromatin fractionation protocol used throughout this work is shown in Fig. 17. Generally, around 90 % of the total nuclear chromatin of isolated immature chicken erythrocyte nuclei is solubilized during nuclear lysis with EDTA following mild micrococcal nuclease fragmentation of chromatin (Delcuve and Davie, 1989). Under these ionic conditions, the chromatin remains essentially unfolded (i.e. in a 10 nm fibre conformation). If the ionic strength of the solution is increased such that the concentration of monovalent ions is near physiological concentrations (e.g., 150 mM), sufficient charges on the chromatin fibre are neutralized to facilitate the folding of the chromatin into more condensed structures (e.g., the 30 nm chromatin fibre) and intermolecular association between fragments resulting in precipitation. In chicken erythrocyte chromatin, approximately 90 % of the EDTA soluble chromatin is precipitated under these conditions. The soluble chromatin consists primarily of mononucleosome-sized particles which resist precipitation (Ridsdale and Davie, 1987; Delcuve and Davie, 1989). Additionally, a much smaller population of poly- and oligonucleosomes resist precipitation. This reflects biophysical differences in the presence of H1 histones between this precipitation-resistant poly- and oligonucleosomes and the precipitation-

prone poly- and oligonucleosomes (Ridsdale *et al.*, 1988). As a consequence, fractions which can be isolated which are highly enriched in transcriptionally active and transcriptionally competent sequences. This is achieved by size-resolving the soluble chromatin by gel exclusion chromatography. By choosing a gel filtration resin which adequately separates mononucleosomes (Fig. 17 fractions IV and V, sometimes these are pooled as one fraction) from larger sized material, poly- and oligonucleosomes (Fig. 17 fractions I-III). Fractions I, II, and III are enriched to different extents in transcriptionally active and competent sequences (Delcuve and Davie, 1989; Ridsdale and Davie, 1987). In mature erythrocytes, fraction I is maximally enriched in transcriptionally competent and active sequences (Ridsdale and Davie, 1987). The 50-fold enrichment of transcriptionally competent sequences in this fraction approach theoretical limits of a pure fraction if 2 % of the genome is in a competent conformation (Lewin, 1986). In immature erythrocytes, the active sequences are enriched to a greatest extent in fractions II and III whereas the competent sequences are enriched to the greatest extent in fraction I (Delcuve and Davie). High enrichments of transcribed sequences (> 10 -fold) can be obtained in these cells.

The chromatin which resists solubilization in EDTA is enriched in transcribed sequences (Delcuve and Davie, 1989). The insolubility of transcribed sequences using similar fractionation schemes occurs in a variety (perhaps all) cell types (Gross and Garrard, 1987; see also general introduction). We have also found that in immature erythrocytes, a further enrichment in transcribed sequences can be obtained by extraction of the insoluble nuclear material with 0.6 M NaCl (Fig. 17). The physical mechanism(s) responsible for this insolubility are unknown.

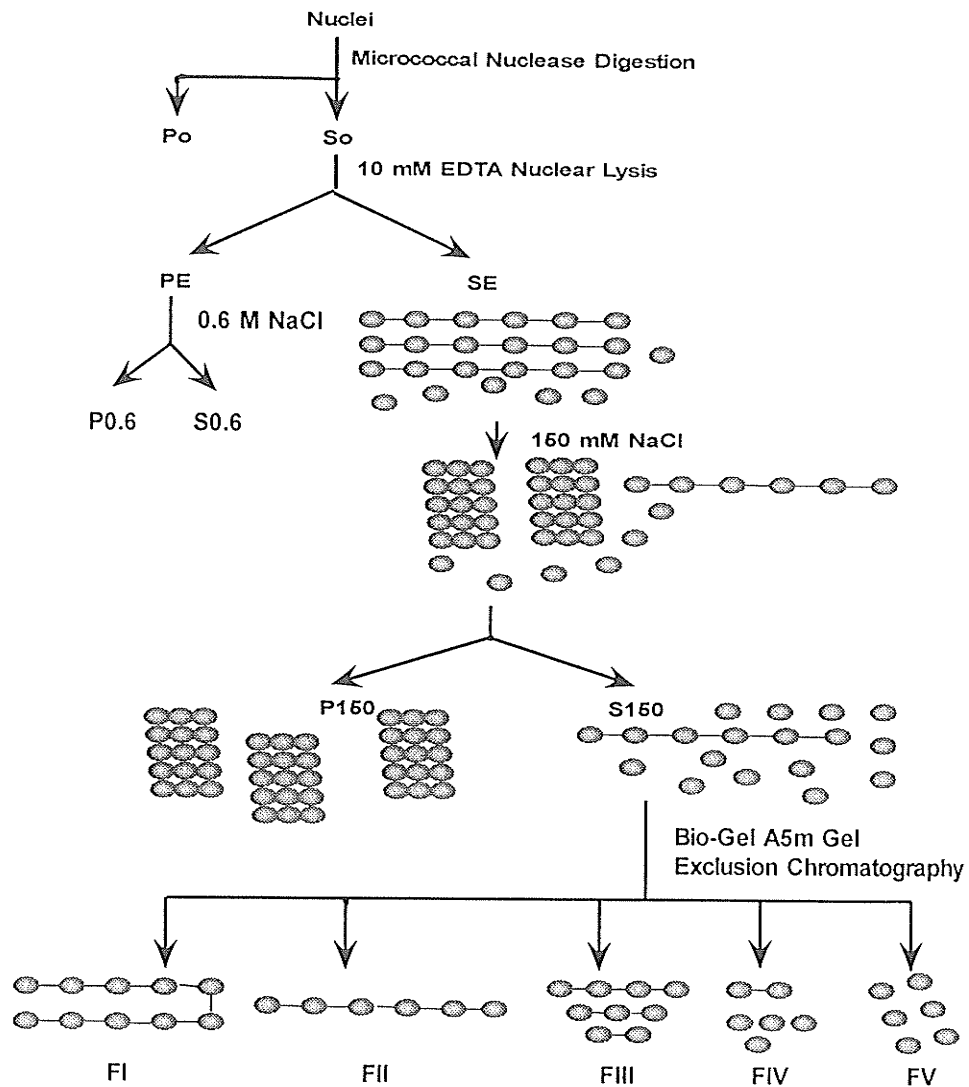


Figure 17. Diagrammatic representation of the fractionation of chicken erythrocyte chromatin. Isolated nuclei were digested with micrococcal nuclease and lysed in 10 mM EDTA. The EDTA soluble chromatin was further fractionated into low salt-soluble and insoluble chromatin fractions. Low salt-soluble chromatin was further size-resolved by gel exclusion chromatography. The EDTA insoluble chromatin was further fractionated into 0.6 M NaCl-soluble and insoluble fractions.

Rationale for studies on metabolic properties of histone acetylation

Highly acetylated histones are complexed to transcriptionally active/competent DNA (Allegra *et al.*, 1987; Zhang and Nelson, 1988a, Hebbes *et al.*, 1988; Ip *et al.*, 1988; Ridsdale *et al.*, 1990). Importantly, genetic analysis of histone H4 acetylation has shown that acetylation of H4 has an essential role in chromosome dynamics (Megee *et al.*, 1990). Histone acetylation has been shown to alter nucleosome structure and to alter the capacity of the H1 histones to condense the transcriptionally active/competent gene chromatin fibre which may in turn facilitate transcription (Norton *et al.*, 1989; Walker *et al.*, 1990; Oliva *et al.*, 1990; Ridsdale *et al.*, 1990).

Histone acetylation is a very dynamic process. This cannot be appreciated in studies which analyze steady state levels of acetylated histones in chromatin preparations. In chicken immature erythrocytes, approximately 4% of the modifiable histone lysine sites participate in active acetylation and deacetylation (Zhang and Nelson, 1986). In these cells there is only one rate of acetylation which has a $t_{1/2}$ of approximately 12 min (Zhang and Nelson, 1988a; see also Fig. 29). However, there are two categories of metabolically active histone acetylation. One type of acetylated histone species becomes hyperacetylated (e.g., the tetraacetylated form of histone H4) in the presence of sodium butyrate, a histone deacetylase inhibitor. Upon removal of the inhibitor, the hyperacetylated histone species are rapidly deacetylated ($t_{1/2}$ = 5 min; Zhang and Nelson, 1988b). We refer to this type of metabolically active acetylation as dynamic, class 1 acetylation. Another population of the metabolically active acetylated histone species only achieve low levels of acetylation (e.g., mono- and diacetylated forms of histone H4) in the

presence of sodium butyrate. In the absence of butyrate, these histones are slowly deacetylated. We refer to this type of acetylation as class 2 acetylation.

The association of the class 1, dynamically acetylated histones with the 0.15 M NaCl or 2 mM MgCl₂ soluble, active gene chromatin fragments has been demonstrated (Zhang and Nelson, 1988a; Ridsdale *et al.*, 1990). However, whether the histones associated with the active gene chromatin fragments bound to the low-salt insoluble residual nuclear material partake in dynamic acetylation remains to be determined. Furthermore, little is known about the chromatin distribution of the class 2 acetylated histones.

In this study, we determined the chromatin distribution of the metabolically active class 1 and class 2 acetylated histone H4 species in chicken immature erythrocytes. We provide evidence that dynamic, class 1 histone H4 acetylation was limited to the transcriptionally active chromatin regions. Dynamically acetylated H4 histone species were associated with the active gene-enriched, salt-soluble chromatin fragments and the active chromatin fragments bound to the residual nuclear material. Class 2 acetylated histone H4 species were found in repressed, active and competent chromatin, with a preference for the latter two chromatin regions.

Results

Dynamically acetylated histones are preferentially associated with transcriptionally active gene chromatin.

In order to label the dynamically acetylated (class 1) histones, chicken immature erythrocytes were pulse-labeled with [³H]-acetate for 15 min followed by a 60 min chase in Swim's S-77 media containing 10 mM

sodium butyrate. Fig. 18B, lane T shows that during this 60-min chase period a portion of the metabolically active histone H4 population became hyperacetylated, with approximately 33.7% of the labeled histone H4 being tetraacetylated. In addition to histone H4, the acetylated species of histones H2A, H2A.Z, H3.2, H3.3, and H2B were also labeled (Fig. 18B, lane T). We observed that of the two histone H3 variants, H3.2 and H3.3, the latter variant preferentially participated in dynamic acetylation. Densitometric analysis of electrophoretic patterns of histones resolved on long AUT polyacrylamide gels (not shown) demonstrated that histone variant H3.3 was labeled to a specific activity 3.2-fold greater than that of histone H3.2, the major histone H3 variant in chicken erythrocytes.

Fig. 18 demonstrates that relative to total histones, the levels of the labeled acetylated histones were elevated in the 0.15 M NaCl-soluble chromatin fragments (fractions S₁₅₀, F_I, F_{II}, F_{III}, and F_{VI+V} which contained salt-soluble mononucleosomes) and fraction P_E. The 0.15 M NaCl-insoluble chromatin fragments (fraction P₁₅₀) had low amounts of the labeled histones, with mono- and diacetylated histone H4 forms being labeled. Labeled, tetraacetylated histone H4 was not detectable in this fraction. The highest concentration of labeled histones was present in the 0.15 M NaCl-soluble oligonucleosomes (F_{II} and F_{III}). Note that the content of labeled histones H2A.Z and uH2B in these fractions was significant. Labeled histone H2A.Z, but not uH2B, was detected in fraction P_E.

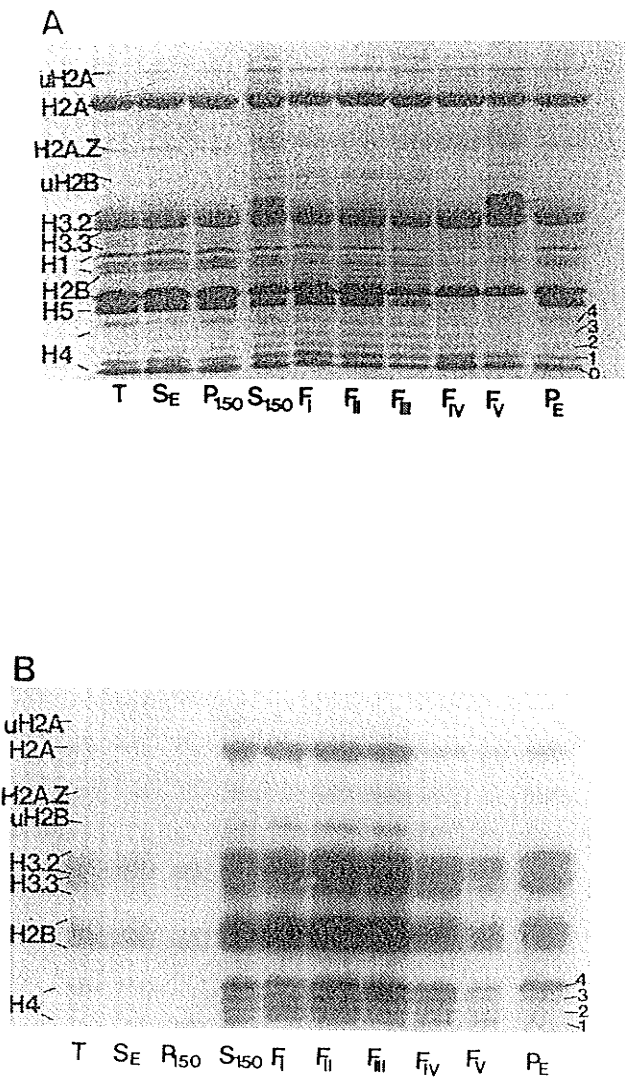


Figure 18. The chromatin distribution of [³H]-acetate labeled histones of chicken immature erythrocytes. Ten µg of acid-soluble protein from each chromatin fraction was electrophoretically resolved on 15% polyacrylamide AUT gels. (A) shows the Coomassie Blue-stained gel pattern and (B) shows the accompanying fluorogram. The acetylated species of histone H4 are denoted numerically 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively. u denotes the ubiquitinated histone species.

To ascertain the extent to which the histones in each chromatin fraction were participating in dynamic class 1 acetylation, we determined the percentage of the labeled histone H4 forms of each fraction that were tetraacetylated (Fig. 19). The small amount of H4 of fraction P₁₅₀ that was metabolically active was of the class 2 acetylation type as indicated by the label being localized in the mono- and diacetylated species (Fig. 19, P₁₅₀15). The histone H4 of the highly competent gene-enriched, 0.15 M NaCl-soluble polynucleosomes (F_I15) was a combination of the class 1 and class 2 acetylation types, with 28.4% of the labeled H4 being tetraacetylated. Histone H4 of the 0.15 M NaCl-soluble oligonucleosomes (F_{III}15) and of the chromatin fibers associated with the residual nuclear material (P_E15) were mainly of the dynamic, class 1 acetylation type, with 43.4 and 44.9% of the labeled H4 being tetraacetylated, respectively. Of the various chromatin fractions, the metabolically active acetylated H4 species of fraction P_E showed the strongest bias towards the dynamic, class 1 acetylation type.

To determine the chromatin distribution of the dynamically acetylated class 1 histones, the percentage of labeled, tetraacetylated histone H4 located in each chromatin fraction was ascertained. Table 1 demonstrates that the distribution of the labeled tetraacetylated H4 species (class 1 acetylation) paralleled that of active DNA among the chromatin fractions. A similar match in the partitioning of competent DNA was not as obvious. The chromatin fragments associated with the residual nuclear material (fraction P_E) contained the majority of the active DNA and labeled tetraacetylated histone H4 species. The remainder of the labeled

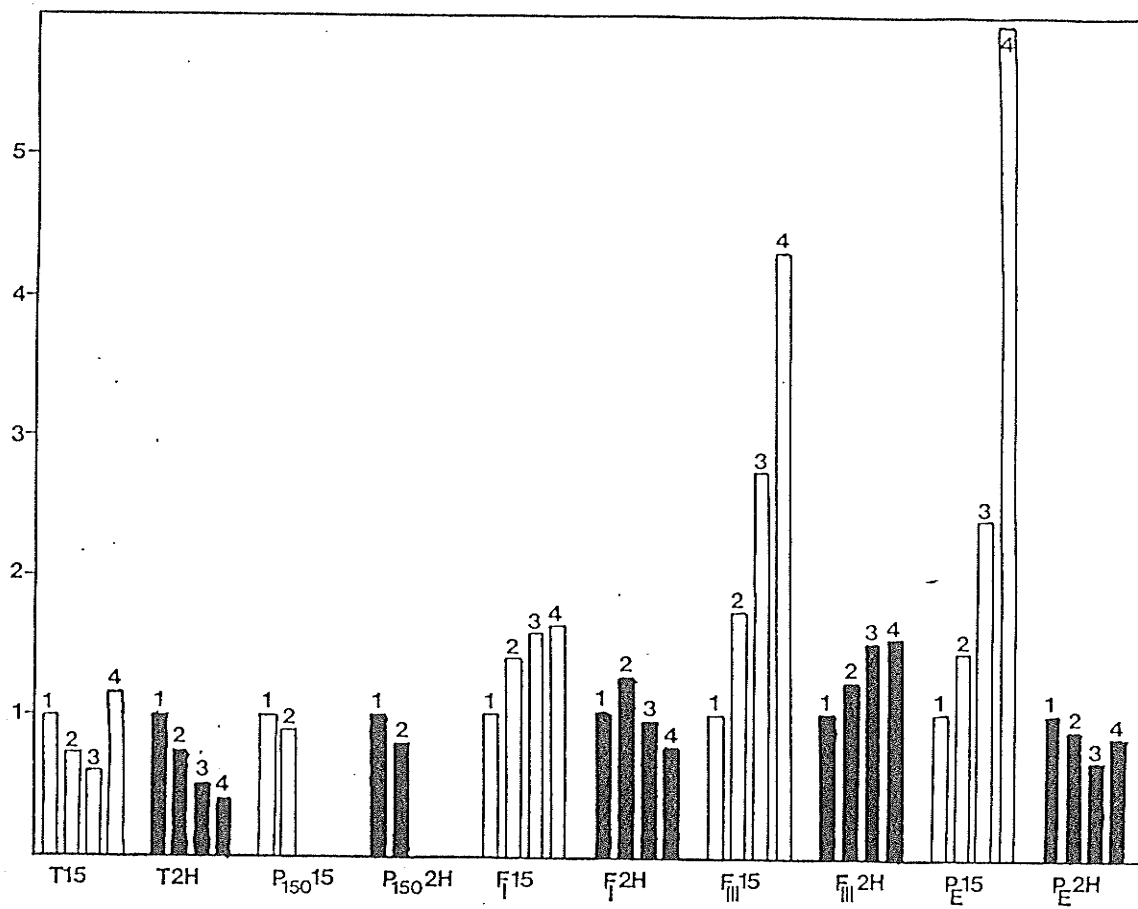


Figure 19. Chromatin distribution of metabolically active class 1 and class 2 acetylated histone H4 species. The chromatin fractions isolated from immature erythrocyte cells were analyzed by fluorography and densitometry following labeling with [³H]-acetate for either 15 min or 2 h, as indicated. The relative distribution of label amongst the mono- (1), di- (2), tri- (3), and tetra- (4) acetylated species of histone H4 was then plotted with the value for monoacetylated (1) histone H4 set arbitrarily at 1.0 for each chromatin fraction.

tetraacetylated histone H4 was located with the active/competent gene-enriched 0.15 M NaCl-soluble chromatin fragments (fraction S₁₅₀). Size-fractionation of these fragments demonstrated that the 0.15 M NaCl-soluble oligonucleosomes (F_{II} and F_{III}) had a greater proportion of the labeled tetraacetylated H4 than the 0.15 M NaCl-soluble polynucleosomes, illustrating the parallel partitioning of class 1 acetylated histones and active DNA. Similar results were obtained when we repeated these experiments with mature erythrocytes.

Metabolically active class 1 acetylated histone species in fraction P_E copurify with active gene chromatin.

The chromatin fragments associated with the residual nuclear material contained competent and repressed DNA as well as an enrichment in active DNA. Fig. 20A shows that the salt elution characteristics of bulk DNA and active DNA sequences from the P_E fraction differ (data kindly provided by Dr. G. Delcuve). Bulk chromatin was eluted more readily than the transcriptionally active histone H5 gene chromatin. We used this differential extractability of bulk versus active gene chromatin to obtain a greater level of enrichment of active gene chromatin. Fig. 20C shows that following the treatment of the fraction P_E with 0.6 M NaCl, the amount of labeled histones associated with the insoluble chromatin (fraction P_{0.6}) was elevated. A striking 51.1% of the labeled H4 of fraction P_{0.6} was tetraacetylated. Moreover, the percentage of total labeled tetraacetylated H4 (dynamic, class 1 acetylation type) in fraction P_{0.6} correlated with the enrichment in active gene chromatin fragments (Table 1).

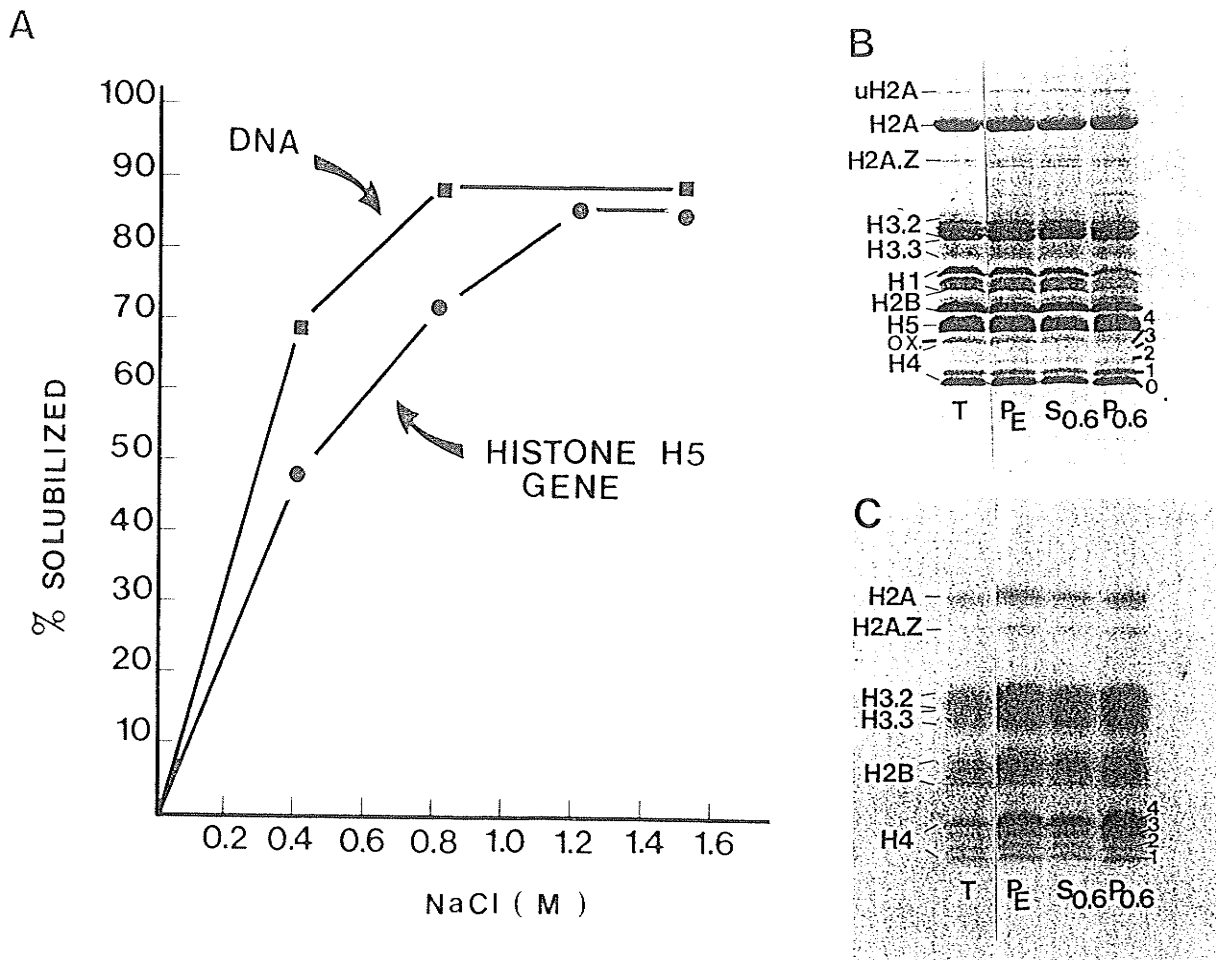


Figure 20. Dynamically acetylated histones copurify with the active gene sequences of the insoluble residual nuclear material. (A) Chromatin fraction P_E was extracted with NaCl at varying concentrations and the levels of bulk DNA and histone H5 DNA sequences solubilized from the P_E fraction were determined by diphenylamine assay and slot blot hybridization, respectively (salt fractionation, bulk DNA quantitation, and slot blot quantitation of histone H5 sequence content data kindly provided by Dr. G. Delcuve). (B) Chromatin fraction P_E was extracted with 0.6 M NaCl and insoluble ($P_{0.6}$) and soluble ($S_{0.6}$) fractions were obtained. Approximately 10 μ g of acid-soluble protein from the various chromatin fractions was electrophoretically resolved on 15% polyacrylamide AUT gels. (C) is the accompanying fluorogram for (B). 0, 1, 2, 3, and 4 represent the un-, mono-, di-, tri-, and tetraacetylated species of histone H4, respectively. ox represents an oxidized unacetylated species of histone H2B. U denotes the ubiquitinated histone species.

Chromatin distribution of class 2 acetylated histone species

In order to label the class 2 acetylated histones, chicken immature erythrocytes were pulse-labeled with [^3H]-acetate for 2 h followed by a 60 min chase in Swim's S-77 media containing 10 mM sodium butyrate. Incubation of cells in the absence of sodium butyrate resulted in the loss of the 0.15 M NaCl solubility of the active/competent gene polynucleosomes. Incubation of the cells with sodium butyrate was necessary to restore this solubility (Ridsdale *et al.*, 1990). The slow rate of deacetylation of the class 2 acetylated histones and the rapid deacetylation of the class 1 acetylated histones results in the preferential labeling of the class 2 acetylated histones during this 2 h labeling period. Fig. 19 shows that the predominating labeled H4 species of unfractionated chromatin (T2h) were the mono- and diacetylated forms. For all of the chromatin fractions except the 0.15 M NaCl-soluble oligonucleosomes (fraction F_{III}2h), the labeled mono- or diacetylated H4 species were at a higher level than the labeled tetraacetylated H4 (Fig. 19).

The content of the labeled histones of each chromatin fraction was similar to those shown in Fig. 18, with 0.15 M NaCl-soluble oligonucleosomes (fractions F_I and F_{III}) having the greatest concentration of labeled histones and the 0.15 M NaCl-insoluble chromatin fragments having the lowest levels (not shown). The chromatin distribution of the labeled mono- and diacetylated histones (class 2 acetylated histone forms) was different from that of class 1 acetylated (tetraacetylated H4) histone (Table 1). Although the 0.15 M NaCl-soluble poly- and oligonucleosomes (fractions F_I, F_{II} and F_{III}) were enriched in class 2 acetylated histones (5.6-, 7.5- and 4.6-fold, respectively), the 0.15 M NaCl-insoluble chromatin fragments contained a substantial amount of the labeled mono- and diacetylated

histone H4 species (Table 1). Further, these labeled histone forms were only slightly enriched (1.3-fold) in the chromatin fragments complexed to the insoluble nuclear material (fraction P_E). These observations suggest that class 2 acetylated histones were associated with repressed, competent and active gene chromatin fragments, with a preference towards competent gene chromatin fragments. Obviously, the chromatin distribution of the class 2 acetylated histones did not parallel that of the active DNA.

Table 1. Chromatin distribution of the tetraacetylated histone H4 species in chicken immature erythrocytes

Histones from the chromatin fractions isolated from cells pulse-labeled for 15 min or 2 h were electrophoresed on 15% AUT polyacrylamide gels and subjected to fluorography. The fluorogram was analyzed by densitometry and the relative proportion of labeled acetylated histone H4 to total labeled histone H4 was determined. The percentages of active and competent chromatin in the 0.15 M NaCl soluble fractions were determined from data presented in Delcuve and Davie (1989). The abbreviations used are: comp., competent; H4A4, labeled tetraacetylated histone H4; H4A1 + A2, mono- and diacetylated labeled histone H4; ND, not detectable.

Fraction	%DNA	%Competent	%Active	% H4A4 (15 min)	% H4A1 + A2 (2h)
S150	7.1	41.7	29.4	33.4	26.8
F _I	0.5	14.4	2.0	2.4	2.8
F _{II}	1.3	18.0	11.0	13.1	9.7
F _{III}	1.6	4.2	7.4	19.5	7.3
PE	21.0	30.5	76.3	73.5	26.5
S _{0.6}	16.3	23.8	44.2	39.3	14.2
P _{0.6}	4.7	6.7	32.1	35.6	8.7
P ₁₅₀	71.9	29.0	9.6	ND	52.8

Discussion

Chicken immature erythrocytes contain two categories of metabolically active acetylated histones. Both groups of histones are acetylated at the same rapid rate but they differ in both the extent of acetylation and the rate of deacetylation (Zhang and Nelson, 1988 a,b). We provide evidence that the dynamically, class 1 acetylated histones, which attain high acetylation levels and are rapidly deacetylated, are complexed principally to active DNA. Parallel chromatin distributions of the active DNA sequences and labeled tetraacetylated histone H4 species were observed, with the 0.15 M NaCl-soluble oligonucleosomes and the chromatin fragments bound to the insoluble nuclear material containing the majority of the active DNA and labeled tetraacetylated histone H4. The strong bias of the dynamically acetylated class 1 histones to active gene-enriched chromatin regions was not apparent with the class 2 acetylated histones. These metabolically active acetylated histones, which are acetylated to low levels and are slowly deacetylated, were located in repressed, competent and active chromatin regions. However, it is important to note that the class 2 acetylated histones were more abundant in active/competent gene-enriched chromatin fractions. The enrichment of the class 2 acetylated histones in the 0.15 M NaCl-soluble poly- and oligonucleosomes was 8.0-fold greater than the 0.15 M NaCl-insoluble, repressed gene chromatin fraction. This implies that the majority of the histones associated with the 0.15 M NaCl-insoluble chromatin fragments do not participate in metabolically active acetylation and they remain at the acetylation levels observed on Coomassie Blue-stained AUT gels.

The spectrum of labeled acetylated histones of the active gene-enriched chromatin fractions, the 0.15 M NaCl-soluble oligonucleosomes and the insoluble chromatin associated with the residual nuclear material, were similar but some differences were noted. Labeled histones H2A, H2A.Z, H3.2, H3.3, H2B, and H4 were present in both chromatin fractions. Labeled ubiquitinated H2B was found in the salt-soluble oligonucleosomes but was found at only low levels in two dimensional gels of the residual nuclear material. Although the 0.15 M NaCl-soluble polynucleosomes contained H2A.Z, it was labeled to a much lower specific activity than the H2A.Z of the 0.15 M NaCl-soluble oligonucleosomes. Labeled H2A.Z thus appears to be predominantly localized in the active but not the competent or the repressed chromatin. It is of interest to note that although the amount of histone H3.2 is considerably higher than that of histone H3.3, the level of labeled histone H3.3 was similar to that of H3.2. Waterborg (1990) reported a similar observation that the alfalfa minor histone H3 variant H3.2 was also preferentially acetylated. Alfalfa histone H3.2 and chicken erythrocyte histone H3.3 are replacement variants (see Wu *et al.*, 1986). The amino acid sequences of these H3 replacement variants are very similar to the replication-linked H3 species and these minor differences are far removed from the sites of acetylation. Waterborg (1990) suggested that alfalfa histone H3.2 was the preferred substrate of the histone acetyltransferase because it was part of the transcriptionally active gene chromatin. Consistent with this hypothesis, we have observed that histone H3.3 was modestly enriched in transcriptionally active gene-enriched chromatin fractions of chicken erythrocyte (Ridsdale and Davie, 1987). Furthermore, we have presented evidence that histones of transcriptionally active nucleosomes of the chicken immature erythrocyte chromatin

preferentially exchange with newly synthesized histone H3.3 (see part 3). This remodeling process, which may be dependent upon dynamic acetylation, would increase the number of active gene nucleosomes containing histone H3.3.

We propose that the class 1, dynamically acetylated histones are necessary to maintain a transcriptionally permissive nucleosome structure. Current evidence indicates that the high levels of acetylation are required to alter the structure of the nucleosome (Oliva *et al.*, 1990). The results of Norton *et al.* (1989; 1990) suggest that nucleosomes with highly acetylated histones H3 and H4 retain less negative supercoiled DNA than nucleosomes with unmodified histones. Rapid changes in the level of highly acetylated histones H3 and H4 could result in dynamic alterations in the shape of the histone octamer (Oliva *et al.*, 1990) and in the levels of restrained nucleosomal superhelical DNA. Thus, histone acetylation could alter the torsional stress in a chromosomal loop bearing a transcribed gene(s). Further, rapid modulations of the highly acetylated histones could conceivably modify the degree of condensation of the chromosomal loop by altering the capacity of the H1 histones to compact the chromatin fibre (Ridsdale *et al.*, 1990; Ausio, 1992). Thus, dynamics in acetylation imparts a mechanism to rapidly modulate the structure of the chromatin. Our observation that the majority (approximately 74 %) of the dynamically acetylated chromatin remains insoluble during nuclear fractionation suggests that these chromatin fragments are associated with the nuclear matrix and moreover that dynamic histone acetylation occurs at the nuclear matrix.

Part 2: Studies of Ongoing Histone Methylation in Chicken Immature Erythrocytes

Introduction

At the onset of these experiments, histone methylation was a largely underinvestigated aspect of chromatin structure and posttranslational modifications of histones. Histone N-lysine methylation occurs on histones H3 and H4 (Wu *et al*, 1986). Lysines 9 and 27 of histone H3 and lysine 20 of histone H4 are the primary sites of methylation. Each of these lysine residues may be unmethylated, monomethylated, dimethylated, or trimethylated. There is some controversy over whether these methyl groups turnover very slowly or do not turn over at all. However, an enzyme has been isolated from kidney cells which is capable of removing methyl groups from the lysine residues of these histones, suggesting at least the potential for turnover (reviewed in van Holde, 1989). Little is known of the chromatin distribution of methylated histones. There has been some indication of the association of histone methylation with transcription (Lee and Loh, 1977; Camato and Tanguay, 1982, Desrosiers and Tanguay, 1985; Desrosiers and Tanguay, 1988; Arrigo, 1983). This relationship is most clearly established in cells undergoing heat shock. Heat shock experiments demonstrate a relationship between a dramatic reduction of transcription in the cell and a decrease in methylation of histones H3 and H4 (Camato and Tanguay, 1982; Arrigo, 1983; Desrosiers and Tanguay, 1985). The studies described herein were the first to describe a nonrandom distribution of ongoing histone methylation amongst functionally distinct regions of chromatin. We demonstrate that newly methylated histones H3 and H4 are preferentially located in transcriptionally active gene-enriched

chromatin fractions and depleted in regions of chromatin which contain the bulk of the repressed gene chromatin. Our results have subsequently been duplicated by independent investigators (Reneker and Brotherton, 1991). There is, however, some controversy as to whether or not this applies to other cell systems. A novel and confirmed observation of our studies, however, is that dynamically acetylated histones are preferentially methylated.

We demonstrate that the acetylated species of histone H4 accumulate a disproportionate amount of label. Several investigators have suggested that acetylated histones are associated with transcriptionally active/competent gene chromatin (Ridsdale and Davie, 1987; Allegra *et al.*, 1987; Delcuve and Davie, 1989; part 1) and, using an antibody directed against tetraacetylated histone H4, Hebbes *et al.* (1988) demonstrated directly that highly acetylated histone species (i.e., tri- and tetraacetylated) are localized in active chromatin domains. These highly acetylated histones undergo rapid acetylation and deacetylation (i.e., the acetylation state of these histones is dynamic) (Zhang and Nelson, 1988; Covault and Chalkley, 1980). Approximately 1-2% of the chicken erythrocyte genome is involved in dynamic acetylation (Zhang and Nelson, 1986) and these dynamically acetylated species are localized in active chromatin regions (Zhang and Nelson, 1988; Ip *et al.*, 1988).

We have also investigated the acetylation dynamics of methylated histones using a procedure modified from Zhang and Nelson (1988a). We find that methylated histones are preferentially located in chromatin fragments whose solubility in 0.15 M NaCl is altered by incubation in the presence or absence of the histone deacetylase inhibitor, sodium butyrate. This is consistent with the partitioning of dynamically acetylated

active/competent gene chromatin (Ridsdale *et al.*, 1990). Further, we demonstrate that approximately 30-35% of the labeled methylated histone H4 population undergo a rapid rate of acetylation ($t_{1/2} = 8$ min). In comparison, 98-99% of the total histone H4 population are not acetylated during the course of incubation under these conditions (Zhang and Nelson, 1988a). This observation demonstrates that dynamically acetylated histones are preferentially methylated.

The observed association between histone acetylation and histone methylation indicates that there exists a coupling between histone acetylation and methylation. This hypothesis was tested by incubating chicken immature erythrocytes in the presence of adenosine dialdehyde, an inhibitor of protein methylation.

Results

Methylated species of histones H3 and H4 are located in chromatin regions enriched in transcriptionally active and competent genes

Immature chicken erythrocytes were incubated with L-[methyl- ^3H] methionine in the presence of cycloheximide, an inhibitor of protein synthesis. Under these conditions, the methylated species of histones H3 and H4 are labeled (Sung *et al.*, 1977). The labeled chromatin was subsequently fractionated by a low ionic strength procedure (see Fig. 17). The distribution of DNA among chromatin fractions S_E , S_{150} , P_{150} , and P_E was 83.2 ± 0.6 , 6.1 ± 0.5 , 77.0 ± 0.6 , and 16.8 ± 0.6 % ($n=4$), respectively.

The extent to which the histones of the various chromatin fractions were labeled is shown by the ratio cpm/mg DNA (Fig. 21). Since the mass

of the histones is about equal to that of DNA, this ratio will reflect the specific activities of the histones. Chromatin fractions S₁₅₀ and P_E were enriched in labeled acid-soluble proteins. Relative to fraction T (unfractionated chromatin), chromatin fractions S_E, S₁₅₀, P₁₅₀, and P_E were enriched 0.80-, 2.60-, 0.67-, and 1.78-fold, respectively, in methylated proteins. This is a representative result of seven separate fractionations. Relative to the content of labeled protein in chromatin fraction P₁₅₀, chromatin fractions S₁₅₀ and P_E were enriched 5.4 ± 1.2 ($n = 7$) and 3.4 ± 0.9 ($n = 4$)-fold, respectively, in labeled acid-soluble proteins.

The hyperacetylated species of histone H4 of chromatin fractions S₁₅₀ and P_E are preferentially methylated

The histones were electrophoretically resolved on AUT 15% polyacrylamide gels, and the location of the labeled proteins was determined by fluorography. Radiolabel was incorporated principally in histones H3 and H4 (Fig. 22), in agreement with the results of Sung *et al.* (1977). These methylated histones were enriched in chromatin fractions S₁₅₀ and P_E, with the histones in fraction S₁₅₀ being labeled to a greater extent. In fraction S₁₅₀ proteins, which migrated in the region of H2A.Z, were also labeled (x and y in Fig. 22). Two-dimensional gel electrophoretic (AUT into SDS) analysis of the histones in fraction S₁₅₀ demonstrated that histone H2A.Z was not labeled (not shown).

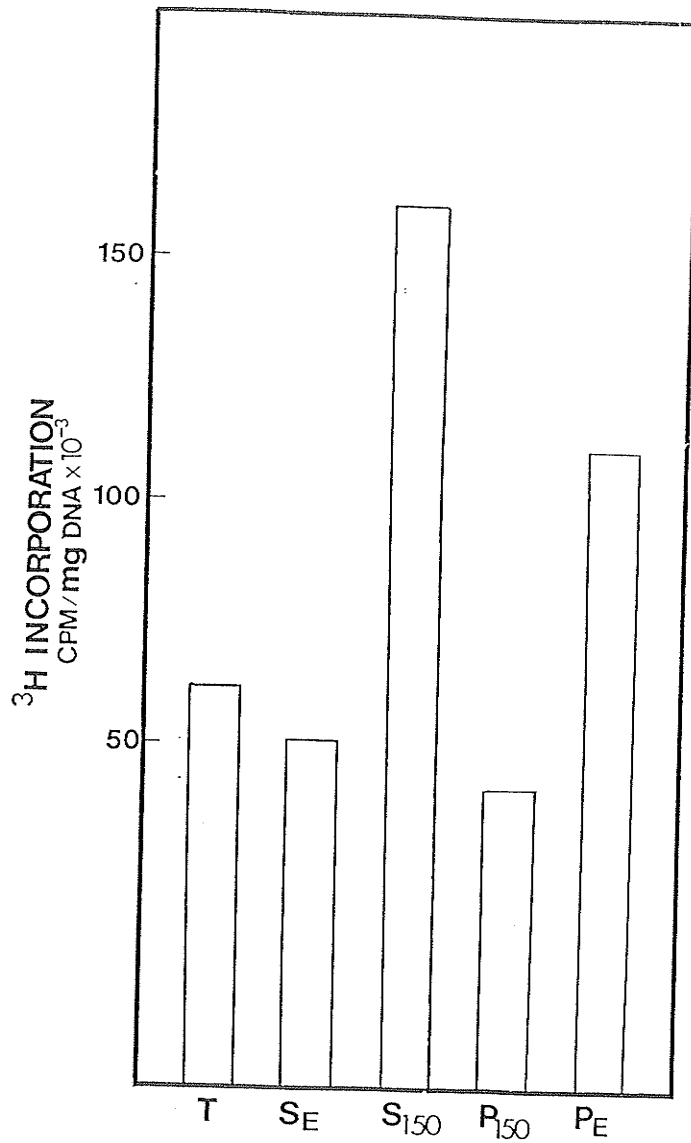


Figure 21. Distribution of methylated proteins among fractions of immature chicken erythrocyte chromatin. Immature chicken erythrocytes were incubated in the presence of cycloheximide and L-[methyl ³H]methionine as described under "Materials and Methods". The chromatin was fractionated and the acid-soluble proteins of each fraction were isolated. Radioactivity was determined by mixing 100 μ l of the protein solution with 5 ml of ACS (Amersham) scintillation fluid.

The acetylated species of histones H3 and H4 of the chromatin fractions S₁₅₀ and P_E were methylated, with histone H3 having a greater level of methylated species (Fig. 22). Fig. 23 shows that the hyperacetylated species (tri- and tetraacetylated) of histone H4 of fraction S₁₅₀ were highly enriched in label. Note also that the unacetylated histone H4 species, which represents the major H4 species of every chromatin fraction, is labeled to the lowest extent. The acetylated species of the histone variants H3.2 and H3.3 were also labeled (Figs. 22 and 23), with two forms of histone H3 being preferentially methylated (Fig. 23). One of these forms, which has the slower cathodal migration, is an acetylated form of histone H3.2 (possibly the diacetylated species), and the other is most likely a hyperacetylated species of histone H3.3. Two-dimensional gel electrophoresis (AUT into SDS) of the histones of fractions S₁₅₀ and P_E confirmed that the hyperacetylated species of histones H3 and H4 were labeled (not shown).

The 0.15 M NaCl-soluble oligonucleosomes are enriched in methylated histone species

Poly- and oligonucleosomes, which are soluble in 0.15 M NaCl, are highly enriched in transcriptionally active (e.g., β -globin and histone H5) and competent (e.g., ϵ -globin) genes, but these chromatin fractions are depleted in repressed genes (e.g., vitellogenin) (Ridsdale and Davie, 1987; Delcuve and Davie, 1989). Among the 0.15 M NaCl-soluble chromatin fragments,

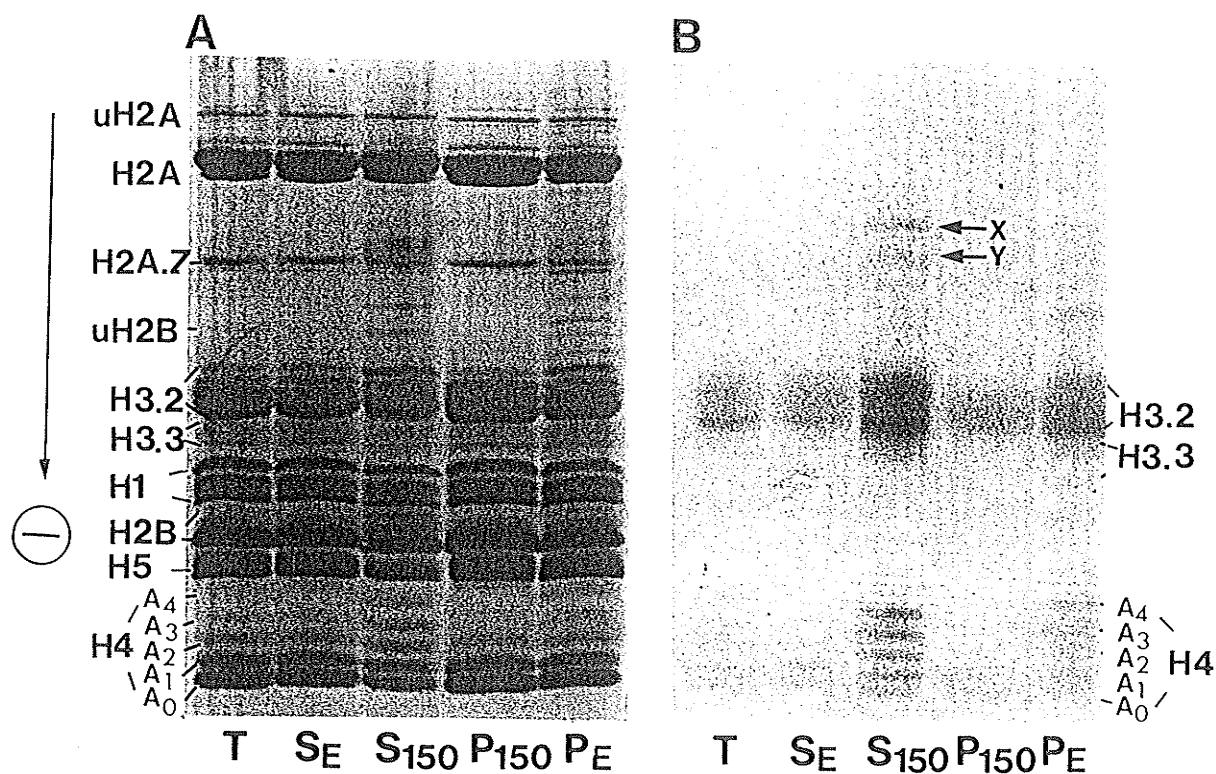


Figure 22. Distribution of the methylated species among the modified and variant forms of the histones H3 and H4. The acid-soluble proteins (100 μ g) of the chromatin fractions were electrophoretically resolved on an AUT-15% polyacrylamide gel. *Panels A and B* show the Coomassie Blue-stained gel pattern and the accompanying fluorogram, respectively. *U* denotes the ubiquitinated histone species. *A*₀, *A*₁, *A*₂, *A*₃, and *A*₄ are the un-, mono-, di-, tri-, and tetraacetylated species of histone H4, respectively. The *arrows* indicate the position of the labeled proteins of fraction S₁₅₀ that migrate in the location of histone H2A.Z.

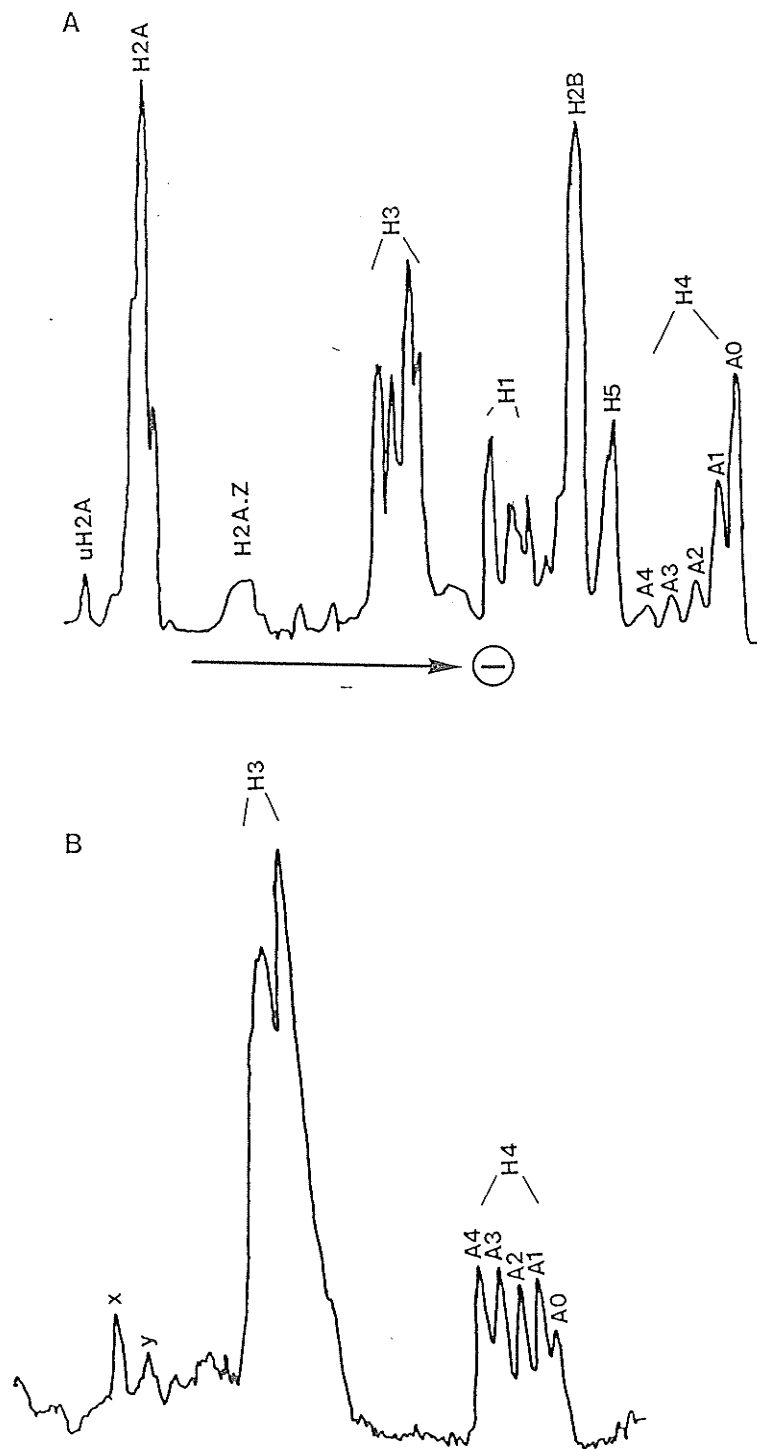


Figure 23. The acetylated species of histone H4 are preferentially methylated. Lane S₁₅₀ of the Coomassie Blue-stained gel pattern and of the fluorogram shown in Fig. 22 were scanned with a densitometer. U denotes the ubiquitinated histone species. A₀, A₁, A₂, A₃, and A₄ are the un-, mono-, di-, tri-, and tetraacetylated species of histone H4, respectively. x and y indicate the position of the labeled proteins that migrate in the region of H2A.Z.

active genes are enriched to the greatest extent in oligonucleosomes (Delcuve and Davie, 1989). The labeled 0.15 M NaCl-soluble chromatin fragments were fractionated by gel exclusion chromatography (Fig. 24), obtaining four fractions F_I, F_{II}, F_{III}, and F_{IV} of decreasing chromatin fiber lengths. The specific activity of the oligonucleosomes of fraction F_{II} (41×10^3 cpm/A₂₆₀) was greater than that of any other chromatin fraction (F_I polynucleosomes, 31×10^3 cpm/A₂₆₀; F_{III} oligonucleosomes, 27×10^3 cpm/A₂₆₀, F_{IV} mononucleosomes, 16×10^3 cpm/A₂₆₀). The histones of the salt-soluble chromatin fragments were electrophoretically resolved on an AUT-polyacrylamide gel (Fig. 25). As seen for the histones H3 and H4 of chromatin fraction S₁₅₀ (Figs. 22 and 23), the acetylated species of histones H3 and H4 of the salt-soluble oligo- and polynucleosomes were labeled (Fig. 25). The labeling of the tetraacetylated histone H4 species of fraction F_{II} was particularly striking. The oligonucleosomes of this fraction are highly enriched in active DNA sequences (Delcuve and Davie, 1989).

The histones of unfractionated chromatin and salt-soluble chromatin fractions (S₁₅₀, F_I, F_{II}, F_{III}, and F_{IV}) were electrophoretically resolved on SDS-polyacrylamide gels, and the specific activities of histones H3 and H4 were determined. Fig. 26 shows the relative levels to which the histones of the salt-soluble chromatin fractions were labeled when compared with the specific activities of the histones of unfractionated chromatin. The enrichment of labeled histones H3 and H4 (10.4 and 11.7, respectively) parallels the enrichment of active DNA sequences (10.3, Delcuve and Davie, 1989) in the salt-soluble oligonucleosomes of fraction F_{II}.

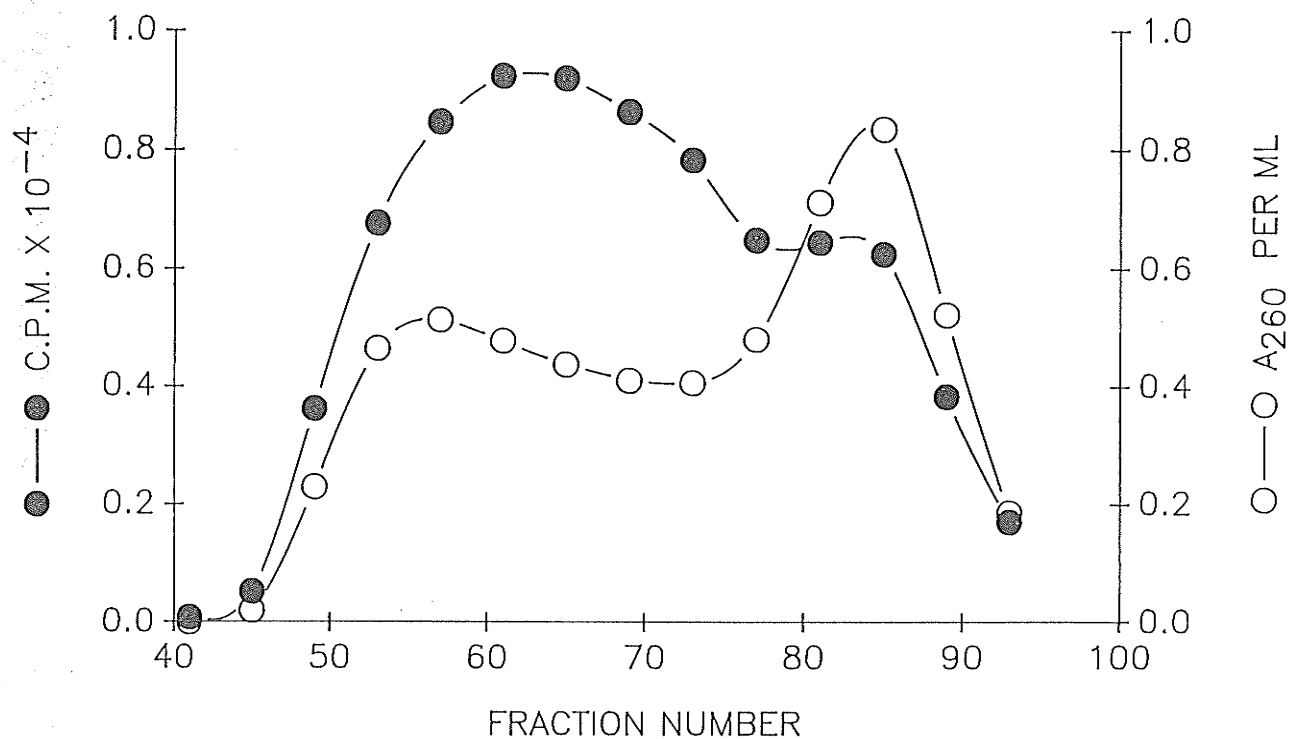


Figure 24. Distribution of the methylated histones among the poly-, oligo-, and mononucleosomes of fraction S₁₅₀. Chromatin fragments of fraction S₁₅₀ were fractionated on a Bio-Gel A-5m column (2.5 x 110 cm). Radioactivity of 250 μ l column fractions mixed with 5 ml of ACS was determined by scintillation counting. The radioactivity and absorbance in 0.5 and 1.0 ml, respectively, are shown. Fractions F_I (49-59), F_{II} (60-69), F_{III} (70-81), and F_{IV} (82-93), which were pooled, contain 0.15 M NaCl-soluble poly-, oligo-, oligo-, and mononucleosomes respectively.

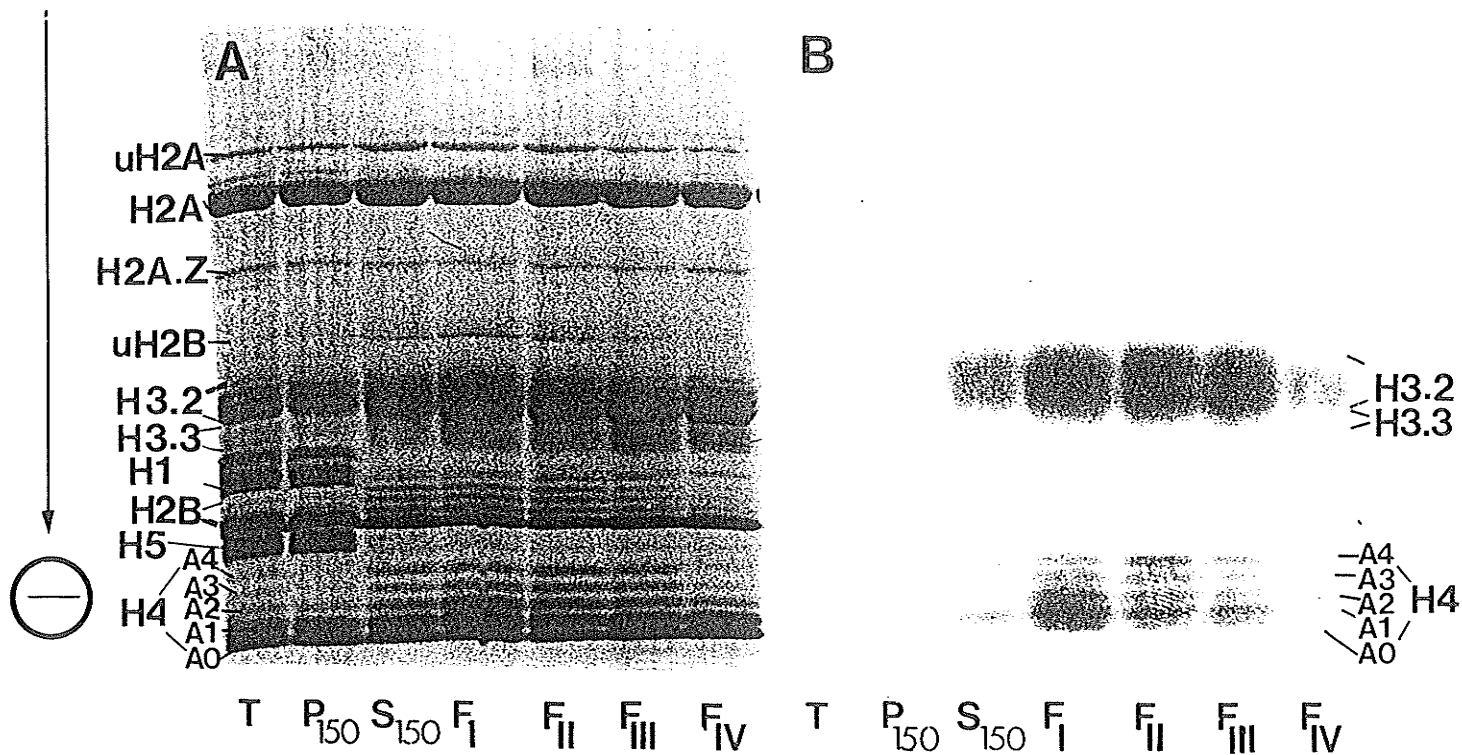


Figure 25. Distribution of the methylated species among the acetylated forms of histones H3 and H4 of the 0.15 M NaCl-soluble chromatin fragments. Chromatin fragments of fraction S₁₅₀ were size fractionated on a Bio-Gel A-5m column as shown in Fig. 17. Histones from fractions S₁₅₀, F_I, F_{II}, F_{III}, and F_{IV} were prepared by hydroxylapatite column chromatography, and histones from fractions T and P₁₅₀ were isolated by acid extraction as described under "Materials and Methods". The histones (approximately 10 μg) were resolved by AUT-15% polyacrylamide gel electrophoresis. Note that the amount of histones of fraction F_I is greater than that of the other histone samples. *Panel A* and *Panel B* show the Coomassie Blue-stained gel (*A*) and accompanying fluorogram (*B*), respectively. *U* denotes the ubiquitinated histone species. A₀, A₁, A₂, A₃, and A₄ are the un-, mono-, di-, tri-, and tetraacetylated species of histone H4, respectively.

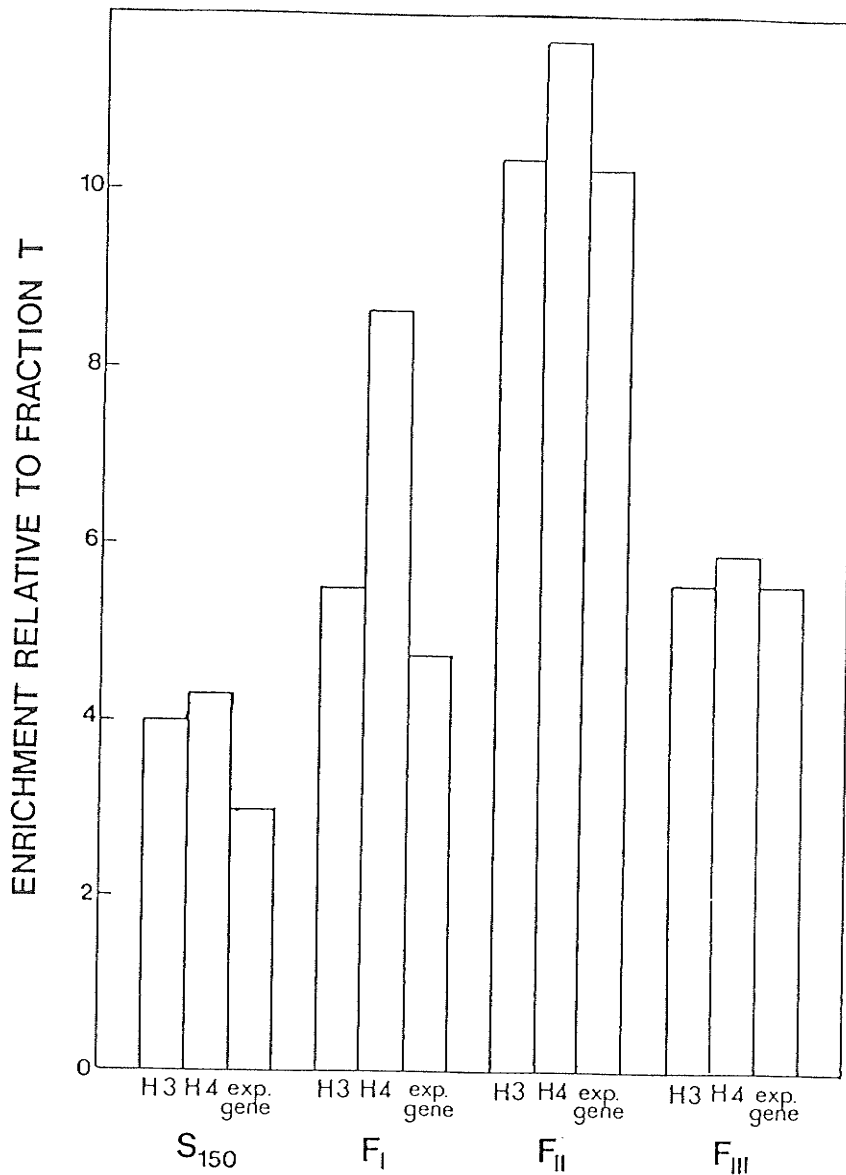


Figure 26. The salt-soluble oligonucleosomes, which are highly enriched in transcriptionally active genes, are highly enriched in methylated species of histones H3 and H4. Histones of unfractionated chromatin and chromatin fractions S₁₅₀, F_I, F_{II}, and F_{III}, were electrophoretically resolved on SDS-15% polyacrylamide gels, and a fluorogram was obtained. Specific activities and enrichments of labeled histone H3 and H4 of these chromatin fractions were determined as described under "Materials and Methods". Enrichment of the labeled histones (H3 or H4) of each chromatin fraction is shown relative to those of unfractionated chromatin. The enrichment of expressed DNA sequences (histone H5 and β -globin) is data from Delcuve and Davie, 1989.

Histone methylation is not dependent on on-going transcription

In order to establish whether histone methylation was dependent on on-going transcription, immature erythrocytes were incubated with actinomycin D and DRB, which are inhibitors of transcription, and cycloheximide prior to the addition of L-[methyl-³H] methionine. The extent of labeling of each chromatin fraction (67.1, 55.3, 170.1, 40.6, and 116.9 cpm/mg DNA for fraction T, S_E, S₁₅₀, P₁₅₀, and P_E) was not appreciably altered by the presence of these inhibitors (compare values with those of Fig. 21).

Among the cell population of anemic chicken blood, a small percentage of the cells is actively undergoing DNA replication and mitosis. To determine whether the arrest of DNA replication affected histone methylation, immature erythroid cells were incubated with aphidicolin, an inhibitor of DNA synthesis, and cycloheximide prior to the addition of radiolabel. This treatment as well as incubation of the cells with actinomycin D, DRB, aphidicolin, and cycloheximide did not alter the specific activity or partitioning of the labeled histones among the chromatin fractions (not shown).

The histone deacetylase inhibitor, sodium butyrate, does not affect histone methylation

Boffa *et al.* (1981) reported that histone methylation of cultured HeLa S3 cells was impaired by the presence of 5 mM sodium butyrate, a histone deacetylase inhibitor. In their study, cells were cultured for 15 h in the presence of sodium butyrate prior to labeling. These authors further demonstrated that incubation under these conditions inhibited cell division

(Boffa *et al.*, 1981). As a result, the reported inhibition of histone methylation by sodium butyrate may be a result of comparing cell populations in two different physiological states. In order to test whether sodium butyrate inhibits methylation in a nonreplicating immature chicken erythrocyte cell population, L-[methyl-³H] methionine was added and mixed into a cell suspension of immature chicken erythrocytes that had been preincubated with cycloheximide in order to inhibit protein synthesis. The cells were then split into two fractions of equal volume and sodium butyrate was added to a final concentration of 10 mM to one fraction. Each fraction was then further incubated for one hour. As can be seen by fluorography (Fig. 27B) sodium butyrate did not influence the incorporation of label into either of histones H3 or H4 during a one hour labeling period. However, significant differences in the distribution of label amongst the acetylated species of H3 and H4 were evident (Fig. 27C and D). Exposures of the film are essentially identical at 1 hr for butyrate treated versus control cells.

Rate of acetylation of methylated histone H4

We have used the procedure of Zhang and Nelson (1988a) to determine the rate of acetylation of methylated histone H4. Cells were labeled for 60 min in the presence of cycloheximide, in DMEM, resuspended in Swim's S-77 media (contains cold methionine) and further incubated for two hours (this prevents labeling of newly methylated species during the sodium butyrate treatment). Cells were then resuspended in Swim's S-77 media and incubated in the presence of sodium butyrate as indicated. Histones isolated from acid extracted nuclei were then analyzed on a 15% polyacrylamide AUT gel. It can be seen that no appreciable difference in the

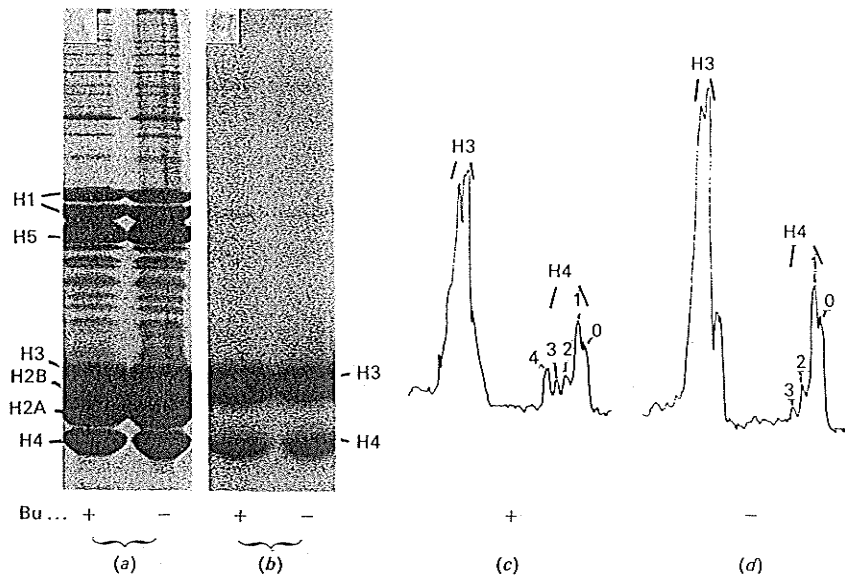


Figure 27. Sodium butyrate has no effect on histone methylation. The acid-soluble proteins (15 μ g) of nuclei isolated from immature chicken erythrocytes for one hour in the presence or absence of 10 mM sodium butyrate were resolved electrophoretically on an SDS 15% polyacrylamide gel. Panels A and B show the Coomassie Blue-stained gel pattern and the accompanying fluorogram, respectively.

amount of total acetylated histone H4 can be detected by Coomassie Blue staining (Fig 28A) indicating that the majority of histone H4 is not acetylated during sodium butyrate treatment. Note that oxidized H2B comigrates with the tetraacetylated species of histone H4 giving a misleading appearance to the quantity of tetraacetylated histone H4 detectable by Coomassie Blue stain. In contrast, fluorography demonstrates that a significant proportion of methylated histone H4 is rapidly acetylated (Fig. 28B) The specific activity of unacetylated and monoacetylated histone H4 can readily be seen to decrease during sodium butyrate treatment and a concomitant increase is seen in the specific activity of the higher acetylated species (particularly striking is that of tetraacetylated histone H4) (Fig. 28B). This clearly demonstrates that dynamically acetylated histone H4 species are preferentially undergoing methylation.

Rate analysis of acetylation of newly methylated histone H4

Figure 29 shows the change in the content of labeled methylated unacetylated histone H4 as a function of incubation time in the presence of 10 mM sodium butyrate. The rate of acetylation and the percentage of unacetylated methylated histone H4 that is acetylated during the course of incubation can be determined from this plot. It can be seen that the majority of acetylation of unacetylated labeled methylated histone H4 takes place within the first twenty minutes of butyrate incubation. From the slope of this line, it can be calculated that these histones are acetylated at a rate of $t_{1/2} = 8$ min. Zhang and Nelson (1988) reported that in immature chicken erythrocytes, monoacetylated histone H4 labeled with ^3H -acetate are acetylated at a rate with a half-life of 12 min. and that there is no slow

rate of acetylation in immature chicken erythrocytes. The calculated rate varied between experiments from a minimum of approximately 4 min. to a maximum of approximately 20 min. Thus, there is some imprecision inherent in this technique with respect to the actual rate of acetylation. What is more important, however, and can be accurately demonstrated using this technique is that there exist two biochemically distinct populations within the unacetylated species of histone H4. One which undergoes rapid acetylation and represents approximately 35% of the population of the labeled methylated unacetylated histone H4 (but a much smaller percentage of total unacetylated histone H4) and one which is essentially refractory to acetylation during the course of incubation in the presence of sodium butyrate. This second population represents only approximately 65% of the labeled methylated species but 98-99% of the total histone H4 population (Zhang and Nelson, 1986). Thus, labeled methylated histone H4 is highly enriched in this population of rapidly and dynamically acetylated histone H4.

The 0.15 M salt-solubility characteristics of chromatin fragments containing methylated histones following incubation in the presence or absence of sodium butyrate

During incubation in the presence or absence of sodium butyrate, histones become hyperacetylated and hypoacetylated, respectively. These changes in acetylation that occur during the course of cell incubation are restricted to a small percentage of the genome (Zhang and Nelson, 1988a) and cannot be observed by Coomassie Blue staining of total histones (Ridsdale *et al.*, 1990). Ridsdale *et al.* (1990) demonstrated that these

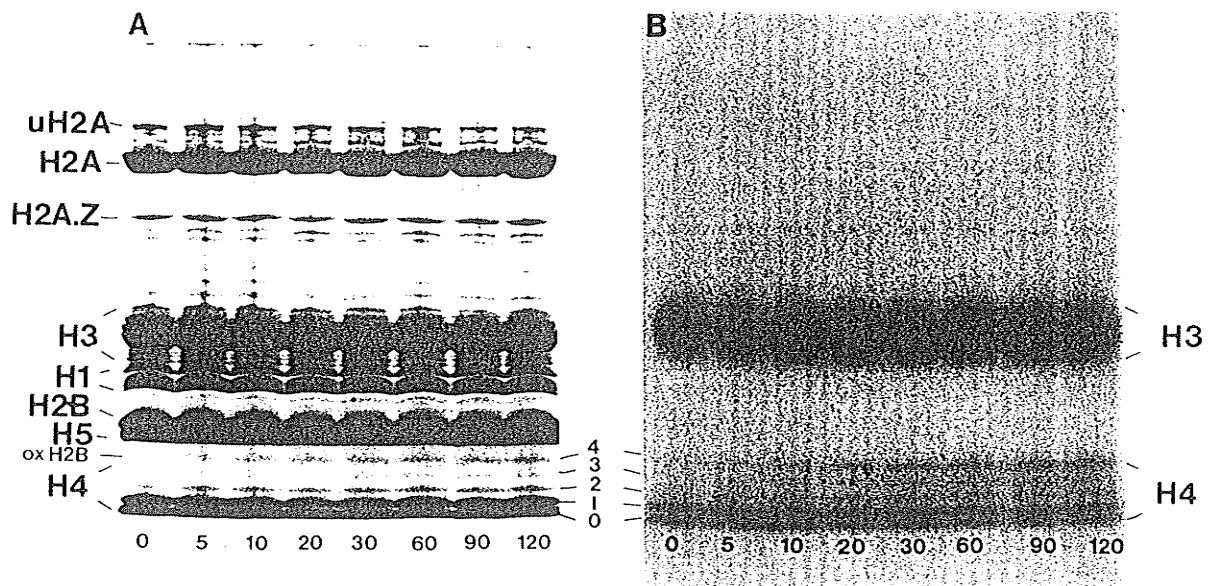


Figure 28. Dynamics of acetylation of newly methylated histone species. The acid-soluble proteins (2500 cpm) of nuclei isolated from immature chicken erythrocytes labeled in the absence of sodium butyrate and then treated with 10 mM sodium butyrate for the times indicated were electrophoretically resolved on AUT 15% polyacrylamide gels. Panels A and B represent the Coomassie Blue-stained gel and fluorogram, respectively. 0, 1, 2, 3, and 4 represent the un-, mono-, di-, tri-, and tetraacetylated species of histone H4. ox H2B represents an oxidized form of histone H2B.

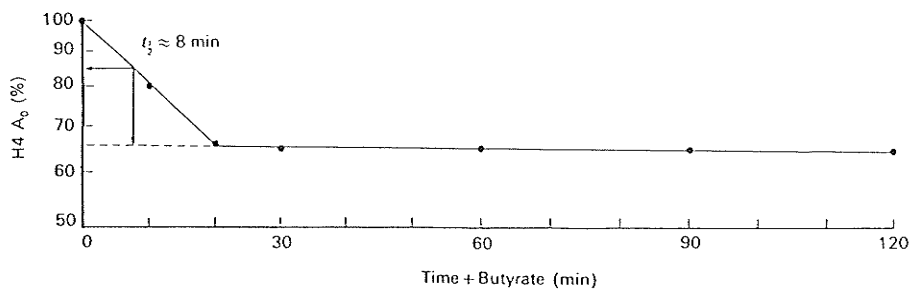


Figure 29. Kinetics of acetylation of methylated histone H4. Fluorograms prepared from cells incubated for increasing time periods in the presence of 10 mM sodium butyrate were scanned using a densitometer. The loss of label from the unacetylated band relative to total labeling of histone H4 was then plotted on semi-logarithmic paper versus time of incubation. The rate of acetylation was determined from this plot and the percentage of unacetylated species undergoing acetylation during incubation was determined similarly.

changes in histone acetylation were primarily occurring on transcriptionally active/competent, but not bulk, gene chromatin and that changes in histone acetylation altered the solubility of active/competent, but not bulk, chromatin in 0.15 M NaCl. For example, incubation of erythrocytes in the presence of sodium butyrate results in hyperacetylation of active/competent, but not bulk, chromatin. This, in turn, resulted in a greater proportion of active/competent, but not bulk, chromatin partitioning in the 0.15 M NaCl-soluble chromatin fraction. Thus, if newly methylated histones are primarily located in dynamically acetylated transcriptionally active/competent chromatin, then incubation in the presence or absence of sodium butyrate should result in changes in the amount of label partitioning with the 0.15 M NaCl-soluble chromatin fraction. The results of such an experiment are seen in Fig. 30. In agreement with the results of Ridsdale *et al.* (1990), we see no change in the level of histone acetylation observable by Coomassie Blue-staining of fractions T or P₁₅₀. However, striking differences can be seen in the levels of acetylated H4 for fraction S₁₅₀ isolated from cells incubated in the presence or absence of sodium butyrate (Fig. 30A). Additionally, it can be seen that fraction S₁₅₀ contains significantly more histone H5 when isolated from cells incubated in the presence of sodium butyrate compared to the minus butyrate incubated cells (Fig. 30A). This is a marker for the loss of poly- and oligonucleosome-sized material in fraction S₁₅₀ as a result of histone deacetylation. This fraction contains mostly This fraction contains mostly mononucleosome-sized material which is not associated with histone H5. The histone H5 is associated with the dynamically acetylated poly- and oligonucleosomes of transcriptionally active/competent gene chromatin and these fragments partition differently between the soluble (S₁₅₀) and insoluble (P₁₅₀)

fractions in the presence and absence of sodium butyrate. Fig. 30B shows the fluorogram of this gel. As can be seen, although there is no significant difference in the specific activity of the histones isolated from total nuclei, higher levels of acetylation in fraction S₁₅₀ correlate with the increased amount of labeled di-, tri-, and tetraacetylated histone H4 and a striking increase in the specific activity of histones H3 and H4 in fraction S₁₅₀. Note that decreased partitioning of active gene chromatin and methylated histones in fraction S₁₅₀ results in a concomitant increase of each in fraction P₁₅₀ (Ridsdale *et al.*, 1990; Fig. 30B). Table 2 shows the enrichment of methylated histones H3 and H4 in fraction S₁₅₀ following incubation in the presence or absence of sodium butyrate. Time 0 represents the enrichment of methylated histones amongst the chromatin fragments soluble in 0.15 M NaCl following labeling in the absence of sodium butyrate (a total of 90 min). Times 60+ and 60- represent a further 60 min incubation in the presence or absence of sodium butyrate. Incubation in the presence of sodium butyrate results in enhanced 0.15 M NaCl-solubility of chromatin fragments containing methylated histones. After 60 min incubation in the presence of sodium butyrate, methylated histones H3 and H4 are enriched 3.5- and 5.1-fold, respectively. In contrast, after a further 60 min incubation in the absence of sodium butyrate 1.6- and 1.1-fold enrichments of histones H3 and H4, respectively, indicates that chromatin fragments containing methylated histones no longer selectively partition in the 0.15 M NaCl-soluble chromatin fractions. This correlates with the loss of polynucleosome-sized material from this fraction, as a result of histone deacetylation, leaving primarily mononucleosome-sized fragments in this fraction (Ridsdale *et al.* 1990).

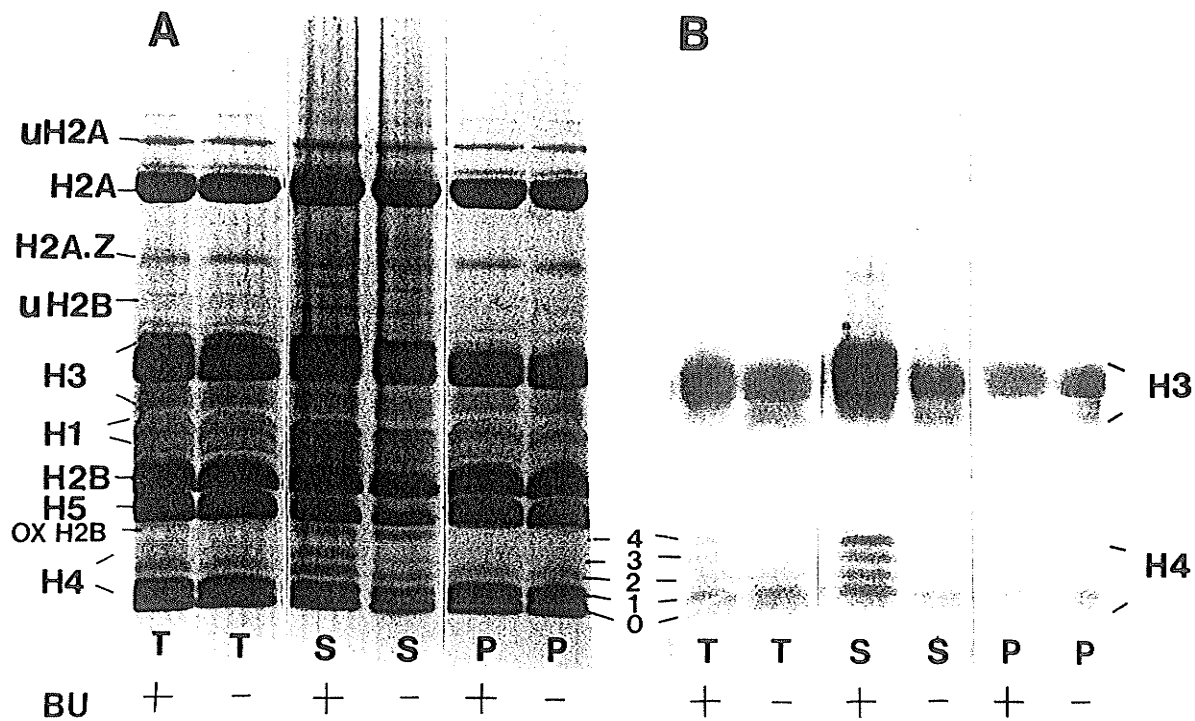


Figure 30. Methylated histones are associated with dynamically acetylated histones. The acid-soluble proteins (50 μ g) of total nuclei (T), EDTA-soluble 0.15 M NaCl-soluble chromatin fraction S₁₅₀ (S) and insoluble chromatin fraction P₁₅₀ (P) fractions were electrophoretically resolved on an AUT-15% polyacrylamide gel. Panels A and B show the Coomassie Blue-stained gel and the fluorogram, respectively. 0, 1, 2, 3, and 4 represent the un-, mono-, di-, tri-, and tetraacetylated species of histone H4, respectively. ox H2B represents the migration of an oxidized species of histone H2B (note that this comigrates with the tetraacetylated species of histone H4). Bu represents the presence (+) or absence (-) of 10 mM sodium butyrate in the incubation media for 60 min.

Table 2. The effect of incubating immature red blood cells with sodium butyrate on the 0.15 M-NaCl solubility of chromatin fragments with newly methylated histone H4. The content of newly methylated histone H4 (Me H4) in low salt-soluble chromatin fragments isolated from immature chicken erythrocytes incubated in the presence (+ Bu) or absence (- Bu) of sodium butyrate was determined. The percentage of total DNA in the 0.15 M-NaCl was determined by absorbance at 260 nm, and the percentage of total labeled methylated histone H4 associated with the low salt-soluble chromatin fragments was determined as described in the methods section. Each value represents the mean \pm S.E.M. from seven measurements.

Fraction	DNA (%)	Me H4 (%)
S ₁₅₀ (+ Bu)	9.8 \pm 0.4	48.3 \pm 3.6
S ₁₅₀ (-Bu)	7.0 \pm 1.6	8.8 \pm 3.3
Ratio (+/-)	1.4	5.5

Adenosine dialdehyde is an inhibitor of histone methylation

To study the effects of adenosine dialdehyde on histone methylation, chicken immature erythrocytes that were pre-incubated with cycloheximide were incubated for 60 min with L-methyl [³H] methionine and 10 mM sodium butyrate in the presence or absence of adenosine dialdehyde, an inhibitor of protein methylation (Najbauer and Aswad, 1990). Fig. 31 shows that adenosine dialdehyde effectively inhibited the incorporation of methyl label into histones H3 and H4.

Inhibition of histone methylation with adenosine dialdehyde does not affect dynamic histone acetylation

To study the effect of adenosine dialdehyde on dynamic histone acetylation, chicken immature erythrocytes were pulse-labeled with [³H] acetate for 15 min followed by a 60 min chase in Swim's medium containing sodium butyrate with or without adenosine dialdehyde. Fig. 31B, lane T, shows that during the chase period in the presence or absence of adenosine dialdehyde a portion of the metabolically active histone H4 population became hyperacetylated, with approximately 31.1% and 29.2% of the labeled histone H4 of adenosine dialdehyde-treated and untreated cells, respectively, being tetraacetylated. We have shown previously that the histone H3 variants H3.2 and H3.3 were actively acetylated and methylated, with histone H3.3 being acetylated and methylated to a greater level than histone H3.2, the major histone H3 variant in chicken erythrocytes. Fig. 32B, lane T, shows that adenosine dialdehyde treatment did not affect the preferential acetate labeling of histone H3.3.

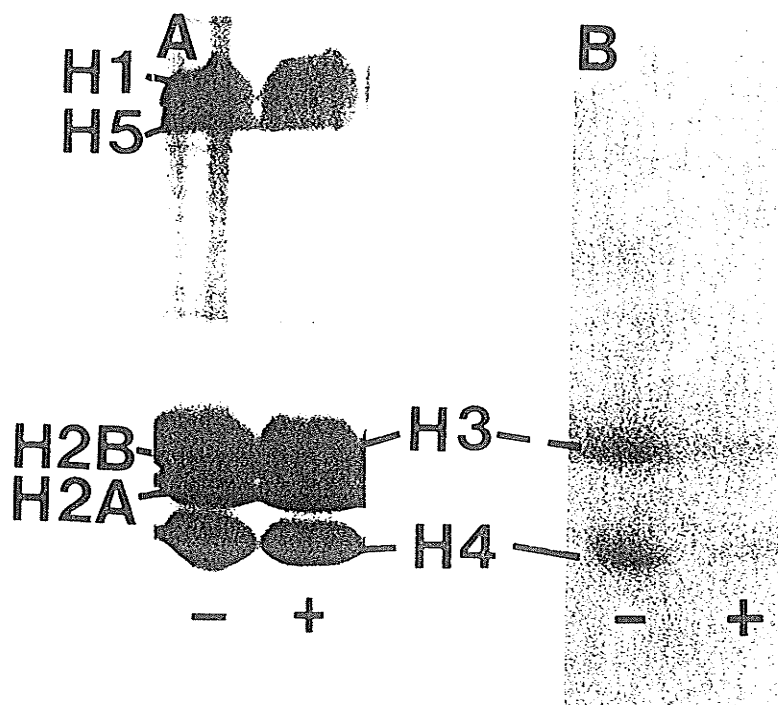


Figure 31. Adenosine dialdehyde is a potent inhibitor of histone methylation. Acid soluble proteins (10 μ g) of nuclei isolated from chicken immature erythrocytes that were labeled with L-[3 H]-methionine following preincubation in the presence or absence of 10 μ M adenosine dialdehyde, were electrophoretically resolved on a SDS/15% polyacrylamide gel. (a) and (b) show the Coomassie Blue-stained pattern and the accompanying fluorogram, respectively.

Chicken immature erythrocyte chromatin was fractionated by a procedure that separates chromatin fragments differing in their content of transcriptionally active and repressed DNA sequences. Transcriptionally active DNA sequences were enriched in the 0.15 M NaCl-soluble chromatin fragments (fraction S₁₅₀) and chromatin fragments associated with the residual nuclear material (fraction P_E) (Delcuve and Davie, 1989). Fig. 32B shows that the extent of the labeling of the histones was the highest for the histones of chromatin fractions S₁₅₀ and P_E. Treating the erythrocytes with adenosine dialdehyde before and during the pulse labeling and chase did not affect the partitioning of the labeled histones among the various chromatin fractions (Fig. 32B). Furthermore, the monoacetylated histone H4 species of fraction P₁₅₀ and the tetraacetylated H4 species of chromatin fractions S₁₅₀ and P_E were the major labeled histone H4 forms of adenosine dialdehyde-treated and untreated cells. For treated and untreated cells, approximately 34.7 and 34.1%, respectively, of the labeled H4 of fraction S₁₅₀ was tetraacetylated, while approximately 39.4 and 41.6%, respectively, of the labeled H4 of fraction P_E was tetraacetylated.

Histone methyltransferase activity is preferentially located in chromatin fractions S₁₅₀ and P_E

The partitioning of histone methyltransferase activity among the chromatin fractions was investigated. Micrococcal nuclease digested nuclei (T), and the chromatin fractions S_E, S₁₅₀, P₁₅₀, and P_E were incubated with S-adenosyl-L-[methyl-³H] methionine for 90 min. The histones were acid extracted, and the radioactivity of the trichloroacetic acid-insoluble

protein was determined. It should be noted that exogenous histones were not added to this assay. Thus, the substrates for the histone methyltransferase are primarily histones in the form of intact nucleosomes. Under these conditions histone H3 was the main acceptor of the methyl groups (not shown). Thus, these assay conditions monitored the activity of the histone H3 methyltransferase (Branno *et al.*, 1983). Fig. 33 shows the amount of labeling (cpm/mg DNA) of the histones in each of the chromatin fractions. Relative to T, chromatin fractions S_E, S₁₅₀, P₁₅₀, and P_E were enriched 0.52-, 2.65-, 0.28-, and 1.36-fold, respectively, in histone H3 methyltransferase activity. This is a representative result of five separate experiments. The histone H3 methyltransferase activity of chromatin fractions S₁₅₀ and P_E was 7.4 ± 2.5 (n=5) and 4.6 ± 0.3 (n=3) -fold greater, respectively, than the enzyme activity of chromatin fraction P₁₅₀ which contains the bulk of repressed DNA. Thus, the active gene-enriched chromatin fractions S₁₅₀ and P_E were enriched in histone H3 methyltransferase activity.

The 0.15 M NaCl-soluble chromatin fragments from a 10- and 20-min digest were fractionated on a Bio-Gel A-5m column (Fig. 34). With increased chromatin fragmentation, there was an increase in the proportion of mononucleosomes to polynucleosomes. Column fractions, which contained the polynucleosomes (F_I), oligonucleosomes (F_{II}), or mononucleosomes (F_{III}), were pooled and assayed for histone methyltransferase activity (Table 3). For the 10-min digest, histone methyltransferase activity was located in each of the fractions, with the polynucleosomes (F_I) having the greatest enzyme activity. Increasing fragmentation of chromatin (20-min digest) resulted in a redistribution of the enzyme activity among the salt-soluble chromatin fragments, with most of

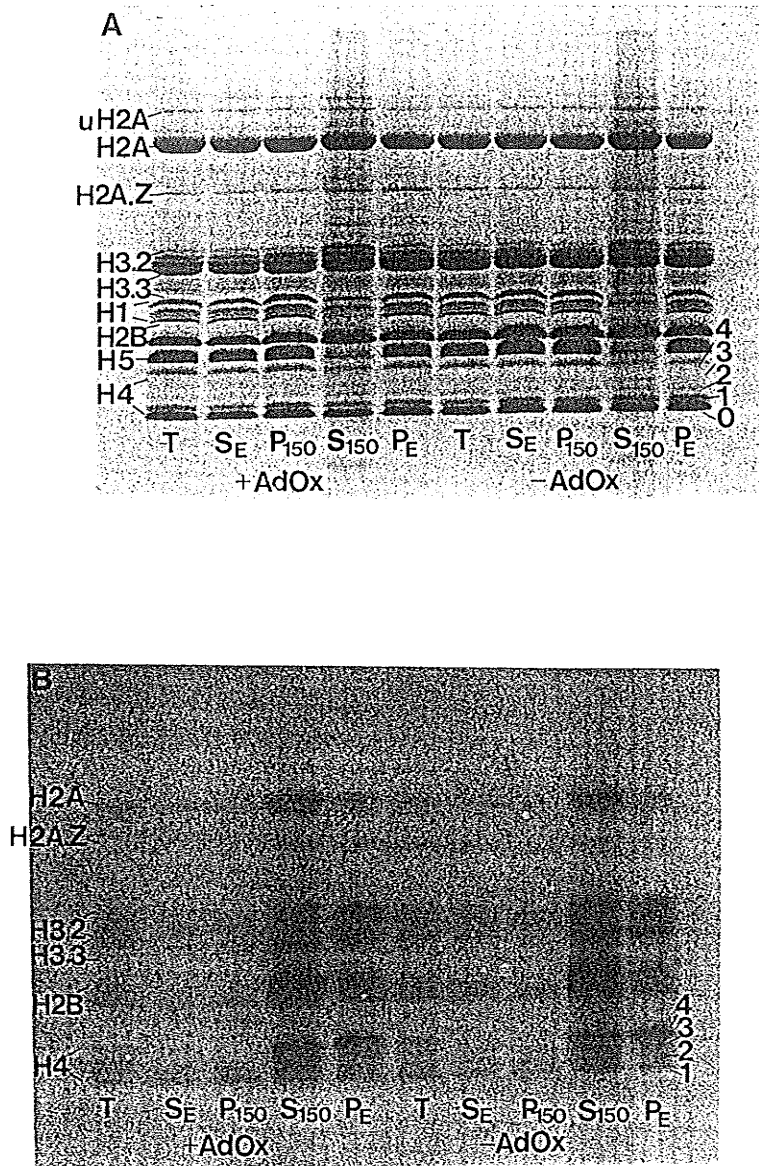


Figure 32. Adenosine dialdehyde does not affect histone acetylation. Acid soluble proteins (10 μ g) of chromatin fractions isolated from chicken immature erythrocytes that were labeled with [3 H]-acetic acid following preincubation in the presence or absence of 10 μ M adenosine dialdehyde, were electrophoretically resolved on an acetic acid-urea-Triton X-100/15% polyacrylamide gel. (a) and (b) show the Coomassie Blue-stained pattern and the accompanying fluorogram, respectively.

the enzyme activity being associated with the mononucleosome fraction (F_{III}) (Table 3). It should be noted that the average enzyme activity of the salt-soluble chromatin fragments (806 and 846 cpm/mg DNA) was similar for both the 10- and 20-min digest. Digestion of the chromatin fragments for longer times (30 min) led to a further increase in the enzyme activity associated with the mononucleosomes, with approximately 98% of the activity of fraction S₁₅₀ being localized in these chromatin fragments. This suggests that histone methyltransferases are complexed to nucleosomes.

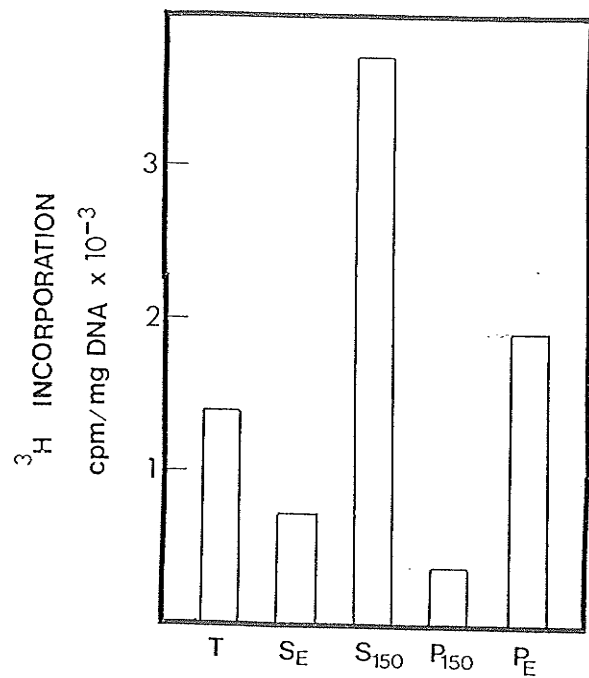


Figure 33. Distribution of histone methyltransferase activity among the chromatin fractions. Immature chicken erythrocytes were incubated for 10 min with micrococcal nuclease as described under "Materials and Methods". Equivalent A_{260} units (20 A_{260} units in 2 ml) of the digested nuclei and chromatin fractions were incubated with S-adenosyl-L-[methyl- ^3H]methionine (0.5 $\mu\text{Ci/ml}$) for 90 min at 37°C . The histones were isolated by acid extraction and the radioactivity of the trichloroacetic acid precipitated histones was determined.

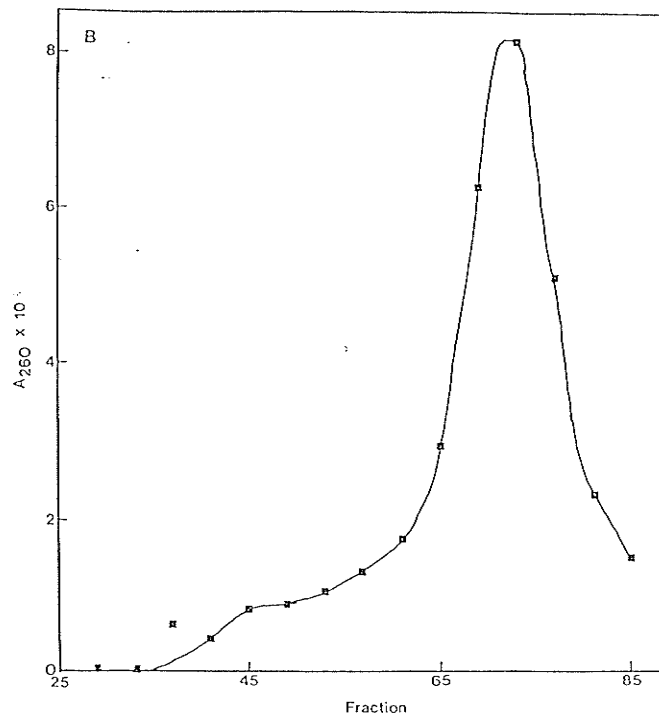
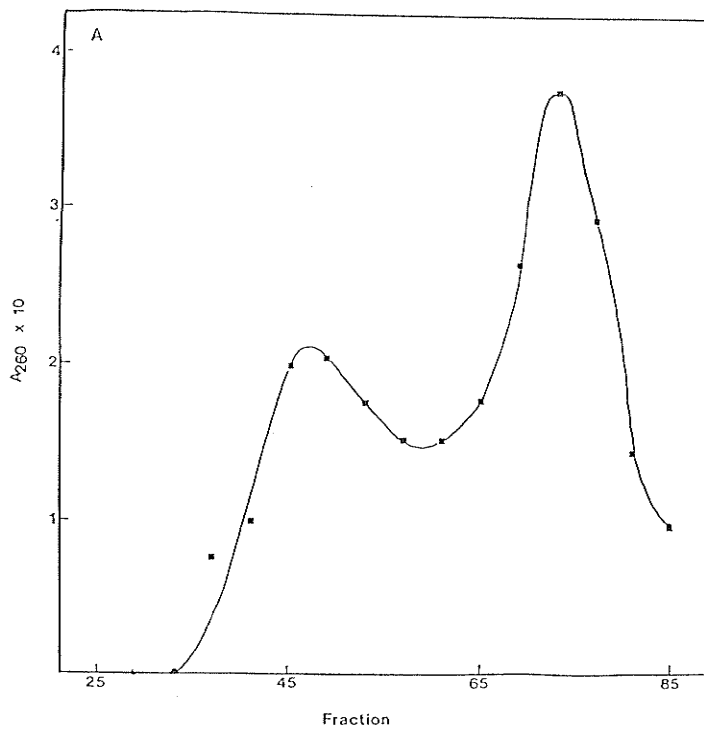


Figure 34. Gel exclusion chromatography of chromatin fraction S_{150} . Chromatin fragments of the S_{150} fraction obtained from immature chicken erythrocyte nuclei incubated for 10 and 20 min with micrococcal nuclease on a Bio-Gel A-5m column (2.5 x 110 cm). Column fractions 33-49, 50-65, and 66-81, which contain polynucleosomes (F_I), oligonucleosomes (F_{II}), and mononucleosomes (F_{III}), respectively, were pooled. For the 10-min digest (A), fractions F_I , F_{II} , and F_{III} contained 29.1, 22.4, and 48.5% of the A_{260} -absorbing material, respectively. F_I , F_{II} , and F_{III} of the 20-min digest (B) had 9.6, 14.1, and 76.3% of the A_{260} -absorbing material, respectively.

Table 3. Distribution of histone H3 methyltransferase activity. Salt-soluble chromatin fragments were fractionated by gel exclusion chromatography (see Fig. 31), and these fractions corresponding to polynucleosomes (F_I), oligonucleosomes (F_{II}), and mononucleosomes (F_{III}) were obtained. Equivalent A₂₆₀ units (8 units in 8 ml) of the column fractions were incubated with S-adenosyl-L-[methyl-³H]methionine (0.25 μCi/ml) for 90 min at 37°C. The histones were isolated, and the radioactivity of the trichloroacetic acid precipitated histones was determined.

Fraction	³ H incorporation <i>cpm/mg DNA</i>	% of activity in S ₁₅₀
10 min digest		
F _I	1012	36.5
F _{II}	838	23.3
F _{III}	669	40.2
20 min digest		
F _I	130	1.5
F _{II}	260	4.3
F _{III}	1044	94.2

Discussion

The data presented herein provides evidence that histone methylation occurs preferentially in transcriptionally active chromatin domains. This was demonstrated by showing parallel enrichments of methylated histones H3 and H4 and transcriptionally active gene sequences using a previously described chromatin fractionation procedure (Delcuve and Davie, 1989) and by the observation that acetylated species of histones H3 and H4 (particularly histone H4) were preferentially methylated.

In contrast to the results of Boffa *et al.* (1981), we demonstrate that the histone deacetylase inhibitor, sodium butyrate, does not inhibit histone methylation. We suggest that the inconsistency between our results and the results of Boffa *et al.* (1981) are due to the use of nonreplicating cells in this study. Sodium butyrate has been shown to inhibit cellular proliferation (Boffa *et al.*, 1981). Thus, in comparing ongoing methylation between HeLa S3 cells in the presence or in the absence of sodium butyrate, two physiologically different cell populations are being compared. One which is replicating and one which is not. The inhibition of methylation by sodium butyrate in their study, then, may simply be a reflection of this difference in the physiological state of the two cell populations. Additionally, these investigators treated their cells with sodium butyrate for 15h and this may effect other aspects of cell physiology as well. In our study, immature chicken erythrocytes isolated from the peripheral blood of anemic birds were used to analyze the effects of sodium butyrate on histone methylation. These cells are no longer proliferating (Williams, 1972) and, as a result, butyrate treatment has no effect on this aspect of cellular physiology in our

cell system. We find that, under these conditions, sodium butyrate does not effect on ongoing histone methylation.

Ridsdale *et al.* (1990) demonstrated active/competent polynucleosomes but not bulk chromatin have different solubility properties in 0.15 M NaCl following incubation of cells in the presence or absence of sodium butyrate. This difference is dependent upon the level of acetylated histones in these chromatin fragments and it was demonstrated that high levels of acetylation altered the capacity of linker histones (histones H1 and H5) to condense these chromatin fragments. We demonstrate in this study that newly methylated histones are located on chromatin fragments whose solubility in 0.15 M NaCl is altered by incubation in the presence or absence of sodium butyrate. Specifically, cells incubated in the presence of sodium butyrate demonstrated a high enrichment in labeled methylated histones H3 and H4 partitioning in the 0.15 M NaCl-soluble fraction but cells incubated in the absence of sodium butyrate demonstrated no significant enrichment in labeled methylated histones H3 and H4 partitioning in the 0.15 M NaCl-soluble fraction. Thus, chromatin fragments containing newly methylated histones behave in a manner consistent with active/competent gene chromatin. It should be noted that histone hyperacetylation induced by sodium butyrate treatment, which is responsible for increased solubility in 0.15 M NaCl, is confined to dynamically acetylated histones that represent only a small proportion of the total genome (Ridsdale *et al.*, 1990; Zhang and Nelson, 1988a).

We demonstrate further in this study that methylated histone H4 is significantly enriched in this rare class of rapidly acetylated histone H4. During the course of incubation in the presence of the histone deacetylase inhibitor, sodium butyrate, the vast majority of histone H4 species are not

acetylated. This is readily illustrated by Coomassie Blue-staining of total acid-soluble proteins where the majority of the histone H4 species remain in the nonacetylated and monoacetylated forms throughout butyrate treatment. In contrast, the methylated histone species redistribute into hyperacetylated forms such that after 60 min of butyrate incubation only 52% of the methylated histones are located in the nonacetylated and monoacetylated species. This compares to 85% located in the nonacetylated and monoacetylated histone H4 species at the onset of butyrate treatment. These methylated histones are acetylated with a $t_{1/2}$ of 8 min. Only 3.7% of the modifiable lysine residues in immature chicken erythrocytes participate in this dynamic acetylation and this involves only 1-2% of the total genome (Zhang and Nelson, 1986; Zhang and Nelson, 1988). Zhang and Nelson (1988) demonstrated that this dynamic acetylation occurs principally within active/competent gene chromatin domains. This provides additional evidence for the association of newly methylated histone species with transcriptionally active/competent chromatin and, further to this, provides an explanation for the striking accumulation of newly methylated species amongst the acetylated species of histone H4.

A question that was important to address as a consequence of our early studies was what is the mechanism responsible for the observed coupling of histone acetylation and histone methylation. Possible explanations for this observation include (a) histone acetylation acts as a signal for the histone methyltransferase, (b) histone methylation triggers histone acetylation, (c) histone acetyltransferases, deacetylases and methyltransferases are co-localized in specific chromatin regions, and (d) selective methylation of dynamically acetylated histones reflects the nuclear organization of transcriptionally active chromatin. We have shown that

unacetylated histones H3 and H4, which did not participate in dynamic acetylation and were associated with transcriptionally active/competent chromatin, were methylated. This observation shows that acetylation of a histone is not a necessary tag for the histone to be methylated. Furthermore, changing the steady state acetylation level of the dynamically acetylated histones affected the 0.15 M NaCl solubility of transcriptionally active chromatin and, presumably, its compaction (Ridsdale *et al.*, 1991). However, the selective methylation of the dynamically acetylated histones was not altered, suggesting that altering the level of compaction of transcriptionally active gene chromatin did not change the accessibility of these histones to the histone methyltransferase. The results presented here show that inhibition of methylation with adenosine dialdehyde does not affect dynamic acetylation of histones H3.2, H3.3 and H4 in chicken immature erythrocytes, demonstrating that histone methylation is not an essential tag for acetylation. Thus, our observations present evidence that acetylation does not favorably predispose histones H3 and H4 for methylation, and vice versa histone methylation does not prime these histones for acetylation. In chromatin fractionation experiments, we found that histone methyltransferase and deacetylase activities did not partition identically among the chromatin fractions. Histone methyltransferase, but not deacetylase, activity was enriched in the 0.15 M NaCl-soluble, transcriptionally active gene-enriched chromatin, while histone deacetylase and, to a much lesser degree, methyltransferase activities were enriched in the low salt insoluble, transcriptionally active gene-enriched chromatin fraction (fraction P_E) which also contained the residual nuclear material (Delcuve and Davie, 1989; see also part 1). Greater than 75% of the total histone deacetylase activity was located in this fraction (see part 4). We

conclude that the two processes, ongoing histone methylation and dynamic histone acetylation, are coupled due to the organization of transcriptionally active gene chromatin within the chicken immature erythroid nucleus. We have performed studies which demonstrate that histone acetyltransferase and deacetylase were components of the internal nuclear matrix (see part 4). We postulate that histone acetyltransferase and deacetylase are involved in the localization of transcriptionally active gene chromatin to specific nuclear compartments by transiently interacting with histones that are complexed with transcriptionally active DNA. The association of histone methyltransferase with transcriptionally active gene nucleosomes would result in the observed linkage between dynamic acetylation and ongoing methylation.

The physiological significance of histone methylation remains to be elucidated. It is important to note that the site of methylation in histone H4 (Lys-20) occurs within a stretch of amino acids that contain at least two sites of posttranslational modifications and at a site where histone-DNA contacts mediate a sharp bend or kink in the path of the DNA within the nucleosome (Ebraldse *et al.*, 1988). Using site-directed mutagenesis, Megee *et al.* (1990) demonstrated that, of the four reversibly acetylated lysines of the N-terminus of histone H4, mutation of Lys-16 altered histone H4 function the most. Additionally, it was found that His-18 was more critical for histone H4 function in yeast than Lys-16 (Megee *et al.*, 1990). These observations suggest strongly that a stretch of the N-terminus of histone H4, which encompasses at least amino acids 16-18 and possibly up to and including Lys-20, may be a site essential for modulation of histone H4 function by altering histone-DNA contacts. Posttranslational modification of this sequence by reversible acetylation and methylation may be the

mechanism whereby function is modulated. The association that we have demonstrated between these two types of posttranslational modification suggests that they may act synergistically.

Since the interpretation of the results of our original study have recently been challenged (Reneker and Brotherton, 1991), I feel it is important to address some of the concerns of these authors with respect to these studies as a whole. Reneker and Brotherton (1991) performed similar experiments to ours in both chicken immature erythrocyte cells and K562 cells (a human erythroleukemia cell line). These authors reproduced our data using immature chicken erythrocytes and found that newly methylated histones were enriched over 3-fold in fraction S₁₅₀ relative to fraction S_E. They, however found that in immature chicken erythrocytes relative to fraction T (total nuclei), fraction P_E was not enriched in methylated histone species (using the same cpm/mg DNA) quantitation as we used in our original study. Because these authors do not describe how they quantitated DNA content (we used a diphenylamine assay rather than the more typical quantitation by absorbance at 260 nm), it is difficult to assess the data with certainty. However, the large proportion of DNA reported by these investigators to be present in fraction P_E (43%) suggests that these investigators used absorbance at 260 nm to quantitate DNA content. We have found that estimation of DNA content by absorbance at 260 nm consistently greatly overestimated the amount of DNA content in fraction P_E as determined by using a nucleic acid specific assay, the diphenylamine assay (Delcuve and Davie, unpublished observations). Under the preparation conditions that we use, and which were used in the study of Reneker and Brotherton (1990), we consistently obtain residual nuclear pellets following EDTA lysis that retain less than 20 % of the total nuclear DNA (not shown,

Delcuve and Davie, unpublished observations). If we assume that approximately 20 % of the total DNA is present in this fraction (see Table 1), then requantitation of their data would result in an approximately 3-fold enrichment of newly methylated histones in fraction P_E. This is similar to the 3.4 ± 0.9 fold enrichment which we originally reported in chicken immature erythroid cells.

Reneker and Brotherton (1991) used the same chromatin fractionation procedure for K562 cells to demonstrate that histone methylation is not associated with transcribed chromatin. They observed that newly methylated histones were enriched in the 0.15 M NaCl soluble chromatin fragments but that active genes were depleted relative to repressed genes in this fraction. The utility of this fractionation procedure is based on the high linker histone density in chicken erythrocyte and perhaps due to the presence of the erythroid specific linker histone variant H5 in the chicken erythrocyte system (Ridsdale *et al.*, 1988). The preferential solubility of active and competent sequences under these conditions is directly dependent upon high acetylation levels associated with these regions of chromatin (Ridsdale *et al.*, 1990). The deployment of this fractionation procedure in tissue-culture cells would not be expected to lead to similar resolution of transcribed and nontranscribed chromatin. Their observation that newly methylated histones were enriched in this chromatin fraction suggests that either newly methylated histones are located in regions of chromatin which are selectively cleaved by micrococcal nuclease to mononucleosome-sized particles or that they occur on polynucleosomes which resist precipitation at physiological salt concentrations (due to lower linker histone densities in these cells, we would expect a greater proportion of the chromatin to be soluble as indeed these investigators found-33%).

Additionally, the chromatin which did precipitate in these cells (6% of the total A₂₆₀ absorbing material) was depleted in newly methylated histones. This suggests that the chromatin which contains higher densities of linker histones and low levels of acetylation in these cells (i.e., repressed chromatin) is not undergoing methylation. Because, in most cell types, the chromatin fraction containing the greatest proportion of transcribed genes is that which remains insoluble following digestion (in this case fraction P_E) (Gross and Garrard, 1987), it is the insoluble fraction which is of greatest interest. Again, the authors use cpm/mg DNA quantitation and given the high percentage of DNA reported to be insoluble (62%), it is likely that DNA content was quantitated using absorbance at 260 nm. If we assume that the actual DNA content is about one-third of that reported, then requantitation of their data would lead to an approximately 1.5-fold enrichment of newly methylated histones in fraction P_E. This would be consistent with the 1.5-fold enrichment in active gene sequences reported by these investigators in this fraction. Thus, if their data is reinterpreted it can be considered to be consistent with our data.

Reneker and Brotherton (1991) used two other criteria to dispute our reported association of actively transcribed chromatin with ongoing histone methylation. First, they used HMG 17, which binds preferentially to actively transcribed nucleosomes (Brotherton and Ginder, 1986), to alter the mobility of active gene mononucleosomes in native gels. Under these conditions, the HMG-bound nucleosomes were found not to be enriched in newly methylated histones but were enriched in actively transcribed genes. However, under these conditions, no enrichment in core histone acetylation is seen in the HMG-bound nucleosomes. This histone modification is clearly associated with transcribed chromatin (van Holde, 1989; Csordas, 1990;

Ausio, 1992) and as such it is apparent that this technique may recognize specific features of a subset of actively transcribed nucleosomes that are not within regions of increased acetylation. The results using this technique must therefore be interpreted with caution. Secondly, the authors used a crosslinking protocol developed by Ip *et al.* (1988) which separates actively transcribed genes that are associated with RNA polymerases on the basis of density from bulk chromatin. Ip *et al.* (1988) used this procedure to demonstrate that dynamically acetylated histones are associated with transcribing chromatin. Reneker and Brotherton (1991) found that the high density polymerase-associated actively transcribed sequences were modestly enriched in newly methylated histone H3.1 (approximately 2-fold versus a 2.5- to 5-fold enrichment in transcribed sequences) but not newly methylated histone H3.2. There was no obvious enrichment in highly acetylated histones in these fractions upon inspection of their AUT gel as was seen in the studies of Ip *et al.* (1988) but these results are more difficult to resolve with our results. Importantly, Reneker and Brotherton (1991) did report a similar striking association between acetylated histones and the incorporation of labeled methyl groups. Although these authors claim that this reflected an association between histone methylation and bulk acetylation (in most cells, inhibition of histone deacetylase with sodium butyrate results in a general increase in acetylhistone isoforms), the disproportionate accumulation of label in the most highly acetylated species suggests that the dynamically acetylated histones, which are associated with transcribed sequences (Boffa *et al.*, 1991; Ridsdale *et al.*, 1990; Ip *et al.*, 1988; see also Part 1), are preferentially methylated in this system. The association of methylation with acetylated isoforms of H3 and H4 suggests that our results with the erythrocyte system, in this respect, may be of a

more global nature. In conclusion, although the association between histone methylation and transcribed chromatin must be considered unresolved (at least outside the chicken erythrocyte system), the association between histone methylation and histone acetylation appears to be a more common phenomenon that was originally demonstrated in our early experiments (see Reneker and Brotherton, 1991). The results imply that these two modifications may act synergistically in the relaxation of histone-DNA contacts and this association is worthy of a more detailed analysis (e.g., determining the methylation state by amino acid analysis and the relationship of the methylated isoforms-mono-, di-, and trimethyllysine with the acetylation state).

Part 3: Exchange of Newly Synthesized Histones into Functionally Distinct Regions of Chicken Erythrocyte Chromatin

Introduction

It is of considerable interest to biochemically characterize transcribed gene chromatin. This is due in part to the need to reconcile the presence of a structure (the nucleosome), whose chief biological function is to package and compact DNA, with the requirement for accessibility to the template during the transcriptional elongation process. It is implied, by nature of its function, that its structure must be altered and that biochemical modifications may be important to allow these structural alterations. Recent studies on nucleosome dynamics demonstrate that at least under some conditions and contrary to common belief, the nucleosome is a dynamic rather than a stable structure (reviewed in Hansen and Ausio, 1991). Biochemical analysis of transcriptionally active/competent gene-enriched chromatin fractions has demonstrated that the compositional features of active/competent gene chromatin differ from those of bulk (Gross and Garrard, 1987). For example, the active/competent gene-enriched, low salt-soluble polynucleosome fraction of chicken immature erythrocytes contains only 2% of the total nuclear DNA but approximately 15% of the total active sequences and approximately 40% of the total competent sequences. In contrast, only 0.3% of the total repressed gene sequences are present in this fraction (Delcuve and Davie, 1989). The active/competent gene-enriched polynucleosomes are enriched in highly acetylated species of histones H2B and H4 (Ridsdale and Davie, 1987; Delcuve and Davie, 1989), ubiquitinated (u) and polyubiquitinated species of H2A and more strikingly

uH2B (Nickel *et al.*, 1989; Delcuve and Davie, 1989), the histones H3 and H4 which are actively undergoing methylation (see Part 2), histone acetyltransferase (Chan *et al.*, 1988) and histone deacetylase activity (see Fig. 43) The analysis of active/competent gene-enriched chromatin fractions isolated from a variety of sources has provided evidence that active/competent DNA is complexed with highly acetylated histones and ubiquitinated histone H2B (Ip *et al.*, 1988; Lin *et al.*, 1989; Nickel *et al.*, 1989). Notable among these studies is the direct demonstration that highly acetylated histones are associated with active, but not repressed, DNA of chicken erythroid cells (Hebbes *et al.*, 1988). These highly acetylated histones are also characterized by their dynamics (Ip *et al.*, 1988; Boffa *et al.*, 1990). A tetraacetylated species of histone H4 has an *in vivo* half-life of 5 min in chicken erythrocyte (Zhang and Nelson, 1988b).

The majority of histone synthesis and incorporation into chromatin is tightly coupled to DNA synthesis during S-phase of the cell cycle (Wu *et al.*, 1986). However, several investigators have demonstrated that a basal amount of histone synthesis occurs in both G1 and G0 phases of the cell cycle (Wu *et al.*, 1983a; Waithe *et al.*, 1983). Non-S-phase histone synthesis differs qualitatively from S-phase synthesis. The proportion of the histone H2A variants H2A.X and H2A.Z (G1 only) and the histone H3 variant H3.3 (G0 and G1) synthesized is increased relative to the S-phase synthesis pattern (Sariban *et al.*, 1985). Although all four core histones are synthesized to a similar extent in the absence of DNA replication, newly synthesized histones H2A and H2B are preferentially incorporated into chromatin by exchanging with nucleosomal histones. The newly synthesized histones H3 and H4 partition to a greater extent with a soluble histone pool that is not tightly associated with chromatin (Wu *et al.*, 1983b;

Louters and Chalkley, 1985; Bonner *et al.*, 1988; Jackson, 1990). Once incorporated into chromatin, the newly synthesized histones are stable for at least several days in G₀-phase cells (Wu *et al.*, 1982). The presence of a process of exchange which can remodel the histone composition of the chromatin over time implies that nucleosomes (or at least a subpopulation of nucleosomes) demonstrate some dynamic behavior with respect to their composition *in vivo*. An obvious question to ask is whether this exchange of histones into and out of nucleosomes was a general phenomenon occurring throughout the genome or whether it was a consequence of or associated with transcription.

Louters and Chalkley (1985) postulated that newly synthesized histones were preferentially exchanging with transcriptionally active chromatin regions. To test this hypothesis, we determined the distribution of newly synthesized histones among chromatin fractions isolated from chicken immature erythrocytes. This cell population consists almost entirely of non-replicating, terminally differentiated erythrocytes arrested in G₀ phase of the cell cycle (Williams, 1972). We demonstrate that newly synthesized histone H5, a histone H1 variant, is deposited randomly onto chromatin. In contrast, newly synthesized histones H2A, H2A.Z, H2B, H3.3, and H4 co-fractionate with transcriptionally active and competent DNA. Moreover, newly synthesized histones H2B and H4 are hyperacetylated and newly synthesized histones H2A and H2B are ubiquitinated, consistent with these histones being localized in transcriptionally active/competent chromatin domains. These observations provide support for the hypothesis that newly synthesized histones preferentially exchange with nucleosomal histones of transcriptionally active/competent chromatin. The results suggest that active/competent

regions of chromatin may be remodeled by exchanging pre-existing nucleosomal histones with a newly synthesized histone pool whose composition varies from the S-phase pattern.

Results

Distribution of newly synthesized nucleosomal histones in chicken erythrocyte chromatin

Chicken immature erythrocytes were incubated with L-[4,5-³H] lysine for 60 min to label the newly synthesized histones. The labeled chromatin was fractionated by a low ionic strength procedure. Four fractions were obtained (see Fig. 17); P_E (EDTA-insoluble chromatin), S_E (EDTA-soluble chromatin), S₁₅₀ (EDTA-soluble, 0.15 M-NaCl-soluble chromatin), and P₁₅₀ (EDTA-soluble, 0.15 M-NaCl-insoluble chromatin), containing approximately 10, 90, 12, and 78%, respectively, of the total nuclear DNA. .

Fig. 35 (lane T) shows that newly synthesized histone H5 was the principal histone synthesized, in agreement with the results of Affolter *et al.* (1987) (see also Fig. 36, panels A and B). Labeled histone H5 was located in all of the chromatin fractions. In contrast to histone H5, the content of labeled nucleosomal histones (e.g., H2A) in the various chromatin fractions was not equivalent, with the nucleosomal histones of chromatin fractions S₁₅₀ and P_E being labeled to the greatest extent.

Fig. 36 shows the labeled proteins of fractions S₁₅₀ and T resolved by two-dimensional gel electrophoresis. In fraction S₁₅₀, histones H1, H5, H2A, H2A.Z, H3, H2B, and H4 and ubiquitinated species of histones H2A

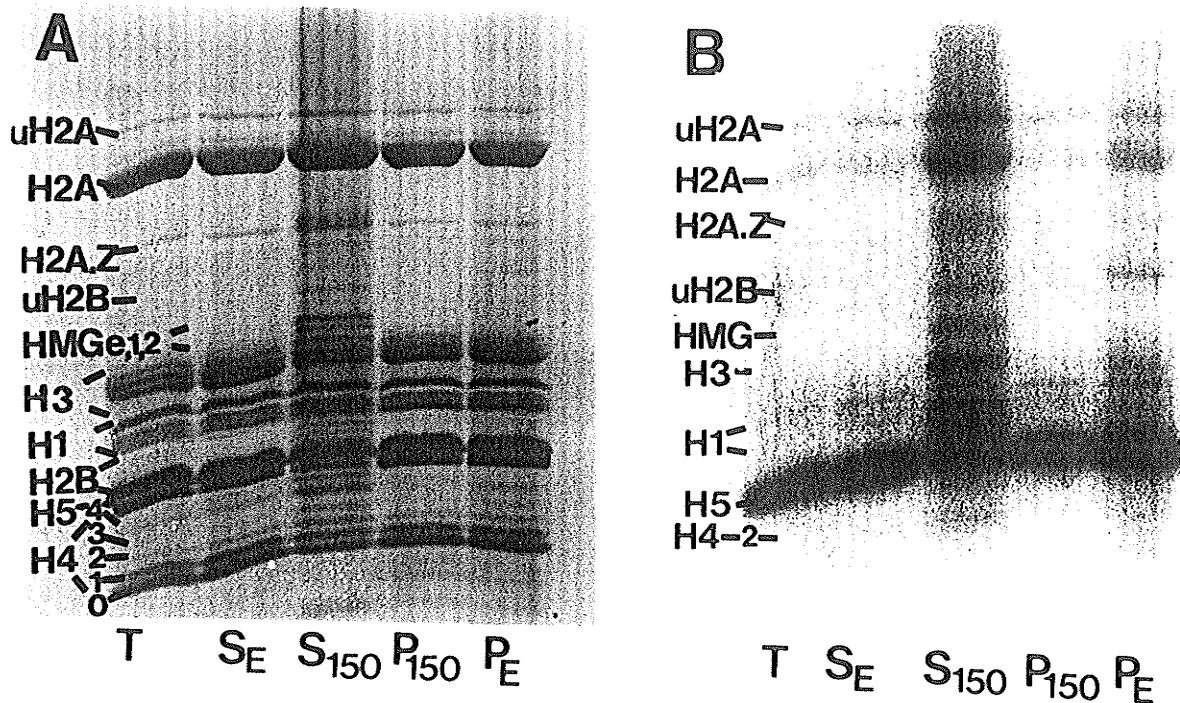


Figure 35. Distribution of newly synthesized histones among the chromatin fractions. Acid-soluble proteins of immature chicken erythrocytes incubated with ^3H -lysine (60 min) were isolated from the chromatin fractions. The proteins (10 μg) were electrophoretically resolved on 15% polyacrylamide AUT gels. Panel A shows the Coomassie Blue-stained gel pattern and panel B shows the accompanying fluorogram. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. The acetylated species of histone H4 are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively. Oxidized histone H2B is indicated as ox.

and H2B (denoted uH2A and uH2B, respectively) were labeled, with histones H2A and H2B being labeled to a greater extent than H3 and H4. The species of histone H3 that incorporated label was identified as H3.3 and acetylated species thereof. The identification of the labeled histone H3 variant as H3.3 was determined by electrophoretically resolving the histones on long AUT polyacrylamide gels which separate the acetylated species of H3.3 from the un-, mono- and diacetylated forms of H3.2 (not shown).

It has been demonstrated that newly synthesized histones can bind to the surface of chromatin without being incorporated into nucleosomes (Seale, 1981; Jackson, 1990). Thus, the acid-soluble, labeled proteins present in the chromatin fractions shown in Fig. 35 may be a combination of nucleosomal and surface-bound histones. The surface bound histones can be removed from chromatin with 0.45 M NaCl (Seale, 1981). In order to analyze only those newly synthesized histones that have exchanged with nucleosomal histones, hydroxylapatite-bound chromatin was washed with 0.45 M NaCl and the histone octamer was recovered in 2 M NaCl. It should be noted that 0.45 M NaCl removes both H1 histones (excluding H5) and HMG proteins from chromatin.

Chicken immature erythrocytes were labeled for 90 min in the presence or absence of actinomycin D, and the chromatin was fractionated. Except for fraction T (acid-extracted nuclear histones), histones were isolated from hydroxylapatite-bound chromatin. In agreement with the results of Louters and Chalkley (1985), Fig. 37 shows that incubation of the cells with actinomycin D did not affect the distribution of labeled histones among the various chromatin fractions. Fig. 37 also shows that similar to the results shown in Fig. 36, fraction S₁₅₀ had the greatest level of labeled nucleosomal histones. This is a representative result of nine experiments.

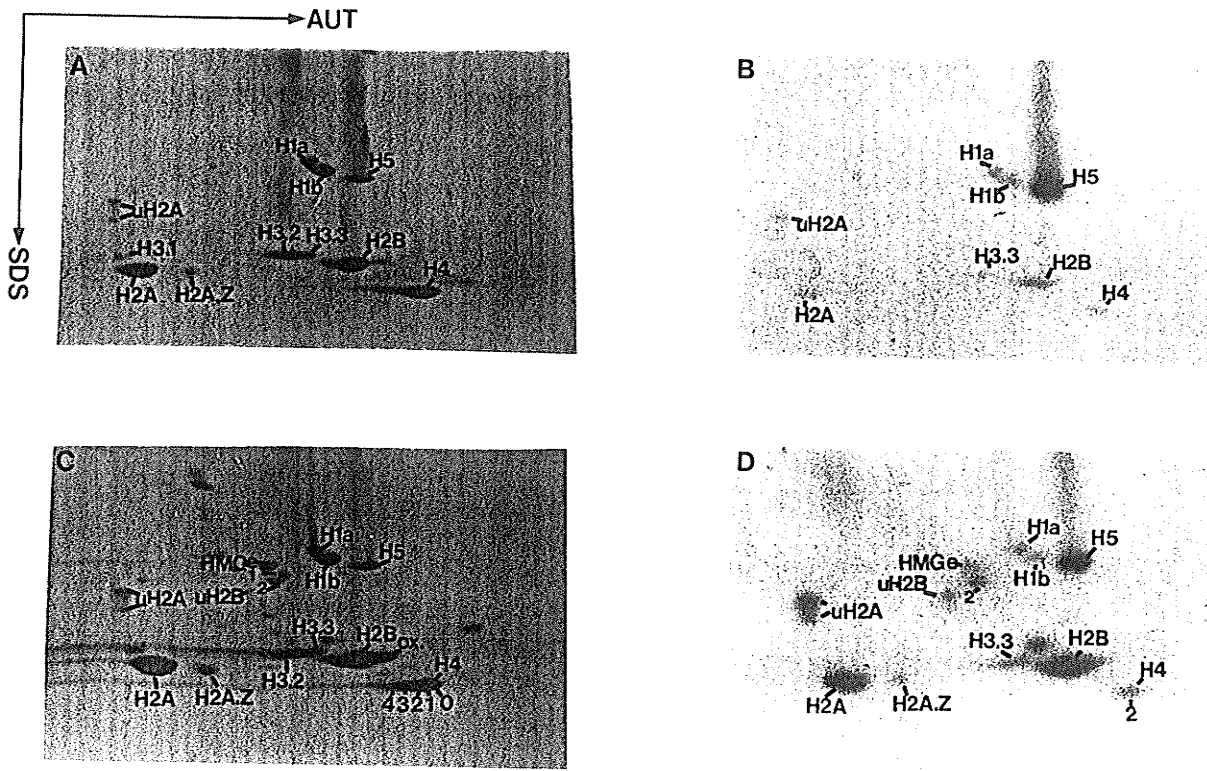


Figure 36 Active/competent gene-enriched chromatin fraction S₁₅₀ is enriched in newly synthesized nucleosomal histones. Acid-soluble proteins of unfractionated chromatin (T) and the salt-soluble, active/competent gene-enriched chromatin fraction S₁₅₀ were electrophoretically resolved by two-dimensional gel electrophoresis (AUT into SDS). Panels A and C are the Coomassie Blue-stained gel patterns containing proteins of T and S₁₅₀, respectively. B is the accompanying fluorogram of A, and D is the fluorogram for C. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. The acetylated species of histone H4 are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively. ox indicates oxidation of histone H2B. Note that on the first dimension AUT gel oxidized H2B comigrates with tetraacetylated H4.

Thus, the observation that fraction S₁₅₀ had the greatest amount of labeled nucleosomal histones appears to be independent of the time that cells were incubated with label and the method of histone isolation.

Fig. 38 shows densitometric scans of fluorograms (solid lines) and Coomassie Blue-stained gel patterns (broken lines) containing histones of chromatin fractions S_E, S₁₅₀ and P₁₅₀. Fraction P₁₅₀ had the lowest level of labeled nucleosomal histones, with histone H2A of fraction P₁₅₀ being labeled approximately 0.4-fold that of H2A in fraction S_E. (Because fraction T was not isolated by hydroxylapatite chromatography, S_E was used as a reference for determining enrichments of labeled histones in the chromatin fractions.) Fraction S₁₅₀, which is enriched approximately 6-fold in active and 4.3-fold in competent genes (Delcuve and Davie, 1989), was enriched approximately 8.3-fold versus fraction S_E in labeled H2A. In fraction S₁₅₀, histones H2A, H2B and their ubiquitinated forms were the major labeled histones. Note that the acetylated forms of H2B were labeled. Label was also incorporated into H2A.Z, H3 and the diacetylated species of H4. This is a representative result of 8 experiments.

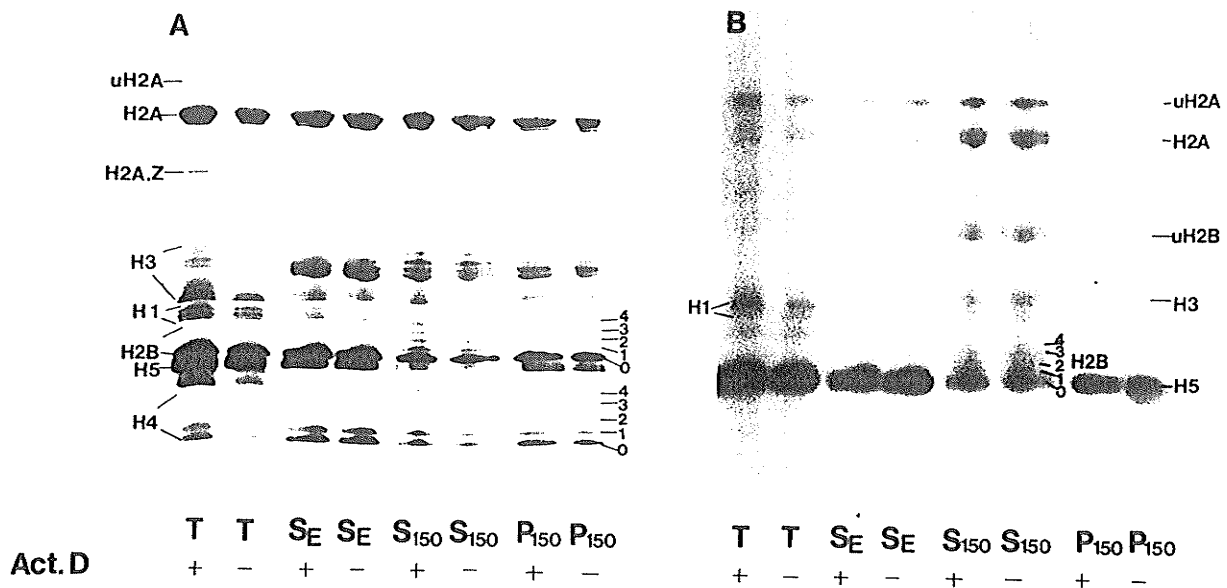


Figure 37. Inhibition of transcription does not affect nucleosomal histone exchange. Immature erythrocytes were incubated for 90 min with ^3H -lysine in the presence (15 $\mu\text{g/ml}$; +) or absence (-) of actinomycin D (Act. D). Acid soluble proteins of unfractionated chromatin (T) and hydroxylapatite-prepared histones of the chromatin fractions were electrophoretically resolved on 15% polyacrylamide AUT gels. Panel A shows the Coomassie Blue-stained gel pattern and panel B shows the accompanying fluorogram. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. The acetylated species of histones H2B and H4 are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively.

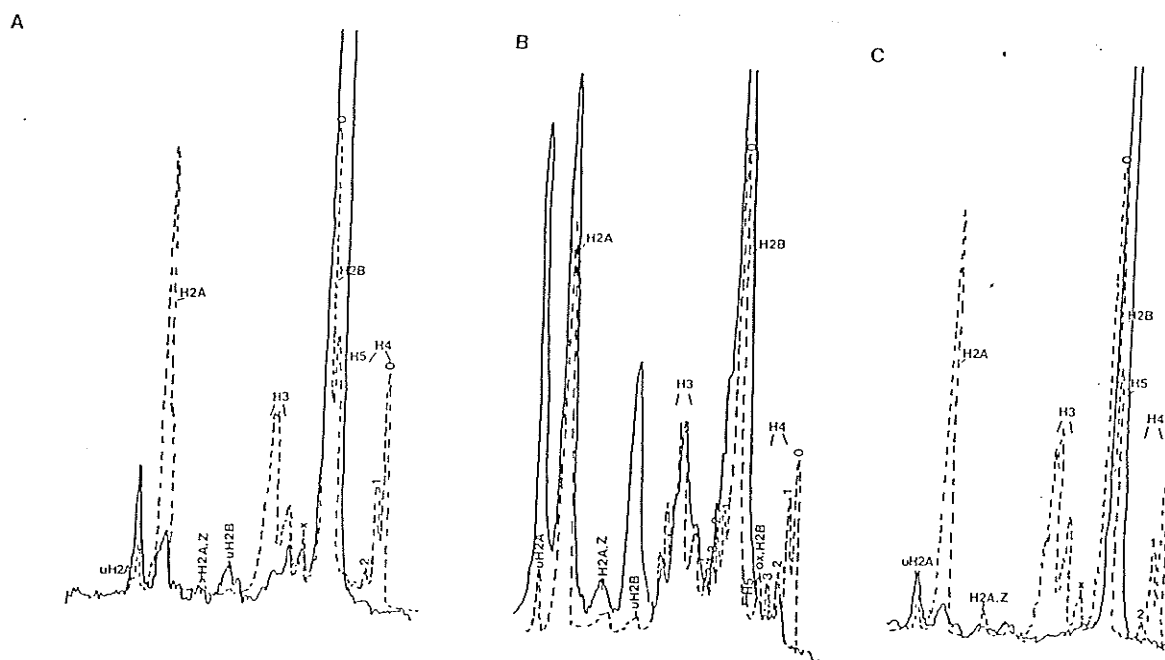


Figure 38. Fraction S_{150} is highly enriched in newly synthesized labeled nucleosomal histones. Hydroxylapatite-prepared histones of chromatin fractions S_E , S_{150} , and P_{150} were electrophoretically resolved on a 15% polyacrylamide AUT gel. Densitometric scans of fluorograms (solid lines) and Coomassie Blue-stained gel patterns (broken lines) containing these histones are shown. The densitometer was adjusted to maximum sensitivity for scanning the fluorograms in order to produce adequate signals from fraction P_{150} . x indicates the migration of an unknown labeled protein. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. The acetylated species of histones H2B and H4 are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively.

Newly synthesized nucleosomal histones are highly enriched in 0.15 M-NaCl-soluble polynucleosomes

To determine whether the labeled nucleosomal histones co-fractionated with the active/competent gene-enriched polynucleosomes, chromatin fragments of fraction S₁₅₀ were size resolved, obtaining four fractions F_I, F_{II}, F_{III}, and F_{IV} of decreasing chromatin fibre lengths. Fig. 39 shows that the labeled nucleosomal histones were enriched in the salt-soluble polynucleosomes (fractions F_I and F_{II}). Fraction P₁₅₀, which contained 78% of the total nuclear DNA, incorporated the least label. The salt-soluble polynucleosomes (F_I and F_{II}) were enriched 17.0-fold in labeled H2A compared to S_E. Of the salt-soluble chromatin fractions, the histones of fraction F_{IV}, which consists primarily of mononucleosome-size particles, were the least labeled. This is a representative result of 6 experiments.

Fig. 40 shows a densitometric scan of a fluorogram (solid lines) and Coomassie Blue-stained gel pattern (broken lines) containing histones of chromatin fraction F_{II}. The following histones were labeled: uH2A, H2A, H2A.Z, uH2B, H3, H2B and its acetylated species (mono-, di-, tri- and tetraacetylated forms), and di- and triacetylated H4. Approximately, 66% of the labeled H2B and 51% of total H2B of fraction F_{II} were acetylated. This is a representative result of 6 experiments. Peptide mapping of uH2A demonstrated that both H2A and ubiquitin were labeled, with approximately 62% of the label being incorporated into H2A (data not shown). After correcting for the contribution of labeled ubiquitin to the total labeling of uH2A, we calculate that approximately 32% of the labeled H2A was ubiquitinated. In contrast, the scan of the Coomassie-Blue stained gel pattern shown in Fig. 40 shows that approximately 8% of the total H2A associated with these chromatin fragments was ubiquitinated.

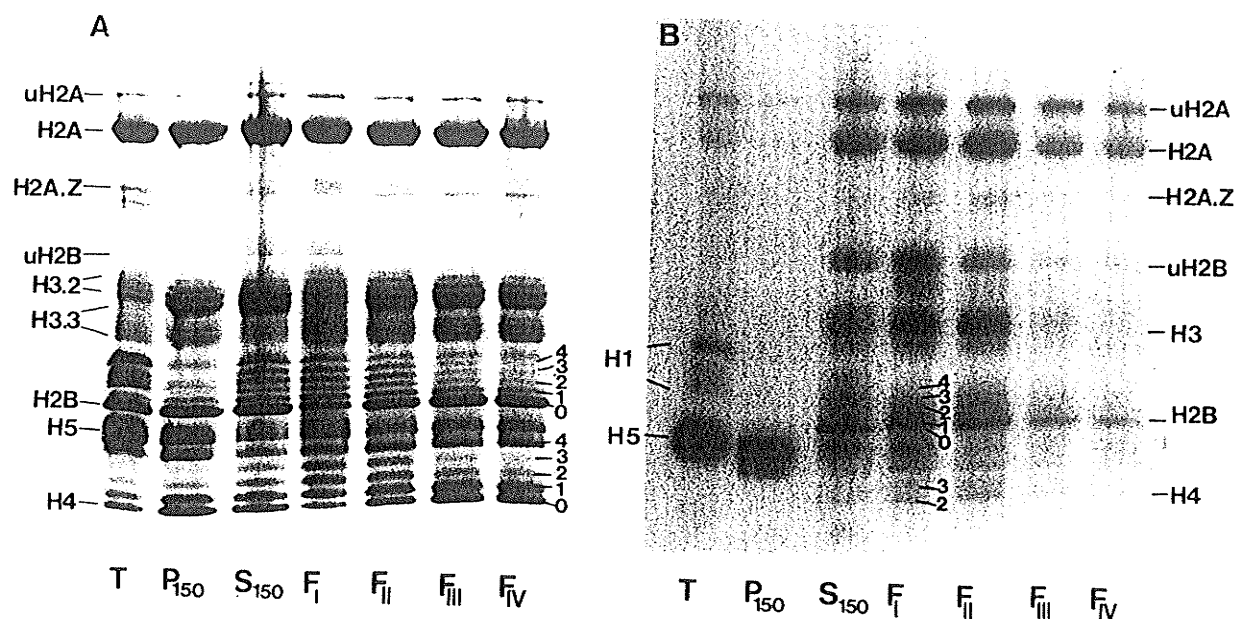


Figure 39. Nucleosomal histones of highly active/competent gene-enriched salt-soluble polynucleosomes are highly enriched in newly synthesized histones. Acid-soluble proteins of unfractionated chromatin (T) and hydroxylapatite isolated histones of the chromatin fractions and size-fractionated salt-soluble chromatin fragments of fraction S₁₅₀ (fractions F_I, F_{II}, F_{III}, and F_{IV}) were electrophoretically resolved on 15% polyacrylamide AUT gels. Panel A shows the Coomassie Blue-stained gel pattern and panel B shows the accompanying fluorogram. The ubiquitin adducts of histones H2A and H2B are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively. ox indicates oxidized H2B. The arrow below H5 in panel B, lane P₁₅₀ shows the position of modified species of H5 which comigrates with H5 on SDS polyacrylamide gels.

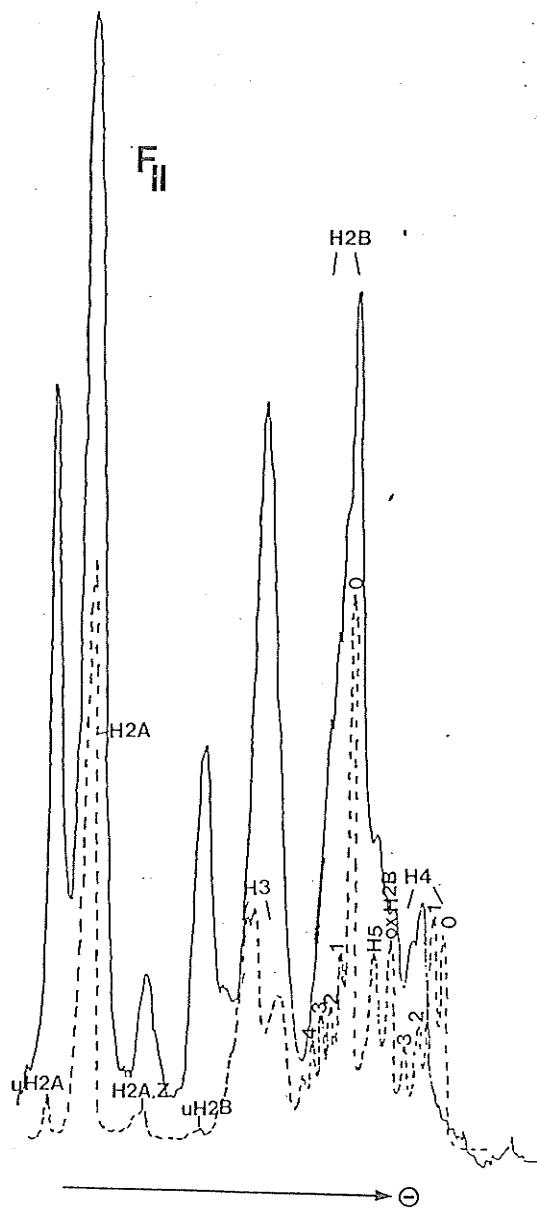


Figure 40. The newly synthesized histones of fraction F_{II} are acetylated and ubiquitinated. Hydroxylapatite-prepared histones of chromatin fraction F_{II} were electrophoretically resolved on a 15% polyacrylamide AUT gel. Densitometric scans of a fluorogram (solid lines) and a Coomassie Blue-stained gel pattern (broken lines) are shown. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. The acetylated species of histones H2B and H4 are denoted as 0, 1, 2, 3, and 4 representing the un-, mono-, di-, tri-, and tetraacetylated species, respectively. oxH2B is oxidized H2B.

Discussion

Our results show that newly synthesized histone H5 is incorporated into all regions of the chromatin of chicken erythrocytes which are arrested in G₀ phase of the cell cycle. The incorporation of newly synthesized histone H5 into chromatin may occur by exchange with preexisting H5 (Louters and Chalkley, 1985) or represent new deposition of this histone onto the chromatin fibre.

This study provides evidence that newly synthesized histones H2A, H2A.Z, H2B, H3.3, and H4 exchange preferentially with the nucleosomal histones of transcriptionally active/competent chromatin domains. The following observations support this conclusion. First, enrichment of labeled nucleosomal histones paralleled the enrichment of active DNA in chromatin fractions S₁₅₀ (salt-soluble chromatin fragments) and F_I/F_{II} (salt-soluble polynucleosomes). The enrichment of labeled histone H2A in fractions S₁₅₀ and F_I/F_{II} of 8.3-fold and 17.0-fold, respectively, is similar to the 6.0-fold and 15.1-fold enrichment of active DNA sequences in these fractions. Second, hyperacetylated species of histones H2B and H4 are labeled. Zhang and Nelson (1986, 1988a) demonstrated that in chicken immature erythrocytes, 3.7% of the modifiable histone lysine sites are undergoing acetylation and deacetylation. Thus, approximately 1-2% of the genome is composed of dynamically acetylated and deacetylated histones, and these histones are complexed with active, but not repressed, DNA (Hebbes *et al.*, 1988; Ridsdale *et al.*, 1990). In the presence of sodium butyrate, which was used in our cell incubations, the dynamically acetylated histones become hyperacetylated (tri- and tetraacetylated forms). The observation that the hyperacetylated species of H2B and H4 of the active/competent

gene-enriched polynucleosomes (fractions F_I and F_{II}) are labeled suggests that newly synthesized histones have exchanged with nucleosomal histones complexed to active/competent DNA. Furthermore, the observation that ubiquitinated histone H2B, which is preferentially located in transcriptionally active chromatin (Nickel *et al.*, 1989), is labeled provides evidence that newly synthesized histones are localized in transcriptionally active/competent chromatin regions.

The amount of labeled uH2A and uH2B in the histones of salt-soluble polynucleosomes is quite striking, with approximately 32% of the labeled H2A being ubiquitinated. In contrast, 8% of the total H2A of the salt-soluble polynucleosomes is ubiquitinated. Other investigators have observed that the ratio of uH2A/H2A in G₁-phase cells or in cells where DNA replication has been inhibited increases 4- to 6-fold compared to the S-phase cells (Jackson and Chalkley, 1985; Jackson, 1990). Jackson (1990) has demonstrated that in the absence of replication the pool of excess histones is susceptible to extensive modification by ubiquitin, resulting in an increase in the amount of the newly synthesized uH2A and uH2B incorporated into chromatin. These results suggest that in the G₀-phase chicken erythrocytes newly synthesized H2A and H2B is ubiquitinated prior to exchange with the nucleosomal histones.

Consistent with the results of others (Louters and Chalkley, 1985; Bonner *et al.*, 1988), we find that more newly synthesized histones H2A and H2B are incorporated into chromatin than newly synthesized histones H3 and H4. A model has been proposed in which the passage of the RNA polymerase displaces one H2A/H2B dimer (Baer and Rhodes, 1983; Loidl, 1988). Although our results do not exclude this possibility, we demonstrate that the incorporation of newly synthesized H2A/H2B and other nucleosomal

histones into chromatin is not dependent upon on-going transcription. Exchange of newly synthesized histones with nucleosomal histones of transcriptionally active/competent chromatin may reflect instability in the active/competent nucleosomes. Ridsdale *et al.* (1988) demonstrated that the nucleosomes of highly active/competent gene-enriched chromatin fragments (fractions F_I and F_{II}) were more readily dissociated than bulk nucleosomes by the intercalating agent, ethidium bromide. We have also demonstrated that histone acetylation and histone ubiquitination facilitate nucleosome dissolution *in vitro* (Nagaraja *et al.*, submitted). Nacheva *et al.* (1989) reported differences in histone binding to DNA with the hsp70 gene upon induction of transcription. Using a low salt fractionation procedure that enriches for active genes, these investigators demonstrated that crosslinking efficiencies of histones to DNA were reduced in transcriptionally active gene-enriched chromatin relative to bulk. Interestingly, they observed that histones H2A and H2B had a greater reduction in crosslinking efficiency to DNA than histones H3 and H4. This may result in histone-DNA complexes of transcriptionally active chromatin existing in a more dynamic state than bulk stable nucleosomes. The instability of the nucleosome within transcriptionally active domains may be necessary to facilitate efficient transcription. A diagrammatic summary of the results is shown in Fig. 41.

Our results have implications for mechanisms of chromatin remodeling. If nucleosomal histone exchange occurs in G1 phase preferentially with active/competent chromatin regions, as we have demonstrated for G0 phase erythrocytes, then this event could account for the enrichment of the histone variants H2A.Z and H3.3 (which are preferentially synthesized in G1) in transcriptionally active/competent

Model for the Exchange of Newly Synthesized Histones into Transcriptionally Competent Gene Chromatin

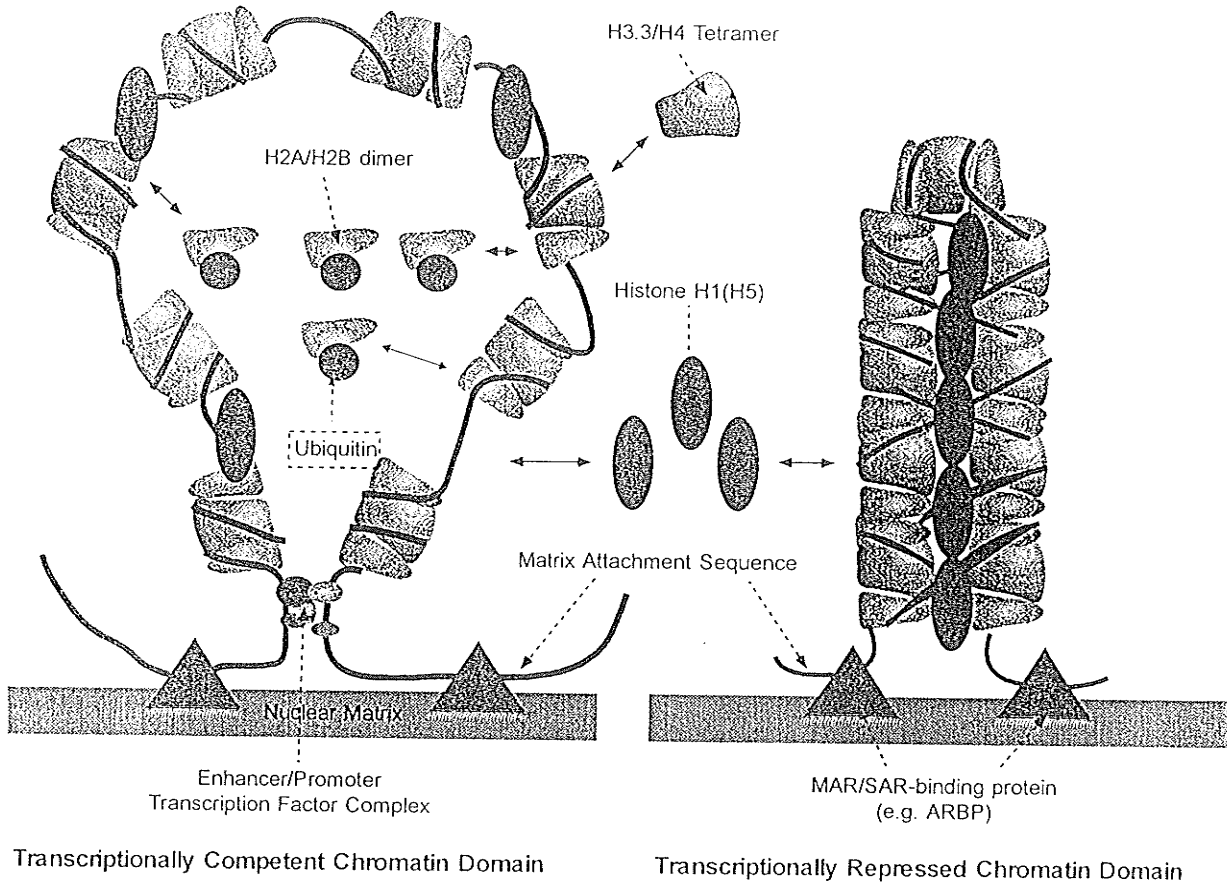


Figure 41. A model for the exchange of newly synthesized histones into functionally distinct regions of chromatin. A nuclear pool of newly synthesized histones enriched in histones H2A.Z and H3.3 is available for exchange with nucleosomal histones. The pool of histone H2A/H2B dimers is extensively ubiquitinated. The transcriptionally competent chromatin domain is structurally different from the repressed chromatin domain. The transcribed domain is in a decondensed chromatin structure and contains nucleosomes which are relatively labile. Thus, histone H2A/H2B dimers and to a lesser extent, histone H3.3/H4 tetramers are able to incorporate into the transcribed domain through an exchange process. The repressed chromatin domain is in a condensed structure and contains relatively stable nucleosomes. These regions are unavailable for the exchange of nucleosomal histones. Newly synthesized histone H1 (H5) is incorporated readily into both transcriptionally active and transcriptionally repressed chromatin domains. This may represent both exchange and new deposition.

chromatin as reported by Ridsdale and Davie (1987). Additionally, early S phase replication of active chromatin regions has been demonstrated (Hatton *et al.*, 1988). This may result in the acquisition of histones from a pool enriched in the histone variants preferentially synthesized in G1 phase (H2A.X, H2A.Z, and H3.3). Therefore, this difference may also contribute to the remodeling of active/competent chromatin regions.

Part 4: Histone Deacetylase is Associated with the Internal Nuclear Matrix

Introduction

We have recently been investigating the mechanisms involved in differentiating the nuclear chromatin into regions undergoing dynamic histone acetylation and regions whose acetylation levels are primarily static during G0/G1 phase of the cell cycle. Dynamic histone acetylation is involved in the regulation of domain topology (Norton *et al.*, 1989; Norton *et al.*, 1990; Thomsen *et al.*, 1991) and the regulation of higher-order chromatin structure (Ridsdale *et al.*, 1990; Ausio, 1992). Experiments using Trichostatin A, a specific inhibitor of histone deacetylase, demonstrate that histone acetylation dynamics are important for the modulation of the nuclear matrix binding of a certain class of MARs by influencing the single-stranded properties of these sequences (Bode *et al.*, 1992; Klehr *et al.*, 1992). Additionally, experiments with this inhibitor have demonstrated that dynamics of histone acetylation are important for normal cell cycle progression (Yoshida *et al.*, 1991). We have observed that the majority of chromatin undergoing dynamic histone acetylation is associated with an insoluble chromatin fraction that contains the nuclear matrix. It has also become apparent that there may be different patterns of histone acetylation associated with different nuclear events (e.g., transcription)(Turner, 1991). Additionally, nonrandom usage of the specific sites of acetylation suggest that the different acetylation sites on individual histones may have unique functions (reviewed in Turner, 1991; Csordas, 1990). Thus, it has become increasingly important to identify and study the regulation of the nuclear

histone acetyltransferases and histone deacetylases. It is important to note on this point that Brosch *et al.* (1992) found that in *Zea mays*, the substrate specificity of a nuclear histone deacetylase was found to be regulated by phosphorylation. Some evidence has been presented in the past for the calf thymus histone deacetylase being phosphorylated (Vidali *et al.*, 1973). Previously, we have demonstrated in chicken erythrocytes that approximately 75 % of both dynamically acetylated chromatin and transcriptionally active gene chromatin are associated with residual insoluble nuclear material (see Table 1). It is well established in these cells that highly acetylated (dynamic) histones are associated with transcribed genes (Hebbes *et al.*, 1988) and as such the dynamically acetylated insoluble nuclear chromatin likely reflects the active gene chromatin population. These results suggested that dynamic histone acetylation may be a solid-state process orchestrated by nuclear matrix-bound histone acetyltransferases and histone deacetylases.

Very recently, several experimental lines of evidence have demonstrated that the nucleus is a highly compartmentalized structure. RNA transcription has been demonstrated to be localized to specific "transcript domains" (Carter *et al.*, 1991; Carter and Lawrence, 1991). RNA is transcribed, processed, and spliced at distinct foci in the nucleus and transported along discrete tracks to the nuclear periphery (Lawrence *et al.*, 1989) and this process is nuclear matrix-associated (Xing and Lawrence, 1991). Importantly, RNA polymerase II has been shown to be associated with the nuclear matrix (Jackson and Cook, 1985) and these results have been confirmed using antibodies specific to RNA polymerase II which demonstrate that this enzyme is confined to discrete nuclear domains (van Driel *et al.*, 1991). These results suggest that transcription and transcript

processing are coupled solid-state processes associated with the nuclear matrix and that the nuclear matrix is involved in the functional compartmentalization of the nucleus

EM studies have questioned the presence of an internal nuclear matrix in chicken erythrocyte. Lafond and Woodcock (1983) used "mild" isolation procedures in an attempt to visualize the nuclear matrix of the chicken mature erythrocyte. These conditions involved DNase I digestion at 4°C and a single extraction of the nuclease-digested nuclei with a 2 M NaCl-containing buffer. Upon visual inspection of these preparations by electron microscopy, they found only the nuclear pore-lamina complex and no evidence for an internal nuclear matrix. Biochemical studies, in contrast, demonstrate the presence of internal nuclear matrix proteins in chicken erythrocytes (Phi-Van *et al.*, 1990; Von Kries *et al.*, 1991)

Although histone acetyltransferases have been solubilized from isolated nuclei and partially purified preparations or preparations purified to apparent homogeneity have been examined, very little work has been done concerning the nuclear localization of this class of enzymes. The literature would suggest that histone acetyltransferases are soluble nuclear enzymes which can associate with chromatin. The ease by which these enzymes are extracted with moderate ionic strength buffers under reducing conditions would suggest that these enzymes are only loosely associated with chromatin. Consistent with this model, Attisano *et al.* (1988) demonstrated that histone acetyltransferase activity was preferentially associated with linker DNA regions of a transcriptionally active gene-enriched chromatin fraction of chicken erythrocyte.

In the following studies we addressed the nuclear location of histone acetyltransferase and histone deacetylase activities. We have utilized both

our standard chromatin fractionation procedure (see Fig. 17) and several different nuclear matrix isolation procedures to establish this association. Using the procedure of He *et al.*, (1990) to prepare nuclear matrices under conditions which preserve nuclear morphology and which separates the nuclei into three major classes of proteins 1) those which are soluble or chromatin bound, 2) those associated with intermediate salt-chromatin depleted nuclear matrices and 3) and those which may be components of the intermediate salt/high salt nuclear matrix core filaments, an underlying intermediate filament-like network, we provide evidence that histone acetyltransferase and histone deacetylase are associated with the nuclear matrix core filaments. We have also determined that sulfhydryl bonds present *in situ* are required for this association. The requirement for disulfide crosslinks to maintain the association of these enzymes to this structure may explain why histone acetyltransferase is readily extracted from nuclei in the presence of intermediate salt concentrations and reducing agents. Our results support a model in which dynamic histone acetylation is mediated by nuclear matrix-associated histone acetyltransferases and histone deacetylases which colocalize and may function in the structural organization of "transcript domains".

Results

Distribution of histone deacetylase activity in chicken immature and mature erythrocyte chromatin.

The analysis of the immature and mature erythroid chromatin distribution of the dynamically acetylated histones demonstrated that the majority of the rapidly deacetylated, class 1 histone H4 was localized with

the chromatin fragments complexed to the insoluble nuclear material (see Table 1). This observation suggested that the majority of the histone deacetylase activity would also be localized in this fraction. Table 4 shows the activity of the histone deacetylase of the chromatin fractions. Fraction P_E was found to possess the majority of the enzyme activity. Of three fractionations, the proportion of the total histone deacetylase activity located in the EDTA-insoluble residual nuclear material (fraction P_E) was highly reproducible, with this fraction containing 77.6 to 78.9% of the enzyme activity.

Zhang and Nelson (1988b) reported that the deacetylation rate of labeled histone H4 of mature erythrocytes was lower than that of immature cells. Consistent with this observation, we found that the histone deacetylase activity of mature erythroid nuclei was 35% less than the enzyme activity of adult immature erythroid nuclei. However, the distribution of the histone deacetylase activity among the mature erythroid chromatin fractions was similar (Table 4).

Whereas treatment of the residual nuclear material (fraction P_E) with 2 M NaCl extracted approximately 90% of the associated chromatin (see Fig. 20), the histone deacetylase activity was not solubilized, suggesting that the enzyme was not bound to chromatin. Also, the enzyme activity remained bound to the nuclear matrices that were treated with 10 mM 2-mercaptoethanol, indicating that the enzyme was not retained by the nuclear matrix via disulfide bonds.

Histone deacetylase is a component of the nuclear matrix.

The observation that histone deacetylase activity was primarily located in fraction P_E suggested that the enzyme may be a component of the nuclear matrix. Nuclear matrices of immature and mature erythrocytes

were prepared by extensively digesting nuclei with DNase I and subsequently extracting the residual nuclear material with 2 M NaCl (see Fig. 42, *high salt matrix*). Table 4 shows that 50 to 57% of the histone deacetylase activity was associated with the nuclear matrix of mature and immature erythrocytes.

There is concern that the direct high-salt extraction of nuclei may induce precipitation of enzymes in the nuclear matrix (see for example, Roberge *et al.*, 1988). To address this, we prepared nuclear matrices by a different method in which DNase I-digested nuclei was extracted with intermediate salt (0.4 M KCl) followed by an extraction with high salt (2 M KCl) (Fig. 42, *intermediate-/high-salt matrix*). This procedure, but not the direct high-salt approach, efficiently solubilized RNA polymerases I and II from the nuclear matrix (Roberge *et al.*, 1988). Table 5 shows that in contrast to RNA polymerase I and II, 42% of the histone deacetylase remained bound to the intermediate-/high-salt prepared nuclear matrices of immature erythrocytes. These results provided strong evidence that approximately half of the histone deacetylase activity of mature and immature erythrocytes was complexed to the nuclear matrix.

We attempted to determine the histone deacetylase activity associated with the nuclear scaffold prepared by the lithium diiodosalicylate (LIS), low ionic strength procedure (Mirkovitch *et al.*, 1984). However, we found that LIS irreversibly denatured the enzyme, and we were unable to detect enzyme activity in either soluble or scaffold fractions (data not shown).

To determine whether the histone deacetylase was a component of the internal nuclear matrix or of the nuclear pore-lamina fraction, nuclear pore-lamina complexes were isolated according to the protocol of Kaufmann

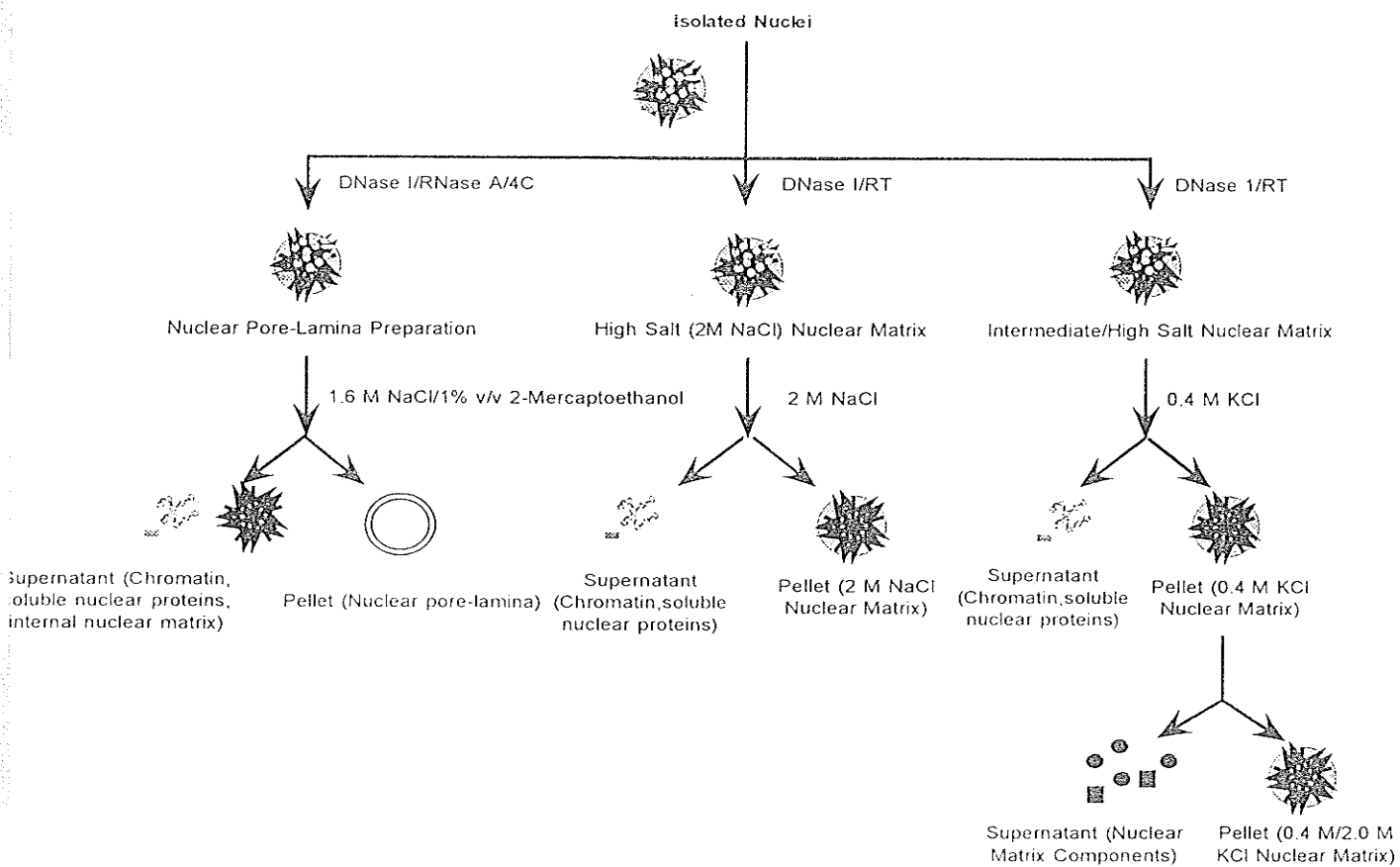


Figure 42. Experimental procedures used to isolate nuclear matrices and nuclear pore-lamina complexes. 4C indicates that DNase I digestions were performed at 4°C whereas RT indicates that digestions were performed at room temperature (approximately 23°C). Details of the procedures can be found in the *materials and methods* section.

TABLE 4

Chromatin distribution of histone deacetylase activity in chicken immature erythrocytes

Four A₂₆₀ units of the various chromatin fractions were assayed for deacetylase activity by incubation with approximately 100 µg of substrate for one hour at 37°C. The released label in ethyl acetate was determined by scintillation counting and the distribution of enzyme was determined by multiplying the dpm released by the percentage of total A₂₆₀ in the chromatin fraction and then dividing by the dpm value for fraction T. For nuclear matrix preparations, the total enzyme activity in the pooled soluble fractions and the matrix were determined, and the distribution among the fractions was the percentage of the combined activities. Each value represents the mean ± S.E.M. from 3 measurements.

Fraction	Immature cells- deacetylase activity	Mature cells- deacetylase activity
T	100.0	100.0
S _E	21.0 ± 0.6	26.4 ± 1.4
S ₁₅₀	7.3 ± 0.2	4.7 ± 0.1
P ₁₅₀	15.2 ± 1.3	22.4 ± 1.4
P _E	78.9 ± 5.1	74.4 ± 3.6
2 M NaCl S (soluble)	50.0 ± 2.0	42.7 ± 4.7
2 M NaCl P (matrix)	50.0 ± 1.8	57.3 ± 5.9

et al. (1983) (Fig. 42, *nuclear pore-lamina preparation*). Treating nuclear matrices with 1% 2-mercaptoethanol-1.6 M NaCl solubilized the internal matrix, leaving the nuclear pore-lamina complexes. This procedure was recently used to solubilize and purify a protein (ARBP) which is responsible for binding DNA to the nuclear matrix (von Kries *et al.*, 1991). Table 5 shows that approximately 95% of the histone deacetylase activity was found in the 1% 2-mercaptoethanol-1.6 M NaCl extract. The nuclear pore-lamina complex contained less than 5% of the histone deacetylase activity. These results demonstrated that like ARBP the histone deacetylase is a specific component of the internal nuclear matrix.

Distribution of histone deacetylase activity amongst the salt-soluble poly- and mononucleosomes.

Fig. 43 shows the elution of enzyme activity *versus* the elution of the 0.15 M NaCl-soluble chromatin fragments from a Bio-Gel A-5m gel exclusion column. Four peaks of enzyme activity were detected. Peaks 1 and 2 were associated with the poly- and oligonucleosomes, respectively. The third peak of enzyme activity eluted with the mononucleosomes, and the fourth peak eluted from the column in a position consistent with the elution of free enzyme. Approximately 28, 15, and 57% of the histone deacetylase activity in fraction S₁₅₀ was present in the 0.15 M NaCl-soluble poly- and oligonucleosomes (peaks 1 and 2), mononucleosomes (peak 3) and free enzyme (peak 4) respectively. The specific enzyme activity of the oligonucleosomes (fraction 30; 14.7 dpm/μg of protein) was greater than that of the polynucleosomes (fraction 18; 12.2 dpm/μg of protein) or mononucleosomes (fraction 40; 10.7 dpm/μg of protein).

TABLE 5**Histone deacetylase activity is a component of the nuclear matrix**

Equivalent proportions of the various fractions from chicken immature erythrocytes were assayed for deacetylase activity. 0.4 M KCl S is the material solubilized following extraction of the digested nuclei with 0.4 M KCl. 2.0 M KCl S is the material solubilized following extraction of the 0.4 M KCl pellet with 2.0 M KCl. 2.0 M KCl matrix is the material remaining after these treatments. Histone deacetylase activity was also assayed from the nuclear fractions following DNase I/RNase A digestion. 1.6 M NaCl/2-ME S is the material solubilized by treating digested nuclei in a buffer containing 1.6 M NaCl/ 1% v/v 2-mercaptoethanol and one subsequent wash with 1.6 M NaCl. This fraction contains internal nuclear matrix proteins in a soluble form. Pellet (nuclear pore-lamina complexes) is the material remaining insoluble following such treatment. The total enzyme activity in the various fractions was determined, and enzyme distribution was quantitated by determining the relative activity of each fraction to the combined total. Each value represents the mean \pm S.E.M. from 3 measurements.

Fraction	% Histone Deacetylase Activity
0.4 M KCl S (Soluble)	38.9 \pm 1.4
2.0 M KCl S (Soluble)	18.7 \pm 1.2
0.4/2.0 M KCl P (nuclear matrix)	42.4 \pm 2.9
1.6 M NaCl/2-Mercaptoethanol (ME) S (Soluble)	96.8 \pm 4.9
1.6 M NaCl/2-ME P (nuclear pore- lamina complex)	3.2 \pm 0.3

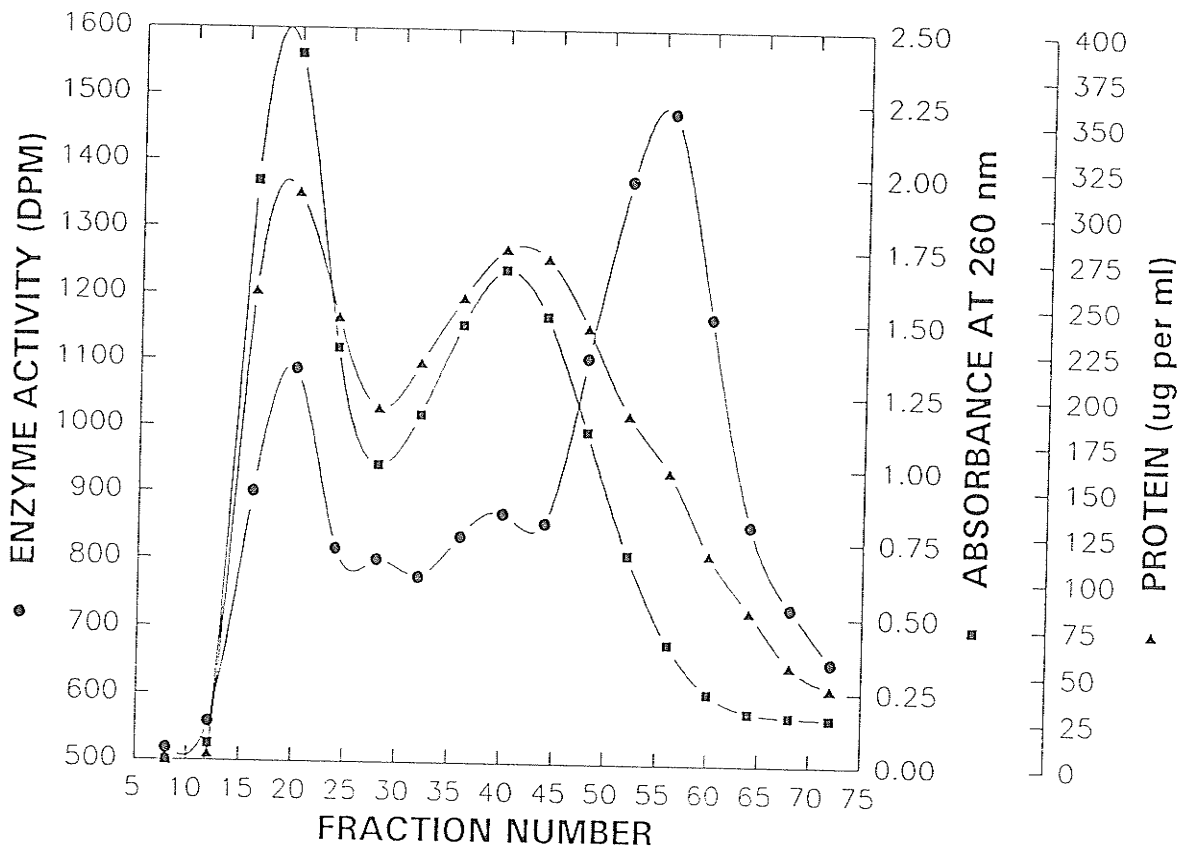


Figure 43. Histone deacetylase is associated with the 150 mM NaCl-soluble poly- and oligonucleosomes. Approximately 3 mg of fraction S₁₅₀ was applied to a Bio-Gel A-5m (2.8 x 20 cm) column. The column was run at a flow rate of 1.2 ml/min in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl, and 1 ml fractions were collected. 290 μ l of the column fraction was then assayed for deacetylase activity.

Identification of two distinct forms of histone deacetylase in chicken immature erythrocyte nuclei

A novel method for the isolation of nuclear proteins was developed as a means of extracting histone deacetylase(s) from isolated nuclei. The technique involves digestion of nuclei with DNase I/RNase A and extraction of the nuclei with a linearly increasing gradient of ammonium sulfate/2-mercaptoethanol from 50 mM Tris-Cl pH 7.0 to 0.25 M ammonium sulfate/1% v/v 2-mercaptoethanol in 50 mM Tris-Cl pH 7.0. It should be noted that the digestion conditions are sufficient to generate empty nuclear shells when challenged with salt plus reducing agents. The gradient extraction was achieved by mixing the digested nuclei with Sephadex G-25 superfine and pouring the mixture into a chromatography column. Figure 44 shows that there were at least two peaks of histone deacetylase activity eluting from the nucleus at different ionic strengths. The first peak consisted of enzyme eluting at below physiological ionic strength whereas the second peak consisted of enzyme eluting at slightly elevated ionic strengths. This second peak of enzyme activity may correspond to the enzyme identified as associated with the internal nuclear matrix using conventional nuclear matrix preparations. Figure 45 shows the analysis of peaks 1 and 2 by gel filtration chromatography on a Sephacryl S300 HR column. The first enzyme peak corresponded to an enzyme form with an approximate molecular mass of 450 KDa. The second enzyme peak corresponded to a much smaller molecular weight protein of approximately 45 KDa. It can be seen from the gel filtration analysis that these two forms of the enzyme were essentially completely resolved during the extraction chromatography procedure. This may reflect different associations with

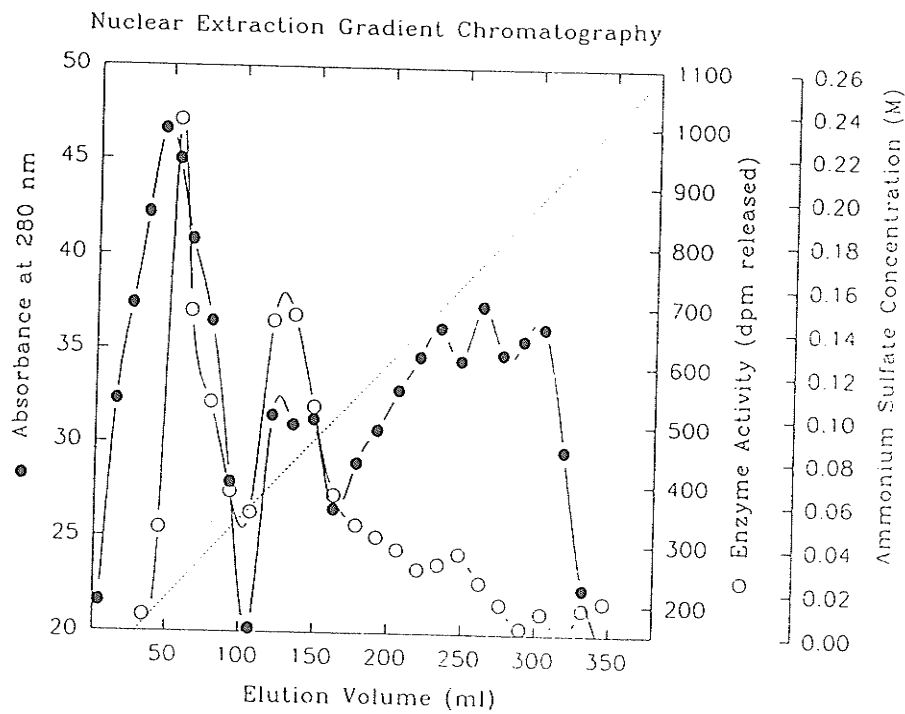


Figure 44. Nuclear chromatography distinguishes two distinct forms of histone deacetylase activity. Nuclei were digested according to the conditions used to generate nuclear pore-lamina complexes (see *materials and methods*). The digested nuclei were mixed with Sephadex G-25 and packed into a 2.3 cm diameter column. The nuclei were then extracted with a linear gradient of 0.0 to 0.25 M ammonium sulfate overnight at 4° C at a flow rate of 0.3 ml/min. The eluted fractions were assayed for protein content and histone deacetylase activity.

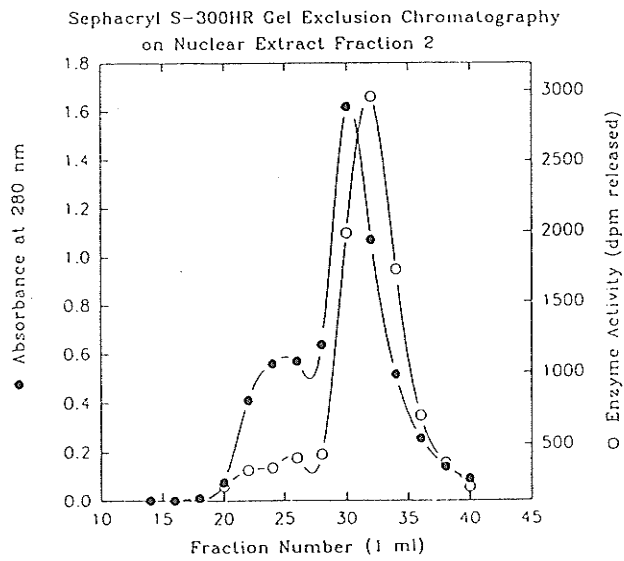
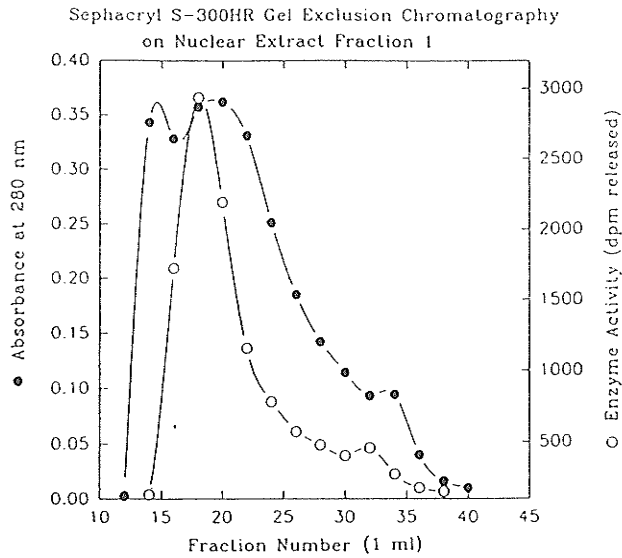


Figure 45. Identification of distinct molecular weight forms of histone deacetylase. The first and second peaks of histone deacetylase activity eluted by nuclear chromatography (see Fig. 44) were individually resolved on a 1.2 x 60 cm Sephacryl S 300 HR column. The column was run at a flow rate of 0.4 ml/min and 1.0 ml fractions were collected. The eluted fractions were assayed for both total protein content and histone deacetylase activity.

other nuclear proteins between the two distinguishable forms of histone deacetylase activity.

Histone deacetylase activity is located with the internal nuclear matrix of chicken liver.

We determined the nuclear distribution of histone deacetylase in chicken liver, a tissue in which the transcriptional activity is considerably higher than that of erythrocytes. The fractionation protocol shown in Fig. 42 was used throughout this study. Nuclear matrices isolated by this procedure may be less susceptible to spurious association with proteins than other nuclear matrix preparations.

Chicken liver nuclei were digested for one hour with DNase I at room temperature (23°C) and then fractionated (Fig. 46). Fig. 47 shows that for all the fractions the histone deacetylase activity was linear with increasing protein concentrations up to between one to two mg protein per reaction. At higher concentrations, inhibition of deacetylase activity was seen. Additionally, Fig. 47 shows that the specific activity of histone deacetylase differed between the four fractions with the 0.4 M KCl/2.0 M KCl insoluble nuclear material (nuclear matrix) having the greatest specific activity (approx. 22500 dpm released/mg protein), followed by the 0.4 M KCl soluble fraction (approx. 4400 dpm released/mg protein), the digestion supernatant (approx. 4000 dpm released/mg protein), and finally the 2.0 M KCl soluble fraction contained the lowest specific activity (approx. 2300 dpm released/mg). Once the linear range of the enzyme activity of each fraction was established, we routinely assayed samples containing between 200 and 500 µg of protein. This ensured an accurate assessment of both the enzyme activity in each fraction and the distribution of enzyme activity amongst the fractions.

We prepared nuclear matrices following incubation of chicken liver nuclei with DNase I at 4°C and room temperature. Nuclease digestions at different temperatures were used because it has been suggested that elevated temperatures may alter the partitioning of material with the nuclear matrix (Kaufmann and Shaper, 1991) or alternatively stabilize the nuclear matrix (Belgrader *et al.*, 1991). Fig. 48 shows that $70.7 \pm 4.5\%$ ($n = 3$ separate experiments) of the total nuclear histone deacetylase activity was found in the nuclear matrices (fraction 2.0 M KCl P, 23°C) prepared from chicken liver nuclei digested at room temperature with DNase I. There was a modest reduction in the amount of histone deacetylase activity ($57.6 \pm 10.2\%$, $n = 3$) partitioning with nuclear matrices (fraction 2.0 M KCl P, 4°C) prepared from nuclei digested at 4°C consistent with the observation that increased temperature stabilizes the internal nuclear matrix (Belgrader *et al.*, 1991).

We have previously shown that histone deacetylase is a component of the internal nuclear matrix but not of the nuclear pore-lamina complex of chicken erythrocyte. Nuclear pore-lamina complexes were isolated from chicken liver nuclei according to the procedure of Kaufmann *et al.* (1983) (see Fig. 42). Fig. 48 shows that $90.8 \pm 2.8\%$ ($n = 2$) of the total nuclear histone deacetylase activity was solubilized in the 1% 2-mercaptoethanol/1.6 M NaCl fraction. Less than 10% of the histone deacetylase activity was retained by the nuclear pore-lamina complex (fraction 2-ME/NaCl P in Fig. 48).

Intermediate-/High-KCl Nuclear Matrix

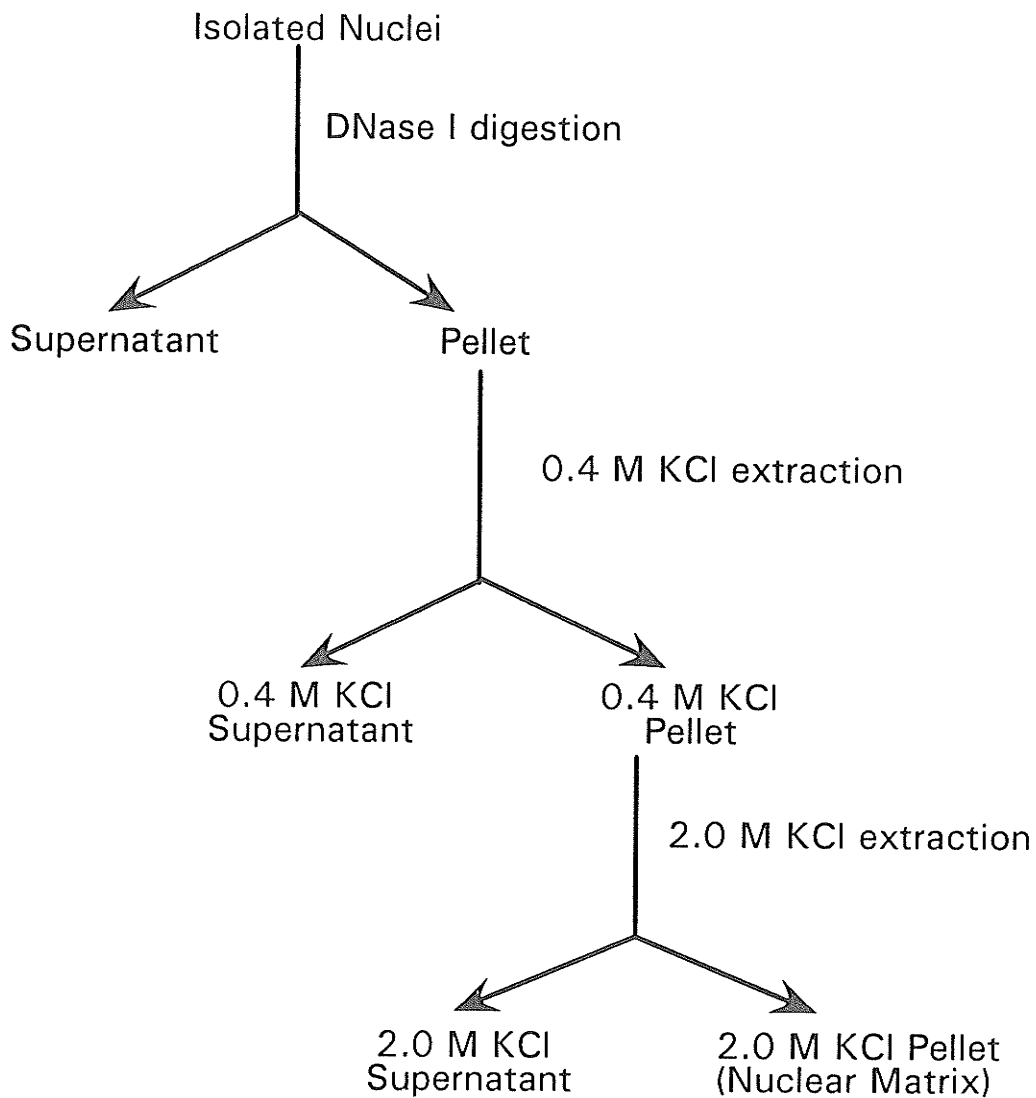


Figure 46. Isolation procedure for intermediate-/high-salt nuclear matrix. Nuclei were isolated, digested with DNase I, and extracted sequentially with buffers containing 0.4 M KCl and 2.0 M KCl. Four fractions were generated and assayed for deacetylase activity as described in *materials and methods*.

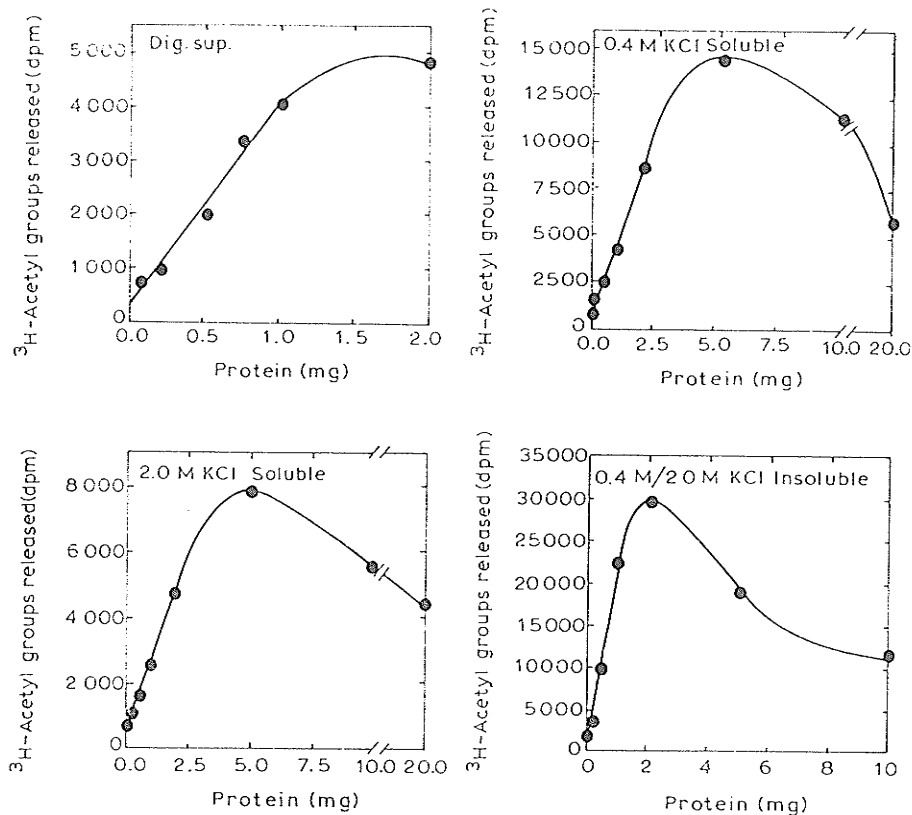


Figure 47. The histone deacetylase assay as a quantitative measure of histone deacetylase activity in nuclear subfractions. Isolated nuclear subfractions (chicken liver, 4°C digest) were characterized over a range of protein concentrations. Protein content in mg represents the amount of protein present in the $300\ \mu\text{l}$ assay volume. ^3H acetyl groups released represents the dpm contained in one-half of the volume of ethyl acetate used to extract the free acetate enzymatically released from a labeled histone substrate. The fraction headings for each of the graphs correspond to the fractions outlined in the flow chart in Fig. 46.

A gentle procedure for the isolation of nuclear matrices has recently been described using ammonium sulfate to extract nuclease digested nuclei (Belgrader *et al.*, 1991). This procedure results in less aggregation and an apparently more preserved nuclear morphology relative to the high salt extraction procedures typically used while extracting 98% of the total DNA, 78% of the total protein and 73% of the total RNA (Belgrader *et al.*, 1991). Additionally, preincubation of nuclei at 37°C results in a stabilization of the internal nuclear matrix prepared under these conditions (Belgrader *et al.*, 1991). This pretreatment results in a quantitatively improved recovery of internal nuclear matrix proteins with the insoluble material but no major changes in the spectrum of proteins associated with this structure. Chicken liver nuclei preincubated in Buffer A for one hour at 37°C or 4°C were digested with DNase I for one-half hour at 4°C in RSB-0.25 M sucrose and then extracted with 0.2 M ammonium sulfate. Fig. 48 shows that greater than 90% of the total nuclear histone deacetylase activity was associated with this nuclear matrix preparation (fraction 0.2 M AS P). These observations suggest that the majority, if not all, of the histone deacetylase activity is localized with the internal nuclear matrix of chicken liver.

The association of the histone deacetylase activity with the chicken erythrocyte internal nuclear matrix is dependent upon temperature.

When chicken erythrocyte nuclear matrices were prepared by digestion with nucleases at room temperature or 37°C using a variety of protocols, histone deacetylase was associated with the internal nuclear matrix. However, chicken erythrocyte nuclear matrices prepared from nuclei incubated with DNase I at 4°C lack an internal nuclear matrix that can be visualized by electron microscopy (Lafond and Woodcock, 1983). We

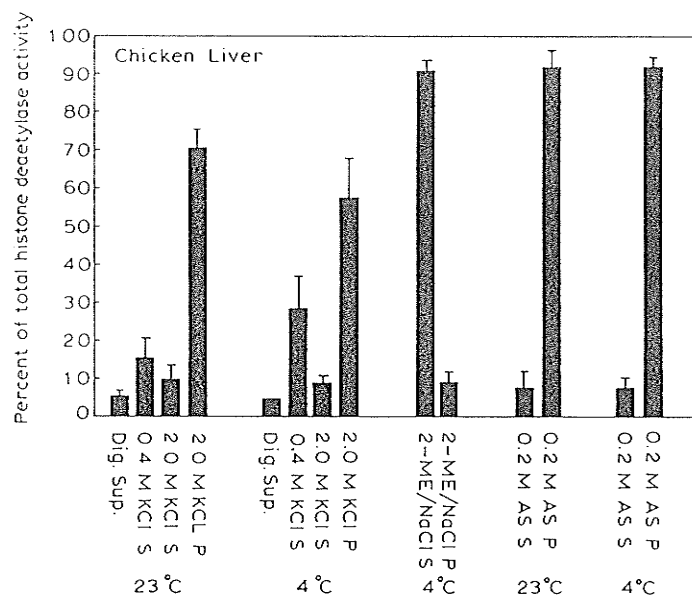


Figure 48. Histone deacetylase activity partitions with the internal nuclear matrix of chicken liver. Isolated chicken liver nuclei prepared by digestion with DNase I at 4°C or at room temperature were fractionated according to the procedure outlined in Fig. 46 or alternatively according to the procedures described by Kaufmann et al. (1983). Belgrader et al. (1991). 2-ME/NaCl S and P represent material solubilized by extraction with 1% v/v 2-mercaptoethanol/1.6 M NaCl (which includes the internal nuclear matrix) and the insoluble material (the nuclear pore-lamina complex), respectively. AS S represents the material solubilized by extraction with 0.2 M ammonium sulfate, and AS P represents the residual material insoluble in 0.2 M ammonium sulfate (the ammonium sulfate nuclear matrix). Between 200 and 500 µg protein per reaction was assayed and the relative distribution of histone deacetylase activity was determined by calculating the total activity in each subnuclear fraction. Each value represents the mean ± standard error from the mean from two or more experiments.

addressed this discrepancy by using histone deacetylase as a marker enzyme for the partitioning of the internal nuclear matrix during nuclear fractionation. Nuclei were digested with DNase I for one hour at 4°C or 23°C. Fig. 50 shows the results obtained from three separate experiments done at each temperature. When digestions were performed at 23°C with DNase I, approximately $50.5 \pm 4.4\%$ of the total nuclear histone deacetylase activity in chicken erythrocyte partitioned with the 0.4 M KCl/2.0 M KCl insoluble material (nuclear matrix). In contrast, when digestions were performed with DNase I at 4°C, over $79.5 \pm 3.3\%$ of the total nuclear deacetylase activity was solubilized by extraction of nuclei with 0.4 M KCl-containing buffer. A further $14.5 \pm 0.7\%$ of the total activity was extracted in 2.0 M KCl-containing buffer. Less than 6% of the histone deacetylase activity remained associated with the 0.4 M KCl/2.0 M KCl insoluble material. These observations suggest that the internal nuclear matrix of chicken erythrocyte is a fragile structure and that incubations at low temperature are sufficient to prepare nuclear matrices devoid of the internal nuclear matrix component. Figure 49 shows electron micrographs of 0.25 M ammonium sulfate and 0.25 M ammonium sulfate/2.0 M NaCl chicken immature erythrocyte matrices (these are homologous to the 0.4 M KCl and 0.4 M/2.0 M KCl matrices above). Considerable internal nuclear structure can be seen in contrast to the results of Lafond and Woodcock (1983).

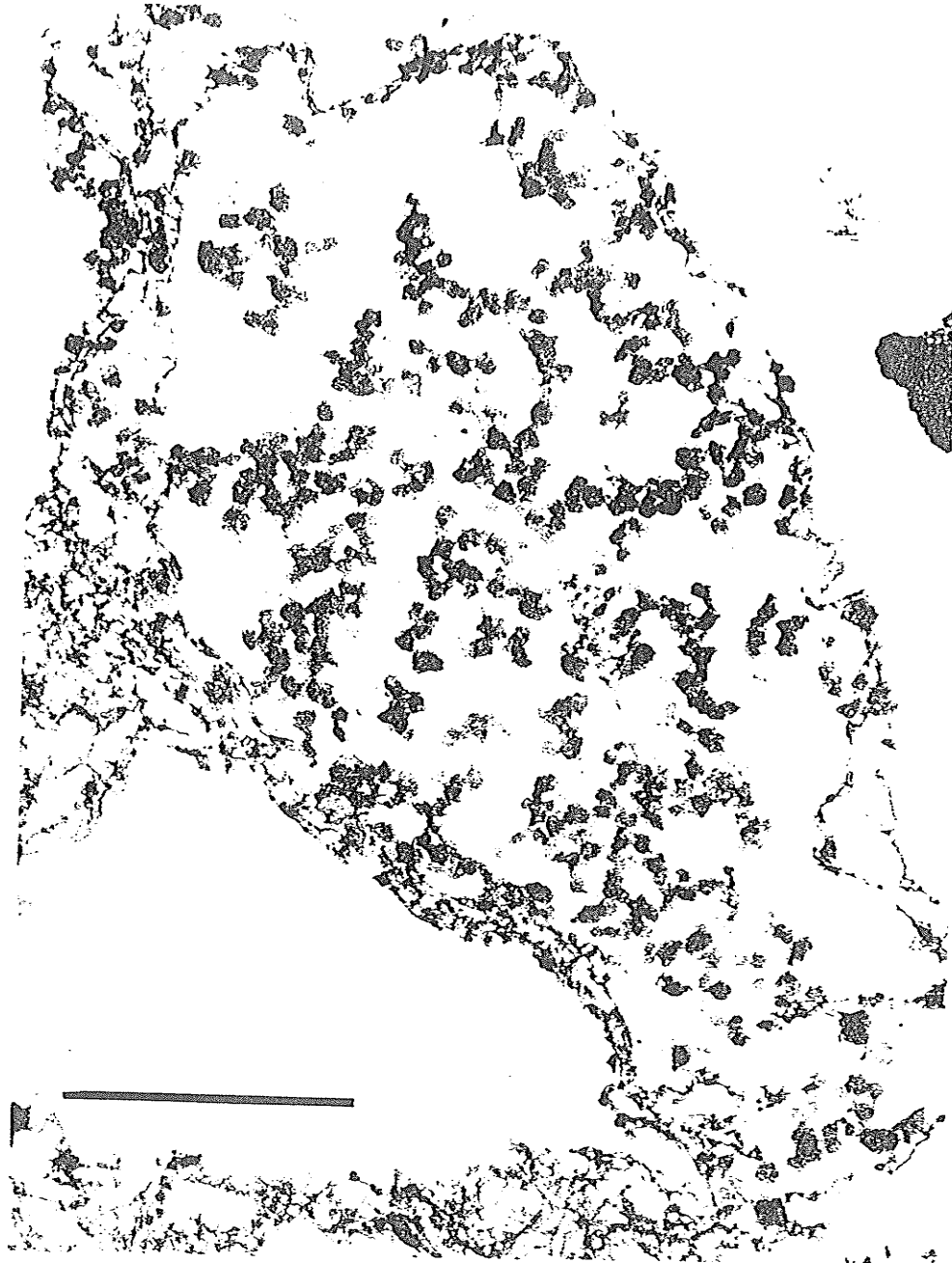


Figure 49. Visualization of the intermediate salt nuclear matrix of immature chicken erythrocyte by electron microscopy. Thin section electron micrographs were prepared from 0.25 M ammonium sulfate/DNase I/Triton X-100 nuclear matrices of chicken erythrocytes that had been fixed with 2.5 % gluteraldehyde. The magnification shown is 4400 x. (Data kindly provided by Dr. J.B. Rattner (University of Calgary)).

Histone deacetylase is a nuclear matrix in trout liver.

We next determined whether the association of the histone deacetylase activity with the internal nuclear matrix was species-specific. Nuclear matrices were prepared from trout liver nuclei that were incubated with DNase I at either 4°C or room temperature. Fig. 51 shows that, regardless of the digestion conditions used, the majority ($84.8 \pm 2.5\%$ at room temperature and $71.2 \pm 5.0\%$ at 4°C) of the histone deacetylase activity partitioned with the nuclear matrix (fraction 2.0 M KCl P). Further, trout liver nuclear pore-lamina complexes retained a low amount of the total histone deacetylase activity, with approximately 98% of the enzyme activity being present in the 1% 2-mercaptoethanol/1.6 M NaCl extract (not shown). Thus, we conclude that the association of histone deacetylase with the internal nuclear matrix is not a species-specific phenomenon.

Neoplastic transformation of trout liver does not alter the association of histone deacetylase with the nuclear matrix.

Neoplastic transformation of a cell is a consequence of the abnormal expression of certain genes. The aberrant expression of these genes may be a result of modification at the gene level and/or changes in chromatin and nuclear structure. Transformed cells have been shown to have altered nuclear matrix composition (Fey and Penman, 1988; Brancolini and Schneider, 1991; Getzenberg *et al.*, 1991; Kuzmina *et al.*, 1984). We investigated whether neoplastic transformation of trout liver altered the association of the histone deacetylase with the nuclear matrix. Aflatoxin B₁-induced trout hepatocellular carcinomas differ from the parent trout liver tissue in that they have an altered H1 subtype composition, more nuclease

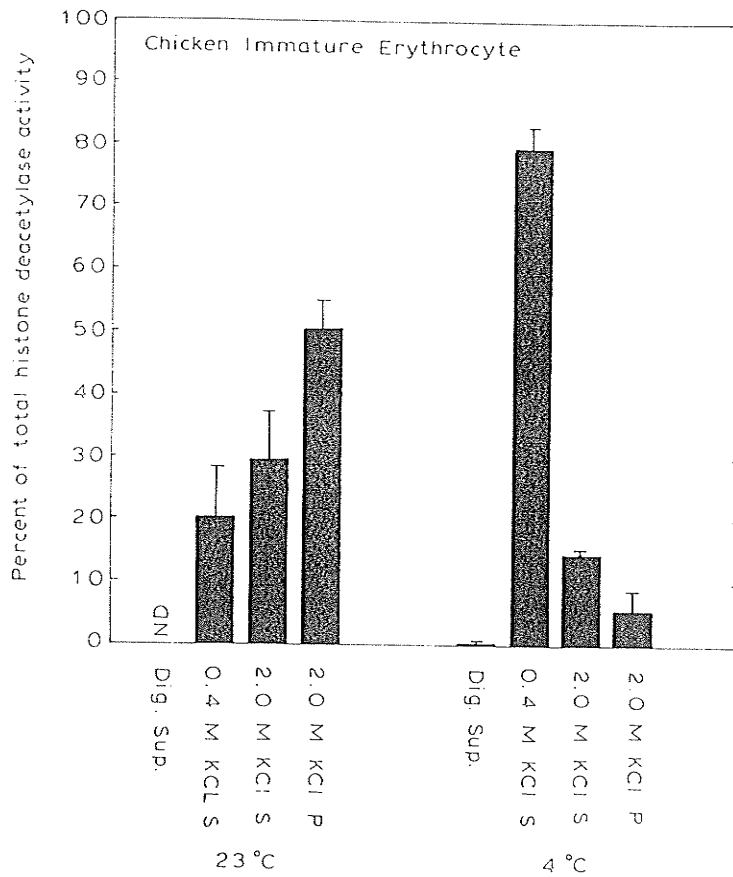


Figure 50. The nuclear matrix of chicken immature erythrocyte is destabilized by digestion at 4°C. Isolated chicken immature erythrocyte nuclei prepared by digestion with DNase I at 4°C or at room temperature were fractionated according to the procedure outlined in Fig. 47. Between 200 and 500 µg per reaction was assayed and the relative distribution of histone deacetylase activity was calculated from the total activity in each subnuclear fraction. ND represents non-detectable activity for the assayed fraction. Each value represents the mean ± standard error from the mean from three experiments.

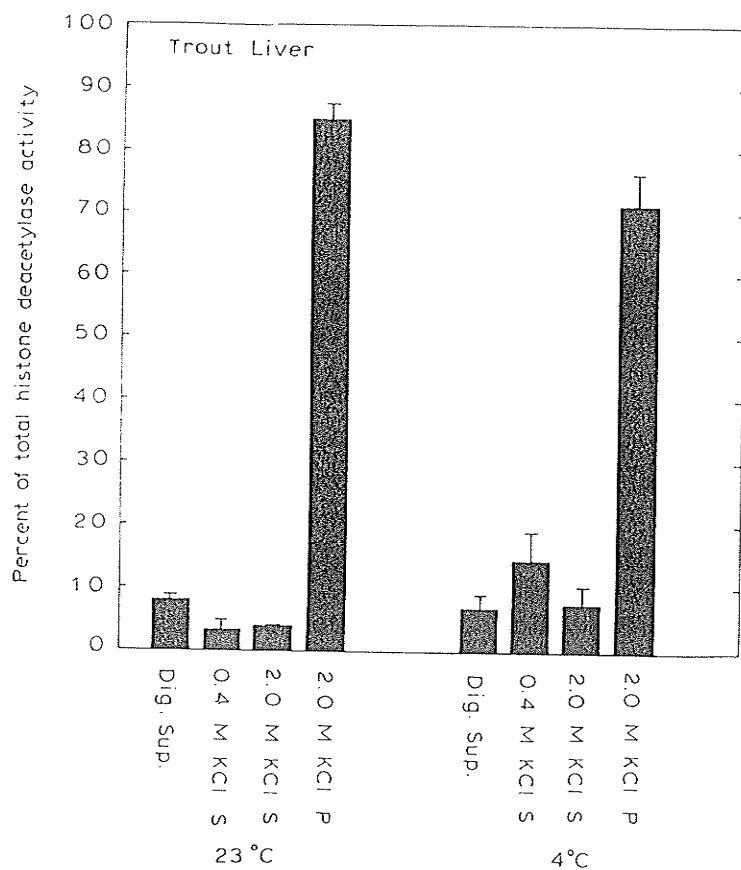


Figure 51. Histone deacetylase is associated with the nuclear matrix of trout liver. Isolated trout liver nuclei prepared by digestion with DNase I at 4°C or at room temperature were fractionated according to the procedure outlined in Fig. 46. Between 200 and 500 µg per reaction was assayed and the relative distribution of histone deacetylase activity was calculated from the total activity in each subnuclear fraction. Each value represents the mean ± standard error from the mean from two experiments.

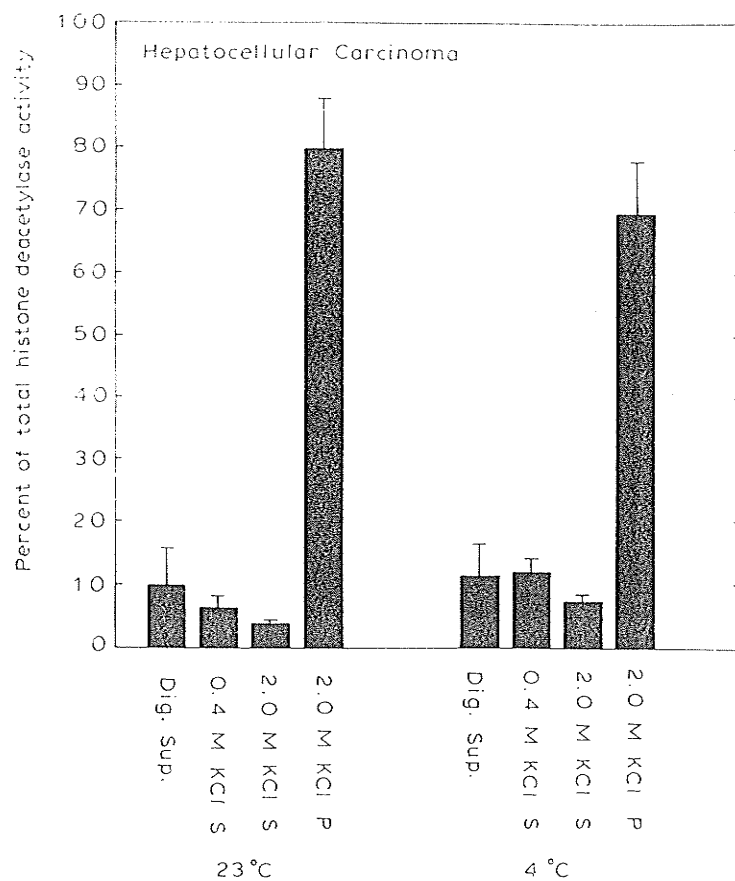


Figure 52. The nuclear matrix in trout hepatocellular carcinoma is resistant to low temperature destabilization. Trout aflatoxin B₁-induced hepatocellular carcinoma nuclei that were incubated with DNase I at 4°C or at room temperature were fractionated according to the procedure outlined in Fig. 46. Between 200 and 500 µg per reaction was assayed and the relative distribution of histone deacetylase activity was determined by extrapolating the total activity in each subnuclear fraction. Each value represents the mean ± standard error from the mean from two fractionations with individual tumors.

sensitive chromatin, and moderately or greatly enlarged hyperchromatic nuclei (Davie and Delcuve, 1991; Sinnhuber *et al.*, 1977). Nuclei isolated from individual hepatocellular carcinomas were incubated with DNase I at 23°C and 4°C. Fig. 52 shows that the distribution of histone deacetylase activity amongst the various nuclear subfractions from hepatocellular carcinoma and liver was similar. Of the total histone deacetylase activity, $79.8 \pm 7.9\%$ (n = 2) and $69.5 \pm 8.5\%$ (n = 2) was associated with the 0.4 M KCl/2.0 M KCl insoluble material (tumor nuclear matrix) at room temperature and 4°C, respectively. The partitioning of the histone deacetylase activity among the nuclear fractions of the individual tumors and livers were similar.

Histone Acetyltransferase and Histone Deacetylase are Functional Components of the core filaments of the internal nuclear matrix

Distribution of nuclear proteins during nuclear fractionation

Trout liver nuclei were isolated under isotonic conditions, and nuclear matrices were isolated by a mild procedure that has been shown to preserve nuclear matrix morphology and distribution of specific RNAs (Nakayasu and Berezney, 1991; Xing and Lawrence, 1991, He *et al.*, 1990). The nuclei were digested with DNase I followed by an extraction of the nuclease-fragmented chromatin with 0.25 M ammonium sulfate. Nuclear matrices of HeLa and MCF-7 cells prepared by this method contain an internal matrix of thick filaments. Further extraction of the nuclear matrices with 2 M NaCl exposes an internal nuclear matrix composed of a network of thinner fibers, referred to as the core filaments of the nuclear matrix (He *et al.*, 1990). There is now evidence that this network is comprised of intermediate-filament proteins homologous to those of the cytoskeleton (He *et al.*, 1990; Jackson and Cook, 1988; Yang *et al.*, 1992; Mirzayan *et al.*, 1992). This sequential intermediate and high salt extraction protocol reduces or eliminates the precipitation that may occur with a subset of the nuclear proteins during the standard high salt nuclear matrix isolation (He *et al.*, 1990). Fig. 53 shows the distribution of protein amongst the various nuclear fractions and the protein composition of each fraction. Fig. 53a shows that approximately 80% of the total nuclear protein was extracted in buffers containing 0.25 M ammonium sulfate (S1) following nuclease digestion. Another approximately 10% of the total nuclear protein was subsequently extracted with 2.0 M NaCl (S2) and the remaining 10% of the nuclear protein was found in the 0.25 M ammonium sulfate/2.0 M NaCl matrix (NM2). The distribution of total nuclear protein amongst these

fractions was similar to that previously reported for HeLa and MCF-7 cells (He *et al.*, 1990). Fig. 53b shows that the protein composition varied amongst the nuclear fractions. The nuclear matrix core filament fraction (NM2) was enriched in high molecular weight proteins. Fig. 53c shows that the 0.25 M ammonium sulfate soluble fraction contained most of the histone H1 and core histone proteins. Although there were approximately equal amounts of H1 and core histone proteins per μg of protein in both the S1 and S2 fractions, the total amount of protein was approximately 8-fold greater in the S1 fraction. The nuclear matrix core filament fraction was essentially devoid of the H1 and core histones. This provides evidence for the effectiveness of the solubilization of non-matrix proteins.

Histone acetyltransferase activity is associated with the internal nuclear matrix and the nuclear matrix core filaments

Fig. 54 shows that histone acetyltransferase activity was associated with the trout liver nuclear matrix. The 0.25 M ammonium sulfate nuclear matrix (NM1) retained 92.8% of the total histone acetyltransferase activity. Extraction of this nuclear matrix with 2.0 M NaCl liberated approximately 14.0% of the total histone acetyltransferase activity. The remaining 83.2% of the total histone acetyltransferase activity was associated with the 2.0 M NaCl nuclear matrix (NM2). Similar results were obtained with chicken erythrocyte and chicken liver demonstrating that this association was neither tissue- nor species-specific (data not shown). To determine whether the histone acetyltransferase activity was associated with the internal nuclear matrix or nuclear pore-lamina complex, nuclear pore-lamina complexes devoid of internal nuclear matrix were isolated (Kaufmann *et al.*, 1983). Trout liver nuclei digested with DNase I and RNase A were

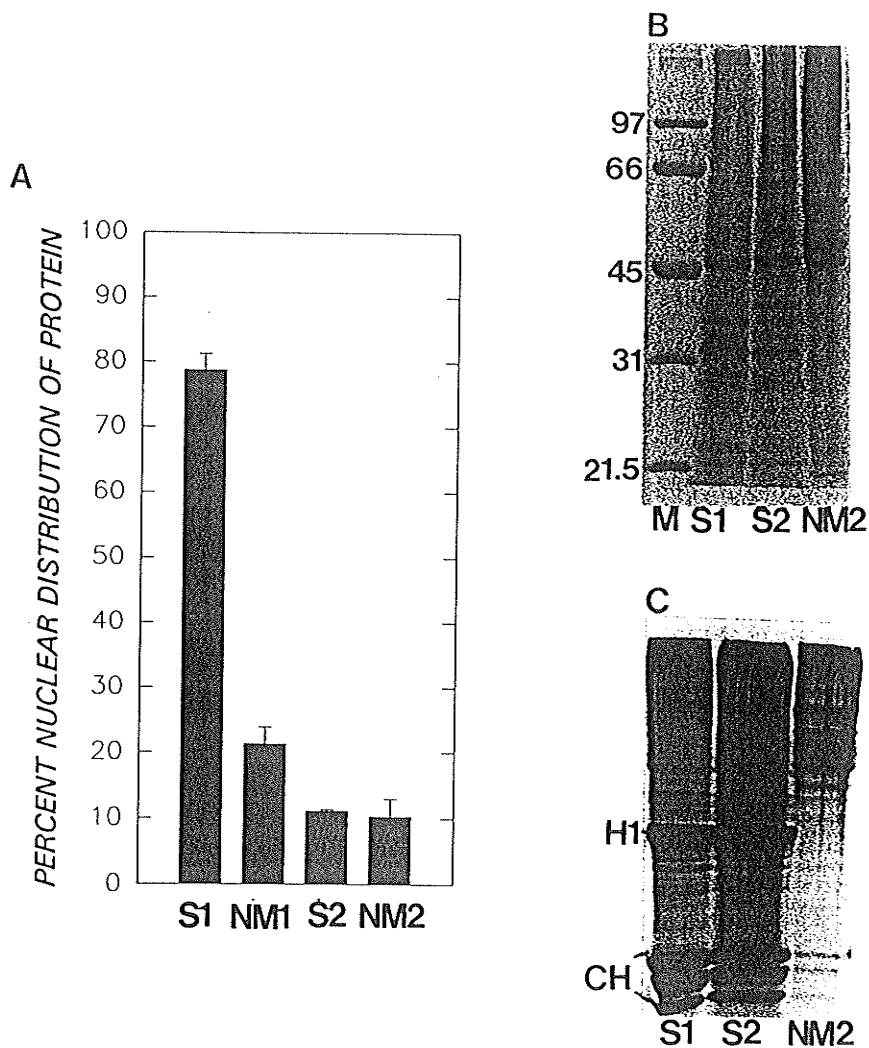


Figure 53 . Protein distribution and composition of isolated nuclear fractions. Fig. 1a shows the protein distribution amongst the various fractions of trout liver nuclei. Protein content was determined by the BioRad protein microassay (BioRad) according to manufacturer's instructions. Each value represents the mean \pm S.E. from the mean from three separate experiments. The fractions are 0.25 M ammonium sulfate soluble (S1), 0.25 M ammonium sulfate insoluble (NM1), 2.0 M NaCl soluble (S2), and 0.25 M ammonium sulfate/ 2.0 M NaCl insoluble (NM2). The values for NM1 were determined by adding the values obtained for S2 and NM2. b and c show the protein content of the 0.25 M ammonium sulfate soluble (S1), 2.0 M NaCl soluble (S2), and the nuclear matrix core filament fraction (NM2). Approximately 10 μ g of protein was resolved on an 8% polyacrylamide SDS gel (b) and 20 μ g of protein was resolved on a 15% polyacrylamide SDS gel (c). Molecular weight markers (BioRad low molecular weight gel standards) are shown for Fig. 1b (lane M). The proteins were stained with Coomassie brilliant blue stain.

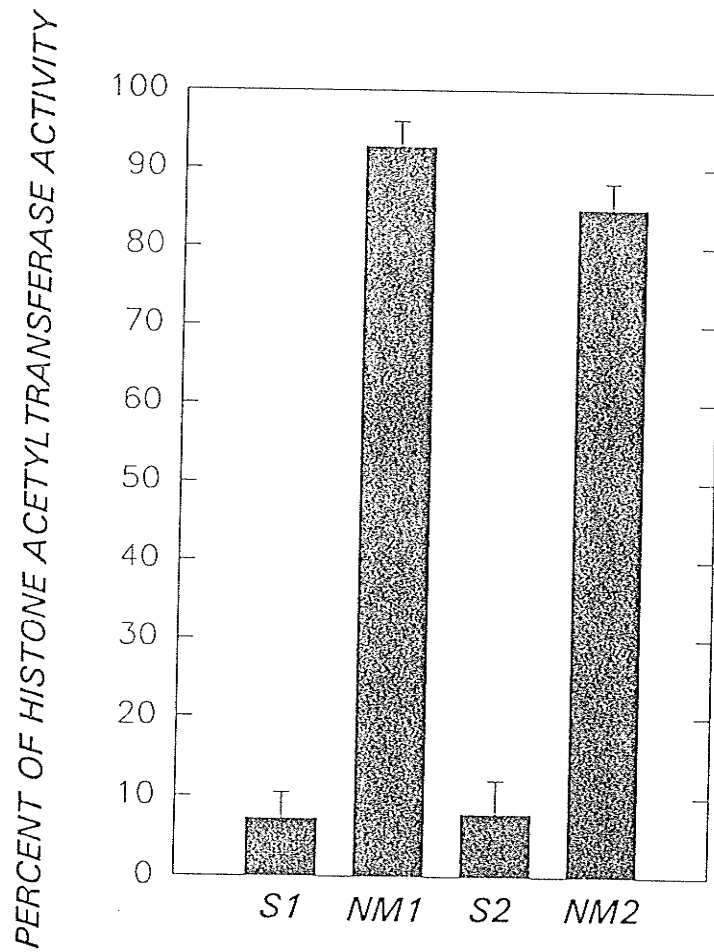


Figure 54. Histone acetyltransferase is a component of the nuclear matrix. Isolated nuclear fractions were assayed for histone acetyltransferase activity as described in *materials and methods*. Each value represents the mean \pm S.E. from the mean from three experiments.

extracted with 1.6 M NaCl and 1% 2-mercaptoethanol. 83.1 ± 4.5 % (S.E.M., n=3) of the total histone acetyltransferase activity was solubilized using this treatment. Thus, nuclear pore-lamina complex preparations were significantly depleted of histone acetyltransferase activity. This result demonstrated that histone acetyltransferase was associated with the internal nuclear matrix core filaments. Fluorographic analysis of the labeled histones recovered from the 2.0 M NaCl nuclear matrix (NM2) that was assayed for histone acetyltransferase activity showed that the enzyme preferred histone H3>H2A=H4>H1 (Fig. 55). The substrate specificity of the nuclear matrix-bound enzyme was similar to those previously reported for nuclear acetyltransferases (Attisano and Lewis, 1990).

Histone deacetylase is a component of the nuclear matrix core filament structure

We have demonstrated that nuclear matrices, but not nuclear pore-lamina complexes, retained the majority (approximately 85%) of the histone deacetylase activity of trout liver. The histone deacetylase activity was also associated with nuclear matrices from chicken liver and chicken erythrocyte demonstrating that this association is neither tissue- nor species-specific. This observation provided evidence that histone deacetylase was a nuclear matrix, a component of the internal nuclear matrix (Nakayasu and Berezney, 1991). To determine whether histone deacetylase activity was associated with the thick filaments or the thinner core filaments (intermediate filament network) of the trout liver internal nuclear matrix, the 0.25 M ammonium sulfate and 0.25 M ammonium sulfate/2 M NaCl liver nuclear matrices were assayed for histone deacetylase activity. Fig. 56 shows that the majority of the histone deacetylase activity was associated with the

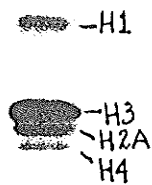


Figure 55. Substrate-specificity of NM2-associated histone acetyltransferase. The 0.25 M ammonium sulfate/2.0 M NaCl nuclear matrices were incubated with 100 μ g of calf thymus histones in the presence of ^3H -acetyl CoA for 60 min at 37°C. Isolated histones were resolved on a 15 % polyacrylamide SDS gel and analyzed by fluorography.

0.25 M ammonium sulfate matrix (NM1). Further extraction of nuclear matrix proteins with 2 M NaCl removed less than 3% of the histone deacetylase activity. Approximately 80% of the total histone deacetylase activity was retained by the 0.25 M ammonium sulfate/2 M NaCl matrix (NM2), demonstrating that this enzyme was associated with the internal nuclear matrix core filaments of trout liver nuclei.

Histone Deacetylase is associated with internal nuclear matrix through disulfide bonds that exist *in situ*

Both nuclear RNA and disulfide bonds that exist *in situ* are essential in maintaining the structural integrity of the internal nuclear matrix (Belgrader *et al.*, 1991; Kaufmann *et al.*, 1983). To investigate the nature of the interaction of the histone acetyltransferase and deacetylase with the internal nuclear matrix, the 0.25 M ammonium sulfate matrix was treated with 2 M NaCl and either RNase A, 2-mercaptoethanol or RNase A plus 2-mercaptoethanol. Fig. 57 shows that approximately 60% of the histone acetyltransferase activity and approximately 80% of the histone deacetylase activity were released from 0.25 M ammonium sulfate matrix with 2 M NaCl and 2-mercaptoethanol. Incubating the 0.25 M ammonium sulfate matrix with RNase A alone and subsequent extraction of the matrix with 2 M NaCl was ineffective in removing either of the enzyme activities from the matrix (data not shown). RNase A plus 2-mercaptoethanol treatment was no more efficient than 2-mercaptoethanol alone in solubilizing the enzymes (Fig. 57). These observations indicate that histone deacetylase and acetyltransferase are bound to the internal nuclear core filament network via disulfide bonds. These disulfide bonds may have formed during the isolation of the nuclear

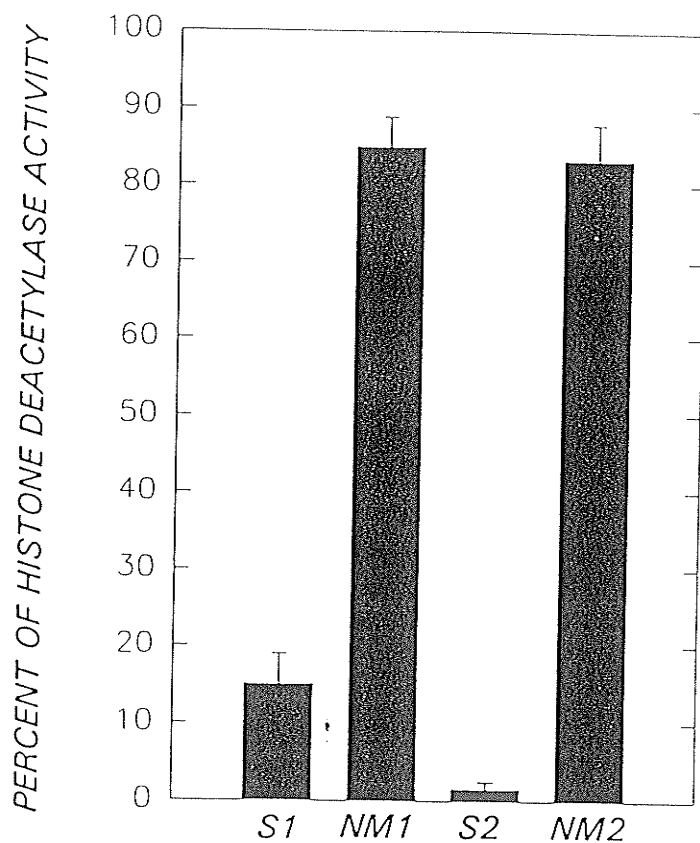


Figure 56. Histone deacetylase is a component of the nuclear matrix core filaments. Isolated nuclear fractions are as described in Fig. 54. Each value represents the mean \pm S.E. from the mean from three experiments.

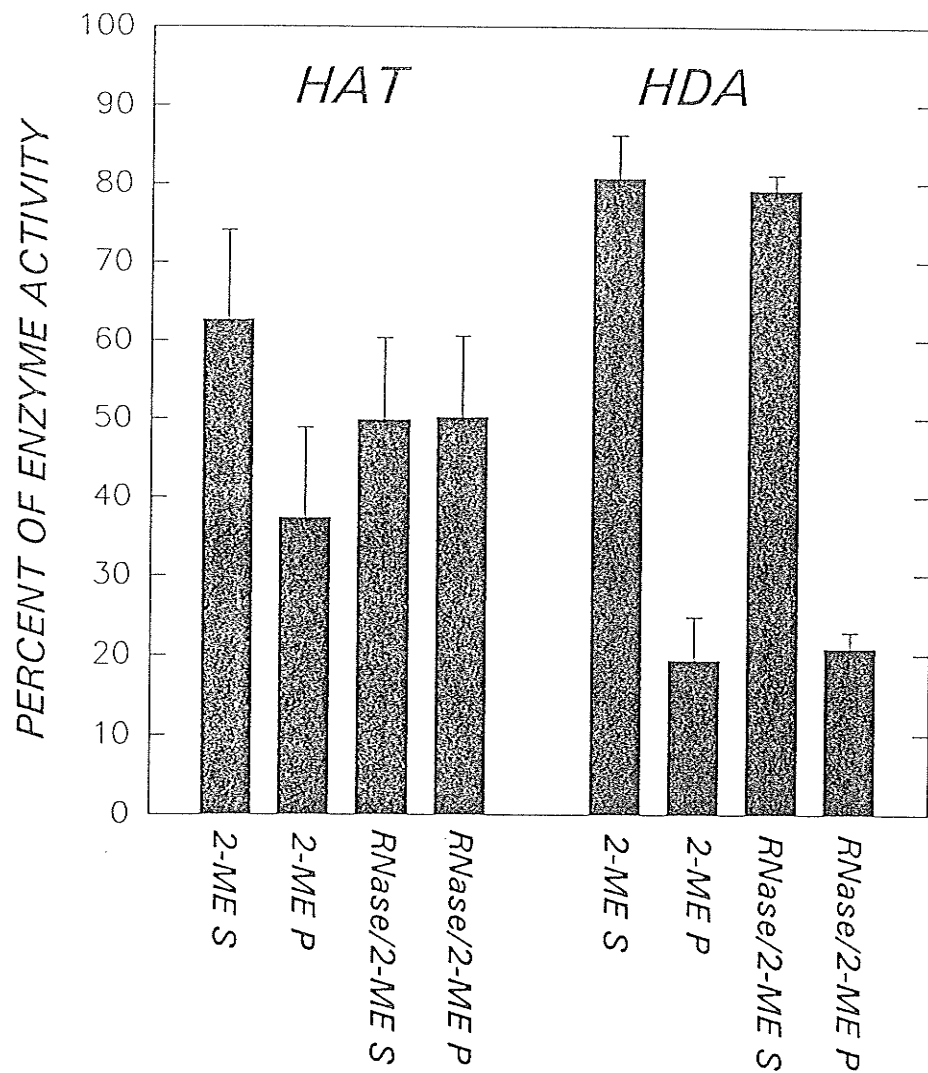


Figure 57. Role of RNA and disulfide crosslinks in the association of histone acetyltransferase and deacetylase with the nuclear matrix. Fraction 2-ME S, RNase/2-ME S, 2-ME P, and RNase/2-ME P represent soluble and insoluble fractions after treatment of the 0.25 M ammonium sulfate matrices with 2-mercaptoethanol and/or RNase A as described in "Materials and methods". HAT and HDA represent histone acetyltransferase and histone deacetylase activities, respectively. Each value represents the mean \pm S.E. from the mean from two or three experiments.

matrix or they may be present *in situ*. To discern between these two possibilities, the 0.25 M ammonium sulfate/2 M NaCl nuclear matrices were isolated in the presence of the sulfhydryl alkylating agent, iodoacetamide. The iodoacetamide treatment had very little effect on the retention of the histone deacetylase activity to the nuclear matrix. For nuclear matrices isolated in the presence of 10 mM iodoacetamide, 67.1 ± 5.3 % (S.E.M., $n=3$) of the total nuclear histone deacetylase activity was retained in the 0.25 M ammonium sulfate/2 M NaCl nuclear matrices. (Because reactive sulfhydryl groups are necessary for histone acetyltransferase activity, the activity of histone acetyltransferase was not determined). This result suggests that the association of the histone deacetylase with the nuclear matrix was not a consequence of disulfide bonds forming during isolation of the nuclear matrix in the absence of iodoacetamide, but depended on disulfide bonds that exist *in situ*.

Discussion

The insolubility of transcriptionally active gene chromatin during nuclear fractionation is a consistent but unexplained phenomena (see Delcuve and Davie, 1989 for a discussion on this point). Dynamic histone acetylation is associated with transcriptionally active gene chromatin (Ip *et al.*, 1988; Table 1). Additionally, the bulk of dynamically acetylated chromatin is insoluble following nuclease digestion and nuclear fractionation (Table 1). Dynamically acetylated chromatin must interact with high frequency with the histone acetyltransferases and deacetylases to maintain its dynamic character. The insolubility of these chromatin fragments suggests that the histone acetyltransferase and deacetylase enzymes may be components of the internal nuclear matrix. We propose that this insolubility is due in whole or in part to the dynamic association with the nuclear matrix associated histone acetyltransferase and histone deacetylase. That is the enzymes, which are targeted to the nuclear matrix perhaps by a mechanism such as that shown for *Su(w^a)* protein (Li and Bingham, 1991), organize the transcribed chromatin on the nuclear matrix. In this respect, these enzymes can be thought to play structural roles within the nucleus. The association of these enzymes with transcriptionally-engaged chromatin may target transcribed chromatin to specific nuclear compartments and may facilitate the processing of hnRNA by colocalizing the template-active chromatin with the nuclear matrix-associated splicing machinery.

Current evidence strongly supports the existence of an internal nuclear matrix that plays a role in the spatial organization of nuclear DNA as well as compartmentalizing and providing a structural framework from which several nuclear processes, such as RNA transcription, transport, and processing, occur (reviewed in Berezney, 1991; Carter and Lawrence, 1991;

van Driel *et al.*, 1991). Additionally, recent results indicate that an underlying intermediate-filament network homologous to that of the cytoskeleton provides the structural framework for the nuclear matrix (He *et al.*, 1990; Jackson and Cook, 1988; Yang *et al.*, 1992; Mirzayan *et al.*, 1992). Our results provide evidence that histone acetyltransferase and deacetylase are components of this intermediate filament network, the internal nuclear matrix core filament structure. These results may seem at odds with the literature which consistently report the solubilization of histone acetyltransferase activities from intact nuclei using moderate (i.e., around 0.5 M) salt concentrations. However, the solubility of nuclear matrix proteins in high salt solutions varies and nuclear matrix proteins are not quantitatively retained with nuclear matrix preparations. Indeed internal nuclear matrices, contrary to common perception, may be quite labile depending upon the method of preparation. Often stabilization steps are required to retain internal nuclear structure (van Driel *et al.*, 1991; Berezney, 1991). Additionally, the stability of internal nuclear matrices during preparation may vary from tissue to tissue and some tissues may have completely labile internal nuclear matrices under some preparation conditions. We have provided biochemical evidence that this occurs with chicken erythrocyte nuclear matrices isolated from cells digested at 4°C with DNase I. Perhaps more important in considering the literature regarding histone acetyltransferase is that the previously reported soluble histone acetyltransferase activities were extracted with buffers containing reducing agents (e.g., Attisano and Lewis, 1990 and references therein). Thus, the apparent discrepancy between this report and the literature may be resolved by our observation that histone acetyltransferase is retained in nuclear matrix preparations by disulfide bonds. (For a discussion of the importance

of disulfide bonds in maintaining internal nuclear matrix structure see recent reviews by van Driel *et al.* (1991) and Berezney (1991)). This observation supports the hypothesis that there exist disulfide bonds *in vivo* which are essential for the integrity of the internal nuclear matrix (Stuurman *et al.*, 1992).

We propose that histone acetyltransferase and deacetylase have dual roles, one being enzymatic catalyzing rapid acetylation-deacetylation and the other being structural. In this respect, these enzymes resemble topoisomerase II (Adachi *et al.*, 1991). A mechanism of nuclear targeting capable of establishing such nuclear organization has a precedence in the targeting domains of DNA methyltransferase (Leonhardt *et al.*, 1992) and the RS domain (Li and Bingham, 1991). Histone acetyltransferase and histone deacetylase play important roles in maintaining the structure of transcriptionally active chromatin. The rapid turnover of acetyl groups of highly acetylated histones affects DNA topology with hyperacetylated histones reducing the linking number change per nucleosome (Norton *et al.*, 1989; Norton *et al.*, 1990; Thomsen *et al.*, 1991). Histone acetylation and DNA topology may also play an important regulatory role in the function of certain MARs by both localizing them to the nuclear matrix and by modulating their single-strandedness by influencing local topology (Bode *et al.*, 1992; Klehr *et al.*, 1992). Further, dynamic histone acetylation favors decondensation of higher order structure (Ridsdale *et al.*, 1990; Ausio, 1992).

The association of dynamic histone acetylation with transcription (Ip *et al.*, 1988; Boffa *et al.*, 1990; Ridsdale *et al.*, 1990; see also Part 1) allow this data to be interpreted in the context of the emerging data on the nuclear compartmentalization of transcription. Novel fluorescent microscopy

experiments have demonstrated that transcription and processing of RNA occur at discrete nuclear foci (Kopczynski and Muskavitch, 1992; Carter *et al.*, 1991; Xing *et al.*, 1991; Carter and Lawrence, 1991; van Driel *et al.*, 1991). Importantly, indirect immunofluorescence experiments show that RNA polymerase II localizes to discrete nuclear domains (van Driel *et al.*, 1991; Elliot *et al.*, 1992) supporting the previously controversial experiments which demonstrated an association of active RNA polymerases with the nuclear matrix (Jackson and Cook, 1985; reviewed in Cook, 1989 and Jackson 1990). Solid state models for gene expression are very attractive because of their inherent efficiency. Solid-state processing and transport of newly synthesized RNA efficiently provide high localized concentrations of the splicing machinery and a mechanism to localize specific transcripts to particular sites within the cytosol (Lawrence and Singer, 1986). Additionally, enzymes can be organized on fixed nuclear structures such that there is the orderly processing of biomolecules in a manner where products of one reaction can serve as substrates for the next. It is difficult to imagine how diffusion-mediated soluble processes would allow such organization. Given the viscosity of the nucleoplasm, diffusion-mediated processes would be expected to be extremely inefficient (Carter and Lawrence, 1991). The solid-state model requires proteins to function in the structural organization of the transcribed genes and nuclear matrix-associated splicing and transport factors. The histone acetyltransferase and deacetylase may function in the structural organization of transcriptionally active genes within "transcript domains". Such a model for histone acetylation is shown in Fig. 58.

Solid-State Model for Transcription-Associated Dynamic Histone Acetylation

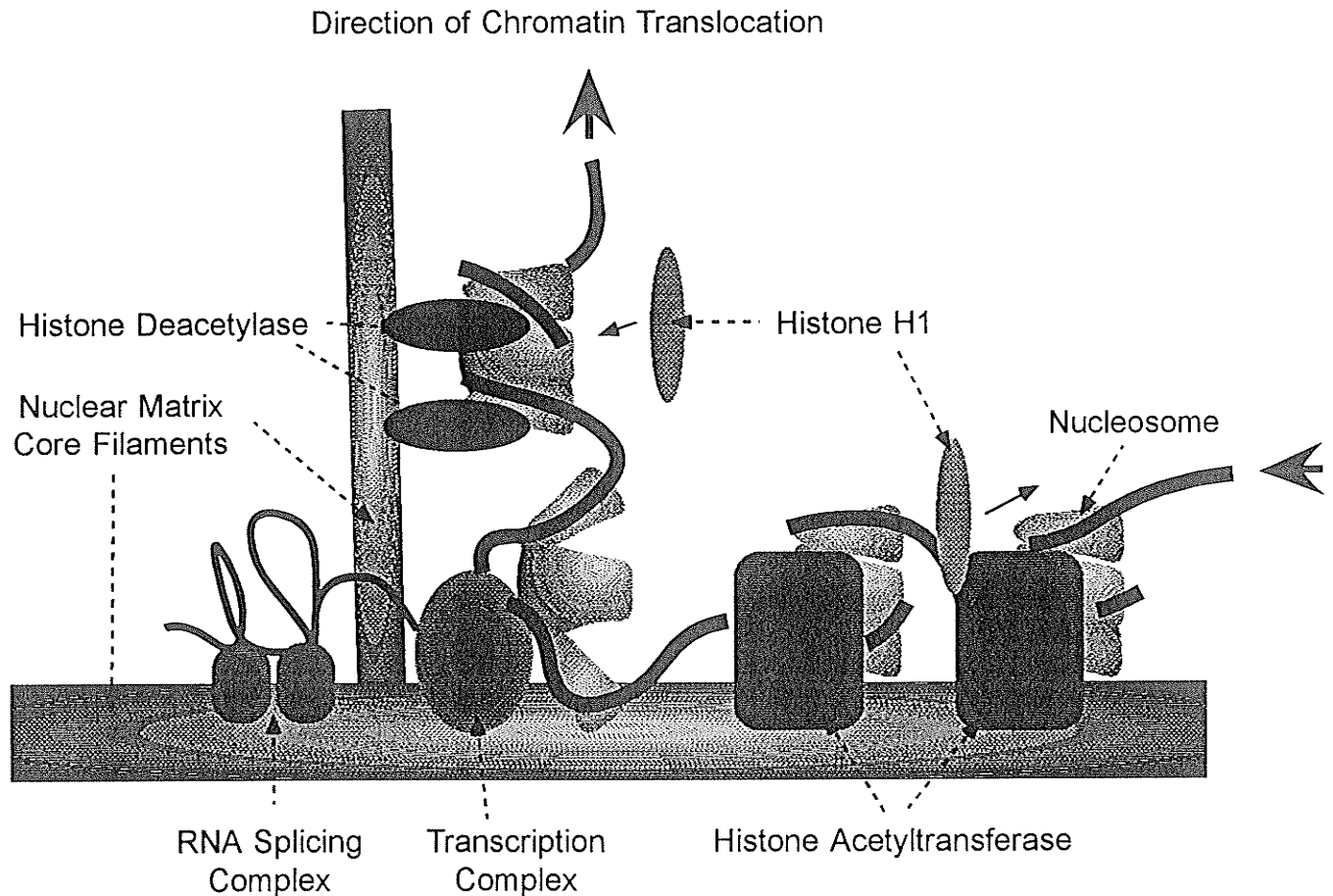


Figure 58. A model for the association of histone acetyltransferases and histone deacetylases with transcribed chromatin. Histone acetyltransferase and histone deacetylase colocalize with RNA polymerases and RNA processing machinery at sites on the nuclear matrix core filaments. The chromatin is translocated past these sites of chromatin processing where the chromatin may be sequentially acetylated, transcribed, and deacetylated. Histone H1 may be released by increased levels of acetylation ahead of the elongating polymerase and may rebind following deacetylation.

Conclusions and Comments

This thesis addressed two types of questions regarding chromatin structure. The first question was: what features distinguish transcriptionally active gene chromatin from bulk chromatin? The second question addressed was: what are the mechanisms responsible for a nonrandom distribution of dynamically acetylated chromatin throughout the genome?

Biochemical Features of Transcriptionally Active Chromatin

Transcriptionally active gene chromatin differs from that of bulk chromatin (Gross and Garrard, 1987; Reeves, 1984; Ausio, 1992). It is likely that some of these biochemical differences may be important for the unfolding of the 30 nm chromatin fibre and for the structural transitions of the nucleosome which must occur during transcription. Currently, there is a great interest in examining gene expression in model *in vitro* transcription systems in which the DNA template is reconstituted into chromatin. It is argued, and current experimental evidence supports, that chromatin contributes significantly to the regulation of gene expression and that transcription factors may have different properties in the context of chromatin templates. The studies of Laybourn and Kadonaga (1991;1992) have been amongst the most informative in this respect. If this argument is correct then it is important to reconstitute chromatin which has biochemical features similar to that of transcribed gene chromatin *in vivo*. This has been essentially ignored to date. Most studies have used bulk histones extracted from nuclei or present in chromatin assembly extract of nuclei.

The studies presented in this thesis made the following contributions to the literature on transcribed gene chromatin. We found that ongoing

histone methylation was associated with the dynamically acetylated chromatin of chicken immature erythrocytes. The literature was and remains confusing surrounding histone methylation. The association of histone methylation and dynamic histone acetylation observed in this and other studies cited herein may be a consequence of a highly compartmentalized nucleus.

We examined the incorporation of newly synthesized histones into the chromatin of immature chicken erythrocytes. This study made an important contribution to our understanding of transcriptionally active gene chromatin. The results demonstrated that transcribed chromatin and chromatin which is transcriptionally competent readily exchange nucleosomal histones with a newly synthesized histone pool. Because this association was not transcription dependent, it is unlikely that the exchange that we observed is due to mechanisms such as displacement of nucleosomes by positive supercoils ahead of the elongating polymerase. Rather, these results imply that the nucleosomes of transcriptionally active and competent chromatin are structurally destabilized such that their composition becomes dynamic. That is, they can be remodeled by exchange with histones which are biochemically different (e.g., histone variants, ubiquitinated histones). In addition, they may exist as hexamers deficient in one H2A-H2B dimer for brief periods of time. The results of our work also suggested that chromatin may be modified by ubiquitination through exchange with a histone pool which is extensively ubiquitinated. It is possible that the structural modifications which result in an ability to exchange histones is also necessary for nucleosome unfolding during transcriptional elongation.

We have investigated histone acetylation dynamics and obtained novel information on the dynamic process of histone acetylation within the

cell. In particular, we provided evidence that the dynamically acetylated species of histones were associated with transcriptionally active gene chromatin and that most of this chromatin was associated with the nuclear matrix. A second class of acetylation, which is characterized by having a very slow turnover rate and occurs predominantly in the lower acetylated isoforms of the core histones, was distributed throughout chromatin but was enriched in transcriptionally active/competent gene chromatin. The results also inferred a third class of acetylation that is metabolically inactive and whose acetylation states do not change during interphase. This is the predominant class of acetylation in repressed gene chromatin. These last two classes of histone acetylation cannot be distinguished by the more common technique of analyzing steady state levels of acetylation on acetic acid-urea polyacrylamide gels.

One of the major contributions of this study to the literature regarding the biochemical composition of transcribed chromatin was our investigation of insoluble chromatin fractions. Investigators have had a tendency to discard all insoluble material because it is difficult to work with. However, it is well established that transcriptionally active chromatin has an insoluble nature during nuclear fractionation. Recent results demonstrate that this reflects an association with the nuclear matrix and this association is transcription-related (Andreeva *et al.*, 1992). We determined that the active gene-enriched residual insoluble nuclear material contained most of the dynamically acetylated chromatin, chromatin which was undergoing histone methylation, and chromatin which was participating in histone exchange. Because very recent literature is beginning to suggest that the internal nuclear matrix may be the site of the transcription process, our biochemical characterization of the residual insoluble nuclear material-associated

chromatin from immature chicken erythrocyte cells is collectively the most thorough study of the biochemical features of this insoluble class of chromatin. The recent studies on the association between the nuclear matrix and metabolic nuclear processes suggests that it may be these chromatin fragments which are the most important to study when trying to understand the relationship between chromatin structure and transcription.

Collectively, this group of work made significant contributions towards the characterization of transcriptionally active gene chromatin. All of the work supports a distinction between transcribed regions of chromatin and transcriptionally repressed regions of chromatin on the basis of nucleosome dynamics and metabolism.

The second question addressed in this thesis is what are the mechanisms responsible for a nonrandom distribution of dynamically acetylated chromatin throughout the genome? In beginning this study, we had a working model in which histone deacetylases and histone acetyltransferases were enzymes in equilibrium between a soluble state and a chromatin-bound state and that transcribed gene chromatin had an abundance of chromatin-bound histone acetyltransferase and histone deacetylase throughout the domain. The finding that the majority of the dynamically acetylated histones and the majority of the histone deacetylase activity partitioned with the insoluble nuclear fraction during nuclear lysis was truly a surprise. However, there has been an explosion of research involving nuclear compartmentalization and the structural role of the internal nuclear matrix in this compartmentalization during the past two years. It is in this context that what seemed surprising two years ago when we began these studies now makes a great deal of sense. We have provided solid evidence that both the histone acetyltransferase(s) and histone

deacetylase(s) have significant pools of activity associated with the internal nuclear matrix. We have provided evidence that this association occurs via disulfide crosslinks that exist *in situ*. There is now considerable evidence for an important role of disulfide crosslinks in maintaining the structural integrity of the internal nuclear matrix (Berezney, 1991) and so this observation is consistent with what may be expected from a native nuclear matrix protein.

The implications of a solid-state process of histone acetylation add a great deal to our understanding of relationships between processes in chromatin. But in doing so, they require a movement away from the more common molecular biology model of nuclear function and a movement toward an emerging model of nuclear compartmentalization on a nuclear matrix that has arisen primarily from fluorescent microscopy studies. Fortunately, there is now a large body of evidence which supports the nuclear matrix model and recent immunofluorescent data (van Driel *et al.*, 1991) now confirm the original observations of Jackson and Cook (1985) that RNA polymerase II is nuclear matrix-associated. In this model, the mechanism of establishing metabolic differences between different regions of the genome (such as we observed for dynamic and nondynamic histone acetylation) is by compartmentalizing the nucleus into regions which are either associated with processing machinery or not. Importantly, protein domains which impart such organization when expressed in living cells have been identified (Li and Bingham, 1991; Leonhardt *et al.*, 1992). Nuclear compartmentalization models about a nuclear matrix provide a simple explanation for the commonly observed association between histone acetylation and transcription. Nuclear compartmentalization models also offer an explanation for associations such as that which we observed

between histone acetylation and histone methylation. Thus, I believe that an important contribution of these studies is that they should encourage the consideration of alternative models of nuclear structure/function and this may be important for the stimulation of new approaches to studying structure-function relationships in eukaryotic nuclei.

A model for the Establishment and Maintenance of the Transcriptionally Active Chromatin Structure

I have summarized the data in the context of a model (Fig. 59) that accommodates all of the results which I have obtained. The figure shows a transcriptionally active domain which is organized around the nuclear matrix. The transcribed domain is localized at a discrete nuclear site containing nuclear matrix-associated processing and transport machinery. Targeting sequences on histone acetyltransferases and histone deacetylases may be in part responsible for this localization. The promoter and enhancer regions are nucleated around an LCR which contains some contacts with the nuclear matrix. Nuclear matrix-associated histone acetyltransferases, histone deacetylases, and RNA polymerases are organized on the nuclear matrix such that the chromatin is acetylated prior to transcribing of the nucleosome. This acetylation destabilizes histone H1(H5) binding and histone H1(H5) may be displaced. Following elongation through the nucleosome, histone deacetylase deacetylates the nucleosomal histones. Newly synthesized histone H1(H5) may then redeposit on the fiber. Generally high levels of histone acetylation throughout the domain (perhaps mediated through additional dynamic contacts with the nuclear matrix)

maintain a decondensed chromatin structure. It is interesting to speculate, in the context of this model, that transcription-dependent ubiquitination of histone H2B is a nuclear-matrix associated event. Generally high levels of posttranslational modification act in synergy to destabilize the nucleosomes facilitating an exchange of principally H2A/H2B dimers with a histone pool extensively ubiquitinated and containing the histone H3 minor variant H3.3. The lability of the histone H2A/H2B dimers may be important for transcription through the nucleosome (see Van Holde *et al.*, 1992; Morse, 1992). A chromatin-bound histone methyltransferase is also present. These processes facilitate the remodeling of the histone composition of the transcribed domain. These processes may be involved in facilitating the transcriptional elongation process.

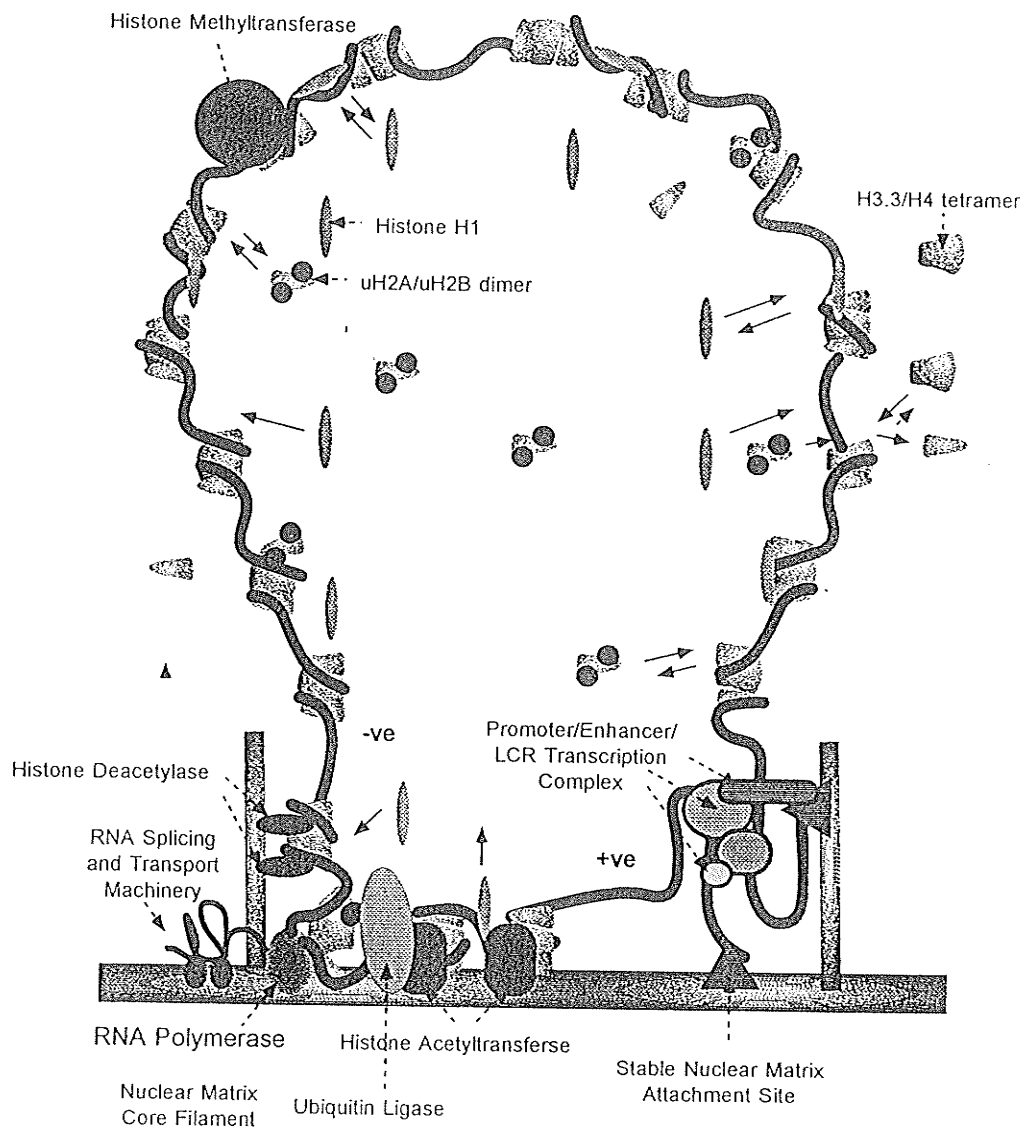


Figure 59. A model for the organization of transcriptionally active gene chromatin. See text for details.

References

- Adachi, Y., Luke M., and Laemmli, U.K. (1991). Chromosome assembly *in vitro*: topoisomerase II is required for chromatin condensation. *Cell* **64**, 137-148.
- Affolter, M., Cote, J., Renaud, J. and Ruiz-Carrillo, A. (1987). Regulation of histone H5 and β^A - globin gene expression during differentiation of the chicken erythroid cell. *Mol. Cell. Biol.* **7**, 3663-3672.
- Allegra, P., Sterner, R., Clayton, D. F., and Allfrey, V.G. (1987). Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. *J. Mol. Biol.* **196**, 379-388.
- Allfrey, V.G. (1980). Molecular aspects of the regulation of eukaryotic transcription-nucleosomal proteins and their postsynthetic modifications in the control of DNA conformation and template function. In *Cell Biology: A Comprehensive Treatise*, Vol. 3 (eds. L. Goldstein and D.M. Prescott). Academic Press Inc.
- Almouzni, G., Mechali, M., and Wolffe, A.P. (1990). Competition between transcription complex assembly and chromatin assembly on replicating DNA. *EMBO J.* **9**, 573-582.
- Andreeva, M., Markova, D., Loidl, P., and Djondjurov, L. (1992). Intracellular compartmentalization of transcribed and nontranscribed c-myc sequences in Namalva-S cells. *Eur. J. Biochem.* **207**, 887-894.
- Aniello, F., Branno, M., Geraci, G., and Tosi, L. (1989). Histone-lysine methyltransferase activity from sea-urchin embryo nuclei. Changes in substrate specificity upon purification. *Biochim. Biophys. Acta* **1008**, 31-38.
- Arents, G., Burlingame, R.W., Wang, B.-C., Love, W.E., and Moudrianakis, E.N. (1991). The nucleosomal core histone octamer at 3.1 Å resolution: A tripartite protein assembly and left-handed superhelix. *Proc. Natl. Acad. Sci.*, **88**, 10148-10152,
- Arrigo, A.-P. (1983). Acetylation and methylation patterns of core histones are modified after heat or arsenite treatment of *Drosophila* tissue culture cells. *Nucleic Acids Res.* **11**, 1389-1404.

- Attisano, L. and Lewis, P.N. (1990). Purification and characterization of two porcine liver nuclear histone acetyltransferases. *J. Biol. Chem.*, **265**, 3949-3955.
- Ausio, J. (1992). Structure and dynamics of transcriptionally active chromatin. *J. Cell Sci.* **102**, 1-5.
- Ausio, J. and van Holde, K.E. (1986). Histone hyperacetylation: its effects on nucleosome conformation and stability. *Biochemistry* **25**, 1421-1428.
- Baer, B.W. and Rhodes, D. (1983). Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes. *Nature* **301**, 482-488.
- Barrack, E.R. and Coffey, D.S. (1980). The specific binding of estrogens and androgens to the nuclear matrix of sex hormone responsive tissues. *J. Biol. Chem.* **255**, 7265-7275.
- Becker, P.B. and Wu, C. (1992). Cell-free system for the assembly of transcriptionally repressed chromatin from *Drosophila* embryos. *Mol. Cell. Biol.* **12**, 2241-2249.
- Becker, P.B., Rabindran, S.K., and Wu, C. (1991). Heat shock-regulated transcription *in vitro* from a reconstituted chromatin template. *Proc. Natl. Acad. Sci.* **88**, 4109-4113.
- Belgrader, P., Siegel, A.J. and Berezney, R. (1991). A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. *J. Cell Sci.* **98**, 281-291.
- Belikoff, E., Wong, L.-J., and Alberts, B. (1980). Extensive purification of histone acetylase A, the major histone N-acetyl transferase activity detected in mammalian cell nuclei. *J. Biol. Chem.*, **255**, 11448-11453.
- Berezney, R. (1991). The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *J. Cell. Biochem.* **47**, 109-123.
- Biegel, D. and Pachter, J.S. (1992). mRNA association with the cytoskeletal framework likely represents a physiological binding event. *J. Cell. Biochem.* **48**, 98-106.
- Bode, J. Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T. (1992). Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* **255**, 195-197.

- Bode, J., Gomez-Lira, M., and Schroter, H. (1983). Nucleosomal particles open as the histone core becomes hyperacetylated. *Eur. J. Biochem.* **110**, 437-445.
- Bodnar, J.W. (1988). A domain model for eukaryotic DNA organization: a molecular basis for cell differentiation and chromosome evolution. *J. Theor. Biol.* **132**, 479-507.
- Boffa, L.C., Gruss, R.J., and Allfrey, V.G. (1981). Manifold effects of sodium butyrate on nuclear function. *J. Biol. Chem.* **256**, 9612-9621.
- Boffa, L.C., Walker, J., Chen, T.A., Sterner, R., Mariani, M.R., and Allfrey, V.G. (1990). Factors affecting nucleosome structure in transcriptionally active chromatin. *Eur. J. Biochem.*, **194**, 811-823.
- Bohm, J., Schlaeger, E.-J., and Knippers, R. (1980). Acetylation of nucleosomal histones *in vitro*. *Eur. J. Biochem.*, **112**, 353-362.
- Bohm, L., Briand, G., Sautiere, P., and Crane-Robinson, C. (1981). Proteolytic digestion studies of chromatin core-histone structure-identification of limit peptides of histones H3 and H4. *Eur. J. Biochem.* **119**, 67-74.
- Bonifer, C., Hecht, A., Saueressig, H., Winter, D.M., and Sippel, A.E. (1991). Dynamic chromatin: the regulatory domain organization of eukaryotic gene loci. *J. Cell. Biochem.* **47**, 99-108.
- Bonner, W.M., Wu, R.S., Panusz, H.T. and Muneses, C. (1988). Kinetics of accumulation and depletion of soluble newly synthesized histone in the reciprocal regulation of histone and DNA synthesis. *Biochemistry* **27**, 6542-6550.
- Boulikas, T. (1990). Poly (ADP-ribosylated) histones in chromatin replication. *J. Biol. Chem.* **265**, 14638-14647.
- Boyle, W.J. and Baluda, M.A. (1987) Subnuclear associations of the v-myb oncogene product and actin are dependent on ionic strength during nuclear isolation. *Mol. Cell. Biol.* **7**, 3345-3348.
- Bradbury, E.M. (1992). Reversible histone modifications and the chromosome cell cycle. *Bioessays* **14**, 9-16.
- Brancolini, C. and Schneider, C. (1991) Change in the expression of a nuclear matrix-associated protein is correlated with cellular transformation. *Proc. Natl. Acad. Sci. USA* **88**, 6936-6940.

- Branno, M., De Francis, V., and Tosi, L. (1983). *In vitro* methylation of histones in sea urchin nuclei during early embryogenesis. *Biochim. Biophys. Acta* **741**, 136-142.
- Brill, S.J. and Sternglanz, R. (1988). Transcription-dependent DNA supercoiling in yeast topoisomerase mutants. *Cell* **54**, 403-411.
- Brosch, G., Georgieva, E.I., Lopez-Rodas, G., Lidner, H., and Loidl, P. (1992). Specificity of *Zea mays* histone deacetylase is regulated by phosphorylation. *J. Biol. Chem.* **267**, 20561-20564.
- Brotherton, T. W., Reneker, J., and Ginder, G.D. (1990). Binding of HMG 17 to mononucleosomes of the avian β -globin gene cluster in erythroid and non-erythroid cells. *Nuc. Acids Res.* **18**, 2011-2016.
- Brotherton, T.W., Covault, J., Shires, A., and Chalkley, R. (1981). Only a small fraction of avian erythrocyte histone is involved in ongoing acetylation. *Nuc. Acids Res.* **19**, 5061-5073.
- Burlingame, R.W., Love, W.E., Wang, B.C., Hamlin, R., Xuong, N.-H., and Moudrianakis, E.N. (1985). Crystallographic structure of the octameric histone core of the nucleosome at 3.3 Angstroms. *Science* **228**, 546-553.
- Camoto, R. and Tanguay, R.M. (1982). Changes in the methylation pattern of core histones during heat shock in *Drosophila* cells. *EMBO J.* **1**, 1529-1536.
- Carmo-Fonseca, M., Pepperkok, R., Sproat, B.S., Ansorge, W., Swanson, M.S., and Lamond, A.I. (1991b). *In vivo* detection of snRNP-rich organelles in the nuclei of mammalian cells. *EMBO J.* **10**, 1863-1873.
- Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S.M.L., Merdes, A., Brunner, C., Zamore, P.D., Green, M.R., Hurt, E., and Lamond, A.I. (1991a). Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.* **10**, 195-206.
- Carter, K.C. and Lawrence, J.B. (1991). DNA and RNA within the nucleus: how much sequence-specific spatial organization. *J. Cell. Biochem.* **47**, 124-129.
- Carter, K.C., Taneja, K.L., and Lawrence, J.B. (1991). Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* **115**, 1191-1202.

- Chan, S., Attisano, L., and Lewis, P.N. (1988). Histone H3 thiol reactivity and acetyltransferases in chicken erythrocyte nuclei. *J. Biol. Chem.* **263**, 15643-15651.
- Chicoine, L.G., Schulman, I.G., Richman, R., Cook, R.G., and Allis, C.D. (1986). Nonrandom utilization of acetylation sites in histones isolated from *Tetrahymena*- evidence for functionally distinct H4 acetylation sites. *J. Biol. Chem.* **261**, 1071-1076.
- Ciejek, E.M., Nordstrom, J.L., Tsai, M.-J., and O'Malley, B.W. (1982). Ribonucleic acid precursors are associated with the chicken oviduct nuclear matrix. *Biochemistry* **21**, 4945-4953.
- Clark, D.J. and Felsenfeld, G. (1991). Formation of nucleosomes on positively supercoiled DNA. *EMBO J.* **10**, 387-395.
- Clark, D.J. and Felsenfeld, G. (1992). A nucleosome core is transferred out of the path of a transcribing polymerase. *Cell* **71**, 11-22.
- Cockerill, P.N., and Garrard, W.T. (1986). Chromosomal loop anchorage of the Kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**, 273-282.
- Cohen, R.B., and Sheffrey, M. (1985). Nucleosome disruption precedes transcription and is largely limited to the transcribed domain of globin genes in murine erythroleukemia cells. *J. Mol. Biol.* **182**, 109-129.
- Compton, D.A., Szilak, I., and Cleveland, D.W. (1992). Primary structure of NuMa, an intranuclear protein that defines a novel pathway for segregation of proteins at mitosis. *J. Cell Biol.* **116**, 1395-1408.
- Cook, P.R. (1988). The nucleoskeleton: artefact, passive framework or active site? *J. Cell Sci.* **90**, 1- 6.
- Cook, P.R. (1989). The nucleoskeleton and the topology of transcription. *Eur. J. Biochem.* **185**, 487-501.
- Cousens, L.S., Gallwitz, D., and Alberts, B.M. (1979). Different accessibilities in chromatin to histone acetylase. *J. Biol. Chem.*, **254**, 1716-1723.
- Covault, J. and Chalkley, R. (1980). The identification of distinct populations of acetylated histone. *J. Biol. Chem.* **255**, 9110-9116.

Croston, G.E., Kerrigan, L.A., Lira, L.M., Marshak, D.R., and Kadonaga, J.T. (1991). Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription. *Science* **251**, 643-649.

Csordas, A. (1990). On the biological role of histone acetylation. *Biochem. J.* **265**, 23-38.

Davie, J.R. and Delcuve, G.P. (1991). Characterization and chromatin distribution of the H1 histones and high-mobility-group non-histone chromosomal proteins of trout liver and hepatocellular carcinoma. *Biochem. J.* **280**, 491-497.

Davie, J.R. and Murphy, L.C. (1990). Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription. *Biochemistry*, **29**, 4752-4757.

Davie, J.R., Delcuve, G.P., Nickel, B.E., Moirier, R., and Bailey, G. (1987). Reduced levels of histone H1^a and H1b, and unaltered content of methylated DNA in rainbow trout hepatocellular carcinoma chromatin. *Cancer Res.* **47**, 5407-5410.

Dawson, B.A., Herman, T., Haas, A.L., and Lough, J. (1991). Affinity isolation of active murine erythroleukemia cell chromatin: uniform distribution of ubiquitinated histone H2A between active and inactive fractions. *J. Cell. Biochem.* **46**, 166-173.

Delcuve, G.P. and Davie, J.R. (1989). Chromatin structure of erythroid-specific genes of immature and mature chicken erythrocytes. *Biochem. J.* **263**, 179-186.

Desrosiers, R. and Tanguay, R.M. (1985). The modifications in the methylation patterns of H2B and H3 after heat shock can be correlated with the inactivation of normal gene expression. *Biochem. Biophys. Res. Commun.* **133**, 823-829.

Desrosiers, R. and Tanguay, R.M. (1989). Transcriptional inhibitors affecting topoisomerase II induce changes in histone methylation patterns similar to those induced by heat shock. *Biochem. Biophys. Res. Commun.* **162**, 1037-1043.

Desrosiers, R., and Tanguay, R.M. (1988). Methylation of *Drosophila* histones at proline, lysine, and arginine residues during heat shock. *J. Biol. Chem.* **263**, 4686-4692.

- Dhar, V., Mager, D., Iqbal, A., and Schildkraut, C.L. (1988). The coordinate replication of the human β -globin gene domain reflects its transcriptional activity and nuclease hypersensitivity. *Mol. Cell. Biol.* **8**, 4958-4965.
- Dickinson, L.A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T. (1992). A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell* **70**, 631-645.
- Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* **11**, 1475-1489.
- Dorbic, T. and Wittig, B. (1987). Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. *EMBO J.* **6**, 2393-2399.
- Druckmann, S., Mendelson, E., Landsman, D., and Bustin, M. (1986). Immunofractionation of DNA sequences associated with HMG-17 in chromatin. *Exp. Cell Res.* **166**, 486-496.
- Duerre, J.A. and Lee, C.T. (1974). *In vivo* methylation and turnover of rat brain histones. *J. Neurochem.* **23**, 541-547.
- Durrin, L.K., Mann, R.K., Kayne, P.S., and Grunstein, M. (1991). Yeast histone H4 N-terminal sequence is required for promoter activation *in vivo*. *Cell* **65**, 1023-1031.
- Dusserre, Y. and Mermod, N. (1992). Purified cofactors and histone H1 mediate transcriptional regulation by CTF/NF-I. *Mol. Cell. Biol.* **12**, 5228-5237.
- Dworetzky, S.I., Wright, K.L., Fey, E.G., Penman, S., Lian, J.B., Stein, J.L., and Stein, G.S. (1992). Sequence-specific DNA-binding proteins are components of a nuclear matrix-attachment site. *Proc. Natl. Acad. Sci.* **89**, 4178-4182.
- Ebralidse, K.K., Grachev, S.A., and Mirzabekov, A.D. (1988). A highly basic histone H4 domain bound to the sharply bent region of nucleosomal DNA. *Nature* **331**, 365-367.
- Einck, L., Fagan, J., and Bustin, M. (1986). Chromatin structure of the cytochrome P450c gene changes following induction. *Biochemistry* **25**, 7062-7068.

- Elgin, S.C.R. (1988). The formation and function of DNase I hypersensitive sites in the process of gene activation. *J. Biol. Chem.* **263**, 19259-19262.
- Elliot, D.J., Bowman, D.S., Abovich, N., Fay, F.S., and Robash, M. (1992). A yeast splicing factor is localized in discrete subnuclear domains. *EMBO J.* **11**, 3731-3736.
- Ericsson, C., Grossbach, U., Bjorkroth, B., and Daneholt, B. (1990). Presence of histone H1 on an active balbiani ring gene. *Cell* **60**, 73-83.
- Felsenfeld, G. (1992). Chromatin as an essential part of the transcriptional mechanism. *Nature* **355**, 219-224.
- Felts, S.J., Weil, P.A., and Chalkley, R. (1990). Transcription factor requirements for *in vitro* formation of transcriptionally competent 5S rRNA gene chromatin. *Mol. Cell. Biol.* **10**, 2390-2401.
- Fey, E.G., and Penman, S. (1988). Nuclear matrix proteins reflect cell type of origin in cultured human cells. *Proc. Natl. Acad. Sci. USA* **85**, 121-125.
- Fey, E.G., Ornelles, D.A., and Penman, S. (1986). Association of RNA with the cytoskeleton and the nuclear matrix. *J. Cell Sci. Suppl.* **5**, 99-119.
- Fujimoto, D. and Segawa, K. (1973). Enzymatic deacetylation of F2A2 histone. *FEBS Lett.* **32**, 59-61.
- Garcea, R.L. and Alberts, B.M. (1980). Comparative studies of histone acetylation in nucleosomes, nuclei, and intact cells: evidence for special factors which modify acetylase action. *J. Biol. Chem.*, **255**, 11454-11463.
- Garcia-Ramirez, M., Dong, F., and Ausio, J. (1992). Role of the histone "tails" in the folding of oligonucleosomes depleted of histone H1. *J. Biol. Chem.* **267**, 19587-19595.
- Garrard, W.T. (1991). Histone H1 and the conformation of transcriptionally active chromatin. *Bioessays* **13**, 87-88.
- Gasaryan, K.G. (1982). Genome activity and gene expression in avian erytroid cells. *Int. Rev. Cytol.* **74**, 95-126.
- Gasser, S.M. and Laemmli, U.K. (1987). A glimpse at chromosomal order. *Trends Genet.* **3**, 16-22.
- Georgieva, E.I., Lopez-Rodas, G., Sendra, R., Grobner, P., and Loidl, P. (1991). Histone acetylation in *Zea mays* II: biological significance of post-

- translational histone acetylation during embryo germination. *J. Biol. Chem.* **266**, 18751-18760.
- Getzenberg, R.H., Pienta, K.J., Huang, E.Y.W., and Coffey, D.S. (1991). Identification of nuclear matrix proteins in the cancer and normal rat prostate. *Cancer Res.* **51**, 6514-6520.
- Giaever, G.N. and Wang, J.C. (1988). Supercoiling of intracellular DNA can occur in eukaryotic cells. *Cell* **55**, 849-856.
- Giles, K.W. and Myers, A. (1965). An improved diphenylamine method for estimation of deoxyribonucleic acid. *Nature* **206**, 93
- Goldknopf, I. L., Sudjaker, S., Rosenbaum, F., and Busch, H. (1980a). Timing of ubiquitin synthesis and conjugation into protein A24 during the HeLa cell cycle. *Biochem. Biophys. Res. Commun.* **95**, 1253-1260.
- Goldknopf, I.L., Wilson, G., Ballal, N.R., and Busch, H. (1980b). Chromatin conjugate protein A24 is cleaved and ubiquitin is lost during chicken erythropoiesis. *J. Biol. Chem.* **255**, 10555-10558.
- Goldman, M.A. (1988). The chromatin domain as a unit of gene regulation. *Bioessays* **9**, 50-55.
- Gonzalez, P.J., Martinez, C., and Palacian, E. (1987). Interaction with RNA polymerase of nucleosomal cores lacking one H2A.H2B dimer. *J. Biol. Chem.* **262**, 11280-11283.
- Granados, E. N. and Bello, J. (1980). Interactions of poly(N^ε, N^ε, N^ε-trimethyllysine) and poly(N^δ, N^δ, N^δ-trimethylornithine) with polynucleotides: salt dissociation and thermal denaturation. *Biochemistry* **19**, 3227-3233.
- Gross, D.S. and Garrard, W.T. (1987). Poising chromatin for transcription. *Trends Biochem. Sci.* **12**, 293-297.
- Gross, D.S. and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. *Ann. Rev. Biochem.* **57**, 159-197.
- Grosveld, F., van Assendelft, G.B., Greaves, C.R., and Kallias, G. (1987). Position-independent high-level expression of the human β -globin gene in transgenic mice. *Cell* **51**, 975-985.
- Grunstein, M. (1990a). Nucleosomes: regulators of transcription. *Trends Gen.* **6**, 395-400.

- Grunstein, M. (1990b). Histone function in transcription. *Ann. Rev. Cell Biol.* **6**, 643-678.
- Grunstein, M. (1992). Histones as regulators of genes. *Sci. Am.* **October**, 68-74B.
- Hansen, J.C. and Ausio, J. (1992). Chromatin dynamics and the modulation of genetic activity. *TIBS* **17**, 187-191.
- Hansen, J.C. and Wolffe, A.P. (1992). Influence of chromatin folding on transcription initiation and elongation by RNA polymerase III. *Biochemistry* **31**, 7977-7988.
- Hatton, K.S., Dhar, V., Brown, E.H., Iqbal, M.A., Stuart, S., Didamo, V.T. and Schildkraut, C.L. (1988). Replication program of active and inactive multigene families in mammalian cells. *Mol. Cell. Biol.* **8**, 2149-2158.
- Hay, C.W., and Candido, E.P.M. (1983a). Histone deacetylase: association with a nuclease resistant, high molecular weight fraction of HeLa cell chromatin. *J. Biol. Chem.* **258**, 3726-3734.
- Hay, C.W., and Candido, E.P.M. (1983b). Histone deacetylase from HeLa cells: properties of the high molecular weight complex. *Biochemistry* **22**, 6175-6180.
- Hayes, J.J. and Wolffe, A.P. (1992). The interaction of transcription factors with nucleosomal DNA. *BioEssays* **14**, 597-603.
- Hayes, J.J., Clark, D.J., and Wolffe, A.P. (1991). Histone contributions to the structure of DNA in a nucleosome. *Proc. Natl. Acad. Sci.* **88**, 6829-6833.
- He, D., Nickerson, J.A., and Penman, S. (1990). Core filaments of the nuclear matrix. *J. Cell Biol.* **110**, 569-580.
- Hebbes, T.R., Thorne, A.W., Clayton, A.L., and Crane-Robinson, C. (1992). Histone acetylation and globin gene switching. *Nuc. Acids Res.* **20**, 1017-1022.
- Hebbes, T.W., Thorne, A.W. and Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *Embo J.* **7**, 1395-1402

- Hershko, A. (1988). Ubiquitin-mediated protein degradation. *J. Biol. Chem.* **263**, 15237-15240.
- Hirose, M. (1988). Effects of histone acetylation on nucleosome properties as evaluated by polyacrylamide gel electrophoresis and hydroxylapatite dissociation chromatography. *J. Biochem.* **103**, 31-35.
- Honda, B. M., Dixon, G.H., and Candido, E.P.M. (1975). Studies of *in vivo* histone methylation in developing trout testis. *J. Biol. Chem.* **250**, 8681-8685.
- Huang, S. and Spector, D.L. (1991). Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes Dev.* **5**, 2288-2302.
- Huang, S. and Spector, D.L. (1992). U1 and U2 small nuclear RNAs are present in nuclear speckles. *Proc. Natl. Acad. Sci.* **89**, 305-308.
- Huang, S.-Y., Barnard, M.B., Xu, M., Matsui, S.-I., Rose, S.M., and Garrard, W.T. (1986). The active immunoglobulin κ chain gene is packaged by non-ubiquitin-conjugated nucleosomes. *Proc. Natl. Acad. Sci.* **83**, 3738-3742.
- Imai, B.S., Yau, P., Baldwin, J.P., Ibel, K., May, R.P., and Bradbury, E.M. (1986). Hyperacetylation of core histones does not cause unfolding of nucleosomes: neutron scatter data accords with disc shape of the nucleosome. *J. Biol. Chem.* **261**, 8784-8792.
- Inoue, A. and Fujimoto, D. (1969). Enzymatic deacetylation of histone. *Biochem. Biophys. Res. Commun.* **36**, 146-150.
- Inoue, A. and Fujimoto, D. (1972). Substrate specificity of histone deacetylase from calf thymus. *J. Biochem.* **72**, 427-431.
- Ip, Y.T., Jackson, V., Meier, J., and Chalkley, R. (1988). The separation of transcriptionally engaged genes *J. Biol. Chem.* **263**, 14044-14052.
- Izban, M.G., and Luse, D.S. (1991). *Genes Dev.* **5**, 683-696.
- Jackson, D.A. (1991). Structure-function relationships in eukaryotic nuclei. *Bioessays* **13**, 1-10.
- Jackson, D.A. and Cook, P.R. (1985). Transcription occurs at a nucleoskeleton. *EMBO J.* **4**, 919-925.

- Jackson, D.A. and Cook, P.R. (1988). Visualization of a filamentous nucleoskeleton with a 23 nm axial repeat. *EMBO J.* **7**, 3667-3677.
- Jackson, D.A., Dickinson, P., and Cook, P.R. (1990). The size of chromatin loops in HeLa cells. *EMBO J.* **9**, 567-571.
- Jackson, V. (1990). *In vivo* studies on the dynamics of histone-DNA interaction: evidence for nucleosome dissolution during replication and transcription and a low level of dissolution independent of both. *Biochemistry* **29**, 719-731.
- Jackson, V. and Chalkley, R. (1985). Histone synthesis and deposition in the G1 and S phases of hepatoma tissue culture cells. *Biochemistry* **24**, 6921-6930.
- Jarman, A.P. and Higgs, D.R. (1988). Nuclear scaffold attachment sites in the human globin gene complexes. *EMBO J.* **7**, 3337-3344.
- Johnson, L.M., Kayne, P.S., Kahn, E.S., and Grunstein, M. (1990). Genetic evidence for an interaction between *SIR3* and histone H4 in the repression of the silent mating loci in *Sacchomyces cerevisiae*. *Proc. Nat'l. Acad. Sci.* **87**, 6286-6290.
- Kamakaka, R.T. and Thomas, J.O. (1990). Chromatin structure of transcriptionally competent and repressed genes. *EMBO J.* **9**, 3997-4006.
- Kaneta, H. and Fujimoto, D. (1974). A histone deacetylase capable of deacetylating chromatin-bound histone. *J. Biochem.* **76**, 905-907.
- Kaufmann, S.H. and Shaper, J.H. (1991). Association of topoisomerase II with the hepatoma cell nuclear matrix: the role of intermolecular disulfide bond formation. *Exp. Cell Res.* **192**, 511-523.
- Kaufmann, S.H., Brunet, G., Talbot, B., Lamarr, D., Dumas, C., Shaper, J.H., and Poirier, G. (1991). Association of poly(ADP-ribose) polymerase with the nuclear matrix: the role of intermolecular disulfide bond formation, RNA retention, and cell type. *Exp. Cell Res.* **192**, 524-535.
- Kaufmann, S.H., Gibson, W. and Shaper, J.H. (1983). Characterization of the major polypeptides of the rat liver nuclear envelope. *J. Biol. Chem.* **258**, 2710-2719.
- Kelner, D.N. and McCarty, K.S. Sr. (1984). Porcine liver nuclear histone acetyltransferase-partial purification and basic properties. *J. Biol. Chem.* **259**, 3413-3419.

- Kerrigan, L.A., Croston, G.E., Lira, L.M., and Kadonaga, J.T. (1991). Sequence-specific transcriptional antirepression of the *Drosophila Kruppel* gene by the GAGA factor. *J. Biol. Chem.* **266**, 574-582.
- Kikuchi, H. and Fujimoto, D. (1973). Multiplicity of histone deacetylase from calf thymus. *FEBS Lett.* **29**, 280-282.
- Kirov, N., Tsaneva, I., Einbinder, E., and Tsanev, R. (1992). *In vitro* transcription through nucleosomes by T7 RNA polymerase. *EMBO J.* **11**, 1941-1947.
- Klehr, D., Schlake, T., Maass, K., and Bode, J. (1992). Scaffold-attached regions (SAR elements) mediate transcriptional effects due to butyrate. *Biochemistry* **31**, 3222-3229.
- Kopczynski, C.C. and Muskavitch, M.A.T. (1992). Introns are excised from the *Delta* primary transcript are localized near sites of *Delta* transcription. *J. Cell Biol.* **119**, 503-512.
- Kornberg, R.D. (1991). Irresistible force meets immovable object: transcription and the nucleosome. *Cell* **67**, 833-836.
- Kuzmina, S.N., Buldyaeva, T.V., Akopov, S.B. and Zbarsky, I.B. (1984). Patterns of the nuclear matrix in differently proliferating and malignant cells. *Mol. Cell. Biochem.* **58**, 183-186.
- Lafond, R.E. and Woodcock, C.L.F. (1983). Status of the nuclear matrix in mature and embryonic chick erythrocyte nuclei. *Exp. Cell Res.* **147**, 31-39.
- Laskey, R.A. and Mills, A.D. (1975). Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**, 335-341.
- Lawrence, J.B. and Singer, R.H. (1986). Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415.
- Lawrence, J.B., Singer, R.H., and Marselle, L.M. (1989). Highly localized tracks of specific transcripts within interphase nuclei visualized by *in situ* hybridization. *Cell* **57**, 493-502.
- Laybourn, P.J. and Kadonaga, J.T. (1991). Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. *Science* **254**, 238-245.

- Laybourn, P.J. and Kadonaga, J.T. (1992). Threshold phenomena and long-distance activation of transcription by RNA polymerase II. *Science* **257**, 1682-1685.
- Lee, M.-S. and Garrard, W.T. (1991a). Transcription-induced nucleosome 'splitting': an underlying structure for DNase I sensitive chromatin. *EMBO J.* **10**, 607-615.
- Lee, M.-S. and Garrard, W.T. (1991b). Positive DNA supercoiling generates a chromatin conformation characteristic of highly active genes. *Proc. Natl. Acad. Sci.* **88**, 9675-9679.
- Leonard, M.W. and Patient, R.K. (1991). Evidence for torsional stress in transcriptionally activated chromatin. *Mol. Cell. Biol.* **11**, 6128-6138.
- Leonhardt, H., Page, A.W., Weier, H.-U., and Bestor, T.H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, **71**, 865-873.
- Levinger, D. and Varshavsky, A. (1982). *Cell* **28**, 375-385.
- Lewin, B. (1986) *Genes II*. John Wiley: New York. page 305.
- Li, H. and Bingham, P.M. (1991). Arginine/serine-rich domains of the *su(w^a)* and *tra* RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell* **67**, 325-342.
- Libertini, L. J., Ausio, J., van Holde, K.E., and Small, E.W. (1988). Histone hyperacetylation-its effects on nucleosome core particle transitions. *Biophys. J.* **53**, 477-487.
- Lin, R., Cook, R.G., and Allis, C.D. (1991). Proteolytic removal of core histone amino termini and dephosphorylation of histone H1 correlate with the formation of condensed chromatin and transcriptional silencing during *Tetrahymena* macronuclear development. *Genes Dev.* **5**, 1601-1610.
- Lin, R., Leone, J.W., Cook, R.G. and Allis, C.D. (1989). Antibodies specific to acetylated histones document the existence of dephosphorylation- and transcription-related histone acetylation in *Tetrahymena*. *J. Cell Biol.* **108**, 1577-1588.
- Ljungman, M. and Hanawalt, P.C. (1992). Localized torsional tension in the DNA of human cells. *Proc. Natl. Acad. Sci.* **89**, 6055-6059.

- Locklear, L., Ridsdale, J.A., Bazett-Jones, D.P., and Davie, J.R. (1990). Ultrastructure of transcriptionally competent chromatin. *Nucl. Acids Res.* **18**, 7015-7024.
- Loidl, P. (1988). Towards an understanding of the biological function of histone acetylation. *FEBS Lett.* **227**, 91-95.
- Lopez-Rodas, G., Georgieva, E.I., Sendra, R., and Loidl, P. (1991). Histone acetylation in *Zea mays* I: activities of histone acetyltransferases and histone deacetylases. *J. Biol. Chem.* **266**, 18745-18750.
- Lopez-Rodas, G., Tordera, V., Sanchez del Pino, M.M., Franco, L. (1989). Yeast contains multiple forms of histone acetyltransferase. *J. Biol. Chem.*, **264**, 19028-19033.
- Lorch, Y., LaPointe, J.W., and Kornberg, R.D. (1987). Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. *Cell* **49**, 203-210.
- Lorch, Y., LaPointe, J.W., and Kornberg, R.D. (1988). On the displacement of histones from DNA by transcription. *Cell* **55**, 743-744.
- Losa, R., and Brown, D.D. (1987). A bacteriophage RNA polymerase transcribes *in vitro* through a nucleosome core without displacing it. *Cell* **50**, 801-808.
- Louters, L. and Chalkley, R. (1984). *In vitro* exchange of nucleosomal histones H2A and H2B. *Biochemistry* **23**, 547-552,
- Louters, L. and Chalkley, R. (1985). Exchange of histones H1, H2A, and H2B *in vivo*. *Biochemistry* **24**, 3080-3085.
- Luderus, M.E.E., de Graaf, A., Mattia, E., den Blaauwen, J.L., Grande, M.A., de Jong, L, van Driel, R. (1992). Binding of matrix attachment regions to lamin B₁. *Cell* **70**, 949-959.
- Lui, L.F. and Wang, J.C. (1987). Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci.* **84**, 7024-7027.
- Lutter, L.C., Judis, L., and Paretto, R.F. (1992). Effects of histone acetylation on chromatin topology *in vivo*. *Mol. Cell. Biol.* **12**, 5004-5014.
- Mahadevan, L.C., Willis, A.C., and Barratt, M.J. (1991) Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell*, **65**, 775-783.

Mann, R.K. and Grunstein, M. (1992). Histone H3 N-terminal mutations allow hyperactivation of the yeast *GAL1* gene *in vivo*. *EMBO J.* **11**, 3297-3306.

Martelli, A.M., Falcieri, E., Gobbi, P., Manzoli, L., Gilmour, R.S., and Cocco, L. (1991). Heat-induced stabilization of the nuclear matrix: a morphological and biochemical analysis in murine erythroleukemia cells. *J. Cell Sci.* **196**, 216-225.

Marvin, K.W., Yau, P., and Bradbury, E.M. (1990). Isolation and characterization of acetylated histones H3 and H4 and their assembly into nucleosomes. *J. Biol. Chem.* **265**, 19839-19847.

Megee, P.C., Morgan, B.A., Mittman, B.A., and Smith, M.M. (1990). Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. *Science* **247**, 841-845

Mirkovitch, J., Mirault, M.-E., and Laemmli, U.K. (1984). Organization of the higher-order chromatin loop:specific DNA attachment sites on nuclear scaffold. *Cell* **39**, 223-232

Mirzayan, C., Copeland, C.S., and Snyder, M. (1992). The *NUF1* gene encodes an essential coiled-coil related protein that is a potential component of the yeast nucleoskeleton. *J. Cell Biol.* **116**, 1319-1332.

Mold, D.E., and McCarty, K.S., Sr. (1987). A Chinese hamster ovary cell histone deacetylase that is associated with a unique class of mononucleosomes. *Biochemistry* **26**, 8257-8262.

Morgan, B.A., Mittman, B.A., and Smith, M.M. (1991). The highly conserved N-terminal domains of histones H3 and H4 are required for normal cell cycle progression. *Mol. Cell. Biol.* **11**, 4111-4120.

Morse, R.H. (1989). Nucleosomes inhibit both transcriptional initiation and elongation by RNA polymerase III *in vitro*. *EMBO J.* **8**, 2343-2351.

Morse, R.H. (1992). Transcribed chromatin. *TIBS* **17**, 23-26.

Nacheva, G.A., Guschin, D.M., Preobrazhenskaya, O.V., Karpov, V.L., Ebralidse, K.E. and Mirzabekov, A.D. (1989). Change in the pattern of histone binding to DNA upon transcriptional activation. *Cell* **58**, 27-36.

Najbauer, J. and Aswad, D.W. (1990) *J. Biol. Chem.* **265**, 12717-12721.

Nakayasu, H. and Berezney, R. (1989). Mapping replicational sites in the eukaryotic nucleus. *J. Cell Biol.* **108**, 1-11.

Nakayasu, H. and Berezney, R. (1991). Nuclear matrices: identification of the major nuclear matrix proteins. *Proc. Natl. Acad. Sci. USA* **88**, 10312-10316.

Nelson, W.G., Pienta, K.J., Barrack, E.R. and Coffey, D.S. (1986) The role of the nuclear matrix in the organization and function of DNA. *Ann. Rev. Biophys. Chem.* **15**, 457-475.

Nickel, B.E., Allis, C.D., and Davie, J.R. (1989). Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. *Biochemistry* **28**, 958-963.

Nickel, B.E., Roth, S.Y., Cook, R.G., Allis, C.D., and Davie, J.R. (1987). Changes in the histone H2A variant H2A.Z and polyubiquitinated histone species in developing trout testis. *Biochemistry* **26**, 4417-4421.

Norton, V.G., Imai, B.S., Yau, P., and Bradbury, E.M. (1989). Histone acetylation reduces nucleosome core particle linking number change. *Cell* **57**, 449-457.

Norton, V.G., Marvin, K.W., Yau, P., and Bradbury, E.M. (1990). Nucleosome linking number change controlled by acetylation of histones H3 and H4. *J. Biol. Chem.* **265**, 19848-19852.

O'Neill, T.E., Roberge, M., and Bradbury, E.M. (1992). Nucleosome arrays inhibit both initiation and elongation of transcripts by bacteriophage T7 RNA polymerase. *J. Mol. Biol.* **223**, 67-78.

Oliva, R., Bazett-Jones, D.P., Locklear, L., and Dixon, G.H. (1990). Histone hyperacetylation can induce unfolding of the nucleosome core particle. *Nucleic Acids Res.* **18**, 2739-2747.

Ornelles, D.A., Fey, E.G., and Penman, S. (1986). Cytochalasin releases mRNA from the cytoskeletal framework and inhibits protein synthesis. *Mol. Cell. Biol.* **6**, 1650-1662.

Pehrson, J.R. and Fried, V.A. (1992). MacroH2A, a core histone containing a large nonhistone region. *Science* **257**, 1398-1400.

Perry, C.A. and Annunziato, A.T. (1991). Histone acetylation reduces H1-mediated nucleosome interactions during chromatin assembly. *Exp. Cell Res.*, **196**, 337-345.

- Perry, M. and Chalkley, R. (1982). Histone acetylation increases the solubility of chromatin and occurs sequentially over most of the chromatin. *J. Biol. Chem.* **257**, 7336-7347.
- Pfaffle, P., Gerlach, V., Bunzel, L., and Jackson, V. (1990). *In vitro* evidence that transcription-induced stress causes nucleosome dissolution and regeneration. *J. Biol. Chem.* **265**, 16830-16840.
- Pfeffer, U., Ferrari, N., Tosetti, F., and Vidali, G. (1989). Histone acetylation in conjugating *Tetrahymena thermophila*. *J. Cell Biol.* **109**, 1007-1014.
- Phi-Van, L. and Stratling, W.H. (1988). The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO J.* **7**, 655-664.
- Phi-Van, L., von Kries, J.P., Ostertag, W. and Stratling, W.H. (1990). The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Mol. Cell. Biol.* **10**, 2302-2307.
- Postnikov, Y.V., Shick, V.V., Belyavsky, A.V., Khrapko, R., Brodolin, K.L., Nikolskaya, T.A., and Mirzabekov, A.D. (1991). Distribution of high mobility group proteins 1/2, E and 14/17 and linker histones H1 and H5 on transcribed and non-transcribed regions of chicken erythrocyte chromatin. *Nuc. Acids Res.* **19**, 717-725.
- Reeves, R. (1984). Transcriptionally active chromatin. *Biochim. Biophys. Acta* **782**, 343-393.
- Reneker, J. and Brotherton, T.W. (1991). Postsynthetic methylation of core histones in K562 cells is associated with acetylation but not with transcriptional activity. *Biochemistry* **30**, 8402-8407.
- Reuter, G., and Spierer, P. (1992). Position effect variegation and chromatin proteins. *Bioessays* **9**, 605-612.
- Richmond, R.G., Finch, T., Rushton, B., Rhodes, D., and Klug, A. (1984). Structure of the nucleosome core particle at a 7 Angstrom resolution. *Nature* **311**, 532-537.
- Ridsdale, J.A. and Davie, J.R. (1987). Chicken erythrocyte polynucleosomes which are soluble at physiological ionic strength and

contain linker histones are highly enriched in β -globin gene sequences. *Nucleic Acids Res.* **15**, 1081-1096

Ridsdale, J.A., Hendzel, M.J., Delcuve, G.P., and Davie, J.R. (1990). Histone acetylation alters the capacity of the H1 histones to condense transcriptionally active/competent chromatin. *J. Biol. Chem.* **265**, 5150-5156.

Ridsdale, J.A., Rattner, J.B. and Davie, J.R. (1988). Erythroid-specific gene chromatin has an altered association with linker histones. *Nucleic Acids Res.* **16**, 5915-5926.

Roberge, M., Dahmus, M.E. and Bradbury, E.M. (1988). Chromosomal loop/nuclear matrix organization of transcriptionally active and inactive RNA polymerases in HeLa nuclei. *J. Mol. Biol.* **201**, 545-555.

Roeder, R.G. (1991). The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.* **16**, 402-408.

Rose, S.M., and Garrard, W.T. (1984). Differentiation-dependent chromatin alterations precede and accompany transcription of immunoglobulin light chain genes. *J. Biol. Chem.* **259**, 8534-8544.

Roth, S.Y. and Allis, C.D. (1992). Chromatin condensation: does histone H1 dephosphorylation play a role. *TIBS* **17**, 93-98.

Roth, S.Y., Shimizu, M., Johnson, L., Grunstein, M., and Simpson, R.T. (1992). Stable nucleosome positioning and complete repression by the yeast $\alpha 2$ repressor and disrupted by amino-terminal mutations in histone H4. *Genes Dev.* **6**, 411-425.

Ruiz-Carrillo, A., Wangh, L.J. and Allfrey, V.G. (1975). Processing of newly synthesized histone molecules. *Science* **190**, 117-128

Saavedra, R.A. (1990). Environmental stimuli and transcriptional activity generate transient changes in DNA torsional tension. *Bioessays* **12**, 125-128.

Sariban, E., Wu, R., Erickson, L. and Bonner, W. (1985). Interrelationships of protein and DNA syntheses during replication of mammalian cells. *Mol. Cell. Biol.* **5**, 1279-1286.

- Schroth, G.P., Chou, P.-J., and Ho, P.S. (1992). Mapping Z-DNA in the human genome: computer-aided mapping reveals a nonrandom distribution of potential Z-DNA-forming sequences in human genes. *J. Biol. Chem.* **267**, 11846-11855.
- Seale, R.L. (1981). *In vivo* assembly of newly synthesized histones. *Biochemistry* **20**, 6432-6437.
- Shepherd, G.R., Hardin, J.M., and Noland, B.J. (1971). Methylation of lysine residues of histone fractions in synchronized mammalian cells. *Arch. Biochem. Biophys.* **143**, 1-5.
- Sinnhuber, R.O., Hendricks, J.D., Wales, J.H. and Putnam, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. NY Acad. Sci.* **298**, 389-408.
- Spector, D.L., Fu, X.D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* **10**, 3467-3481.
- Stein, G.S., Lian, J.B., Dworetzky, S.I., Owen, T.A., Bortell, R., Bidwell, J.P., and van Wijnen, A.J. (1991). Regulation of transcription-factor activity during growth and differentiation: involvement of the nuclear matrix in concentration and localization of promoter binding proteins. *J. Cell. Biochem.* **47**, 300-305.
- Stratling, W.H. (1987) Gene-specific differences in the supranucleosomal organization of rat liver chromatin. *Biochemistry* **26**, 7893-7899
- Stratling, W.H., Dolle, A. and Sippel, A.E. (1986). Chromatin structure of the chicken lysozyme gene domain as determined by chromatin fractionation and micrococcal nuclease digestion. *Biochemistry* **25**, 495-502.
- Stuurman, N., Floore, A., Colen, A., de Jong, L., van Driel, R. (1992). Stabilization of the nuclear matrix by disulfide bridges: identification of matrix polypeptides that form disulfides. *Exp. Cell Res.* **200**, 285-294.
- Sung, M.T., Harford, J., Bundman, M., and Vidalakas, G. (1977). Metabolism of histones in avian erythroid cells. *Biochemistry* **16**, 279-285.
- Sures, I. and Gallwitz, D. (1980). Histone-specific acetyltransferases from calf thymus. Isolation, properties, and substrate specificity of three different enzymes. *Biochemistry* **19**, 943-951.

- Svaren, J. and Chalkley, R. (1990). The structure and assembly of active chromatin. *Trends Gen.* **6**, 52-56.
- Taneja, K.L., Lifshitz, L.M., Fay, F.S., and Singer, R.H. (1992). Poly(A) codistribution with microfilaments: evaluation by *in situ* hybridization and quantitative digital imaging microscopy. *J. Cell Biol.* **119**, 1245-1260.
- Tanguay, R.M. and Desrosiers, R. (1988). Histone methylation and modulation of gene expression in response to heat shock and chemical stress in *Drosophila*. In *Advances in the Post-translational Modifications of Proteins and Ageing*. (V.Zappia, Ed.) pp.353-362. Plenum Press, New York, N.Y.
- Tazi, J. and Bird, A. (1990). Alternative chromatin structure at CpG islands. *Cell* **60**, 909-920.
- Thomas, G., Lange, H.-W., and Hempel, K. (1975) Kinetics of histone methylation *in vivo* and its relation to the cell cycle of Ehrlich ascites tumor cells. *Eur. J. Biochem.* **51**, 609-615.
- Thomsen, B., Bendixen, C., and Westergaard, O. (1991) Histone hyperacetylation is accompanied by changes in DNA topology *in vivo*. *Eur. J. Biochem.* **201**, 107-111.
- Thorne, A.W., Kmiciek, D., Mitchelson, K., Sautiere, P., and Crane-Robinson, C. (1990). Patterns of histone acetylation. *Eur. J. Biochem.* **193**, 701-713.
- Steif, A., Winter, D.M., Stratling, W.H., and Sippel, A.E. (1989). A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature* **341**, 343-345.
- Tidwell, T., Allfrey, V.G., and Mirsky, A.E. (1968). The methylation of histones during regeneration of the liver. *J. Biol.Chem.* **243**, 707-715.
- Travers. A.A. (1992). The reprogramming of transcriptional competence. *Cell* **69**, 573-575.
- Turner, B.M., Franchi, L., and Wallace, H. (1990). Islands of acetylated histone H4 and their relationship to chromatin packaging and transcriptional activity. *J. Cell Sci.* **96**, 335-346.
- Turner, B.M. (1991). Histone acetylation and control of gene expression. *J. Cell Sci.* **99**, 13-20.

- van Daal, A. and Elgin, S.C.R. (1992). A histone variant, H2AvD, is essential in *Drosophila melanogaster*. *Mol. Biol. Cell* **3**, 593-602.
- van Driel, R., Humbel, B., and de Jong, L. (1991). The nucleus: a black box being opened. *J. Cell. Biochem.* **47**, 311-316.
- van Holde, K.E. (1989). *Chromatin*. Springer-Verlag, New York, New York.
- van Holde, K.E., Lohr, D.E., and Robert, C. (1992). What happens to nucleosomes during transcription? *J. Biol. Chem.* **267**, 2837-2840.
- Verheijen R., van Venrooij, W. and Ramaekers, F. (1988). The nuclear matrix: structure and composition. *J. Cell Sci.* **90**, 11-36.
- Vidali, G., Boffa, L.C., and Allfrey, V.G. (1972). Properties of an acidic histone-binding protein fraction from cell nuclei: selective precipitation and deacetylation of histones F2A1 and F3. *J. Biol. Chem.* **247**, 7365-7373.
- Villeponteau, B., Lundell, M., and Martinson, H. (1984). Torsional stress promotes the DNase I sensitivity of active genes. *Cell* **39**, 469-478.
- von Kries, J.P., Buhrmester, H., and Stratling, W.H. (1991). A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. *Cell* **64**, 123-135.
- Waithe, W.I., Renaud, J., Nadeau, P. and Pallotta, D. (1983). Histone synthesis by lymphocytes in G0 and G1. *Biochemistry* **22**, 1778-1783.
- Walker, J., Chen, T.A., Sterner, R., Berger, M., Winston, F., and Allfrey, V.G. (1990). Affinity chromatography of mammalian and yeast nucleosomes: two modes of binding of transcriptionally active mammalian nucleosomes to organomercurial-agarose columns, and contrasting behavior of the active nucleosomes of yeast. *J. Biol. Chem.* **265**, 5736-5746
- Waterborg, J.H. (1990). Sequence analysis of acetylation and methylation in two histone H3 variants of Alfalfa. *J. Biol. Chem.* **265**, 17157-17161
- Wilkitorowicz, J.E. and Bonner, J. (1982). Studies on histone acetyltransferase: partial purification and basic properties. *J. Biol. Chem.* **257**, 12893-12900.
- Williams, A.F. (1972). DNA synthesis in urifid populations of avian erythroid cells. *J. Cell Sci.* **46**, 27-42

Wittig, B., Dorbic, T., and Rich, A. (1989). The level of Z-DNA in metabolically active, permeabilized mammalian cell nuclei is regulated by torsional strain. *J. Cell Biol.* **108**, 755-764.

Wittig, B., Dorbic, T., and Rich, A. (1991). Transcription is associated with Z-DNA formation in metabolically active permeabilized mammalian cell nuclei. *Proc. Natl. Acad. Sci.* **88**, 2259-2263.

Wolffe, A.P. (1991). Implications of DNA replication for eukaryotic gene expression. *J. Cell Sci.* **99**, 201-206.

Workman, J.L., Abmayr, S.M., Cromlish, W.A., and Roeder, R.G. (1988). Transcriptional regulation by the immediate early protein of pseudorabies virus during *in vitro* nucleosome assembly. *Cell* **55**, 211-219.

Wu, H.-Y., Shyy, S., Wang, J.C., and Liu, L.F. (1988). Transcription generates positively and negatively supercoiled domains in the template. *Cell* **53**, 433-440.

Wu, R., Perry, L. and Bonner, W. (1983b). Fate of newly synthesized histones in G1 and G0 cells. *FEBS Lett.* **162**, 161-166.

Wu, R., Tsai, S. and Bonner, W. (1983a). Changes in histone H3 composition and synthesis pattern during lymphocyte activation. *Biochemistry* **22**, 3868-3873.

Wu, R.S., Kohn, K.W., and Bonner, W.M. (1981). Metabolism of ubiquitinated histones. *J. Biol. Chem.* **256**, 5916-5920.

Wu, R.S., Panusz, H.T., Hatch, C.L. and Bonner, W.M. (1986). Histones and their modifications. *CRC Crit. Rev. Biochem.* **20**, 201-263.

Wu, R.S., Tsai, S., and Bonner, W.M. (1982). Patterns of histone variant synthesis can distinguish G0 from G1 cells. *Cell* **31**, 367-374.

Xing, Y. and Lawrence, J.B. (1991). Preservation of specific RNA distribution within chromatin-depleted nuclear substructure demonstrated by *in situ* hybridization coupled with biochemical fractionation. *J. Cell Biol.* **112**, 1055-1063,

Yang, C.H., Lambie, E.J., and Snyder, M. (1992). NuMa: An unusually long coiled-coil related protein in the mammalian nucleus. *J. Cell Biol.* **116**, 1303-1317.

- Yoshida, M. and Beppu, T. (1988). Reversible arrest of proliferation of rat 3Y1 fibroblasts in both G1 and G2 phases by Trichostatin A. *Exp. Cell Res.*, **177**, 122-131.
- Yoshida, M., Nomura, S., and Beppu, T. (1987). Effects of Trichostatins on differentiation of murine erythroleukemia cells. *Cancer Res.* **47**, 3688-3691.
- Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by Trichostatin A. *J. Biol. Chem.* **265**, 17174-17179.
- Yukioka, M., Sasaki, S., Qi, S.-L., and Inoue, A. (1984). Two species of histone acetyltransferase in rat liver nuclei. *J. Biol. Chem.* **259**, 8372-8377.
- Zamore, P.D. and Green, M.R. (1991). Biochemical characterization of U2 snRNP auxiliary factor: an essential pre mRNA splicing factor with a novel intranuclear distribution. *EMBO J.* **10**, 207-214.
- Zeitlin, S., Parent, A., Silverstein, S., and Efstratiadis, A. (1987). Pre-mRNA splicing and the nuclear matrix. *Mol. Cell. Biol.* **7**, 111-120.
- Zhang, D.-E., and Nelson, D.A. (1986). Histone acetylation in chicken erythrocytes-estimation of the percentage of sites actively modified. *Biochem. J.* **240**, 857-862.
- Zhang, D.-E., and Nelson, D.A. (1988a). Histone acetylation in chicken erythrocytes. Rates of acetylation and evidence that histones in both active and potentially active chromatin are rapidly modified. *Biochem. J.* **250**, 233-240.
- Zhang, D.-E., and Nelson, D.A. (1988b). Histone acetylation in chicken erythrocytes. Rates of deacetylation in immature and mature red blood cells. *Biochem. J.* **250**, 241-247.