BIOCHEMICAL ANALYSIS OF CHROMATIN IN SPERMATOGENESIS AND TRANSCRIPTION: THE ROLE OF HISTONE UBIQUITINATION

by

Barbara E. Nickel

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To Rob

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LIST OF ABBREVIATIONS

ACS	aqueous counting scintillant
AUT	acetic acid/urea/Triton X-100
A_{260}	absorbance at 260 nm 1 A_{260} unit = 50 ug of DNA
bp	base pairs
ddH_2O	deionized distilled water
DNase I	deoxyribonuclease I
EDTA	(ethylenedinitrilo)tetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)] tetraacetic acid
HMG	high mobility group
IAC	24:1 (v/v) chloroform and isoamyl alcohol
kbp	kilobase pairs
kDa	kilodaltons
MAR	matrix associated region
5-mC	5-methylcytosine
MNase	micrococcal nuclease
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
RNase	ribonuclease
SAR	scaffold attached region
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

ABSTRACT

Transcriptionally active chromatin and chromatin undergoing replacement by protamines during spermatogenesis is thought to have a disrupted chromatin structure. The role that histone ubiquitination may have in altering chromatin structure is unknown. In early stage rainbow trout testis, chromatin of the germline specific, transcriptionally active protamine genes is enriched in the insoluble nuclear material and has a disrupted nucleosome structure. The repressed protamine gene chromatin of erythrocytes is not enriched in the insoluble nuclear material and exhibits a canonical nucleosome repeat pattern. Ubiquitinated histones from testes at different stages of development and from chromatin fractions enriched for transcriptionally active genes were analyzed by two-dimensional polyacrylamide gel electrophoresis and immunochemical detection of ubiquitin. Naturally occurring polyubiquitinated histone species were observed for the first time. In the final stages of spermatogenesis, where transcription is diminished, levels of histone variant H2A.Z (characterized by peptide mapping and partially sequenced), uH2A.Z and uH2B were reduced. However, uH2A and u_2H2A levels were similar between early and late stage testis. Chromatin from several sources was fractionated and there was a correlation between transcriptional activity and increased levels of uH2A, polyubiquitinated H2A, uH2A.Z and especially uH2B. The arrangement of ubiquitin molecules in polyubiquitinated H2A from trout liver and calf thymus was characterized by peptide mapping and two-dimensional gel analysis of resulting peptides. A seven residue fragment of H2A with two lysine

residues (118 and 119) contains three ubiquitin molecules. Due to steric hindrance it is likely that ubiquitin is attached at only one lysine and additional ubiquitins are linked through lysine residues of a ubiquitin molecule already attached to H2A. In cytoplasmic proteins destined for degradation polyubiquitination is thought to occur at different lysine residues along the protein molecule. If this is the case, the arrangement of ubiquitin molecules on polyubiquitinated H2A may be functionally significant. This arrangement may serve to modify chromatin structure rather than "tag" proteins for degradation.

I. INTRODUCTION

A. Structure of eukaryotic chromatin

Eukaryotic chromosomes package DNA so that approximately 1 m of DNA is contracted by at least 10^5 fold (Lilley and Pardon, 1979). Chromatin, the major component of chromosomes, is a complex of DNA with about equal weight of histones. Histones are relatively small proteins with a very high proportion of positively charged amino acids (lysine and arginine). The basic nature of histones helps them bind tightly to DNA regardless of its nucleotide sequence. In eukaryotic cells precise control of gene expression is required for development, differentiation and metabolism. Regulation of gene expression at the level of transcription is thought to involve changes in chromatin structure and composition, since most of the DNA in the nucleus is too tightly packed to be accessible for transcription. To understand the role chromatin structure may play in transcriptional regulation, it is necessary to be familiar with the hierarchies of chromatin structure (Figure 1).

The lowest level of DNA coiling in chromatin is the nucleosome. A nucleosome consists of approximately 146 basepairs (bp) of double-stranded B-form DNA wrapped into two left-handed superhelical turns around the outside of an octamer of histone proteins arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers (reviewed in Reeves, 1984). The nucleosome core particle is shaped like a short cylinder approximately 11 nm in diameter and 5.7 nm in height with the two parallel turns of DNA being about 2.8 nm apart on the particle surface as shown by x-ray diffraction analysis (Finch et al., 1981,) and electron microscopic image reconstruction techniques (Klug et al., 1980).

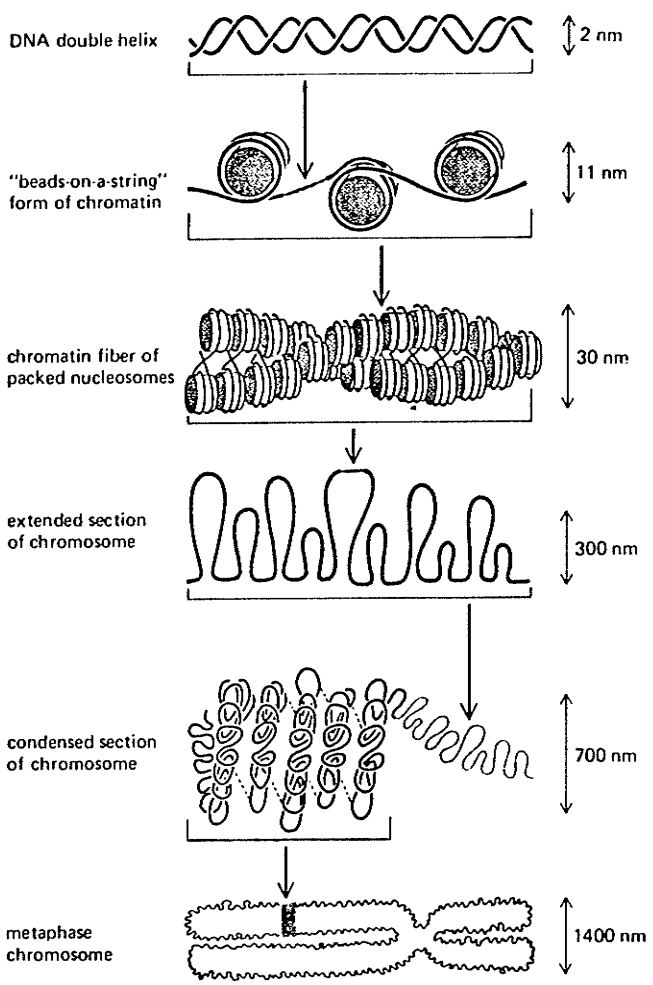

Figure 1. Schematic illustration of different orders of chromatin packing postulated to give rise to the highly condensed metaphase chromosome. (from Alberts et al., 1983)

Chromatosomes are core nucleosomes with an additional 20 bp of DNA and a histone H1 molecule positioned at the entrance and exit of DNA from the particle. Core nucleosomes are joined to each other by linker DNA which can vary between 0 and 80 bp. The arrangement of nucleosomes and linker DNA results in the "beads-on-a-string" structure of the 10 nm fiber that is visualized by electron microscopy (Olins and Olins, 1974).

In a living cell chromatin must be kept in a highly compacted state and therefore, probably is seldom in the form of a 10 nm fiber. When nuclei are very gently lysed onto an electron microscope grid in the presence of divalent cations, most of the chromatin is seen as a fiber with a diameter of about 30 nm. Condensation of the 10 nm fiber results in the formation of the 30 nm fiber. The formation and/or stability of the 30 nm fiber is dependent on the presence of histone H1. Each histone H1 molecule has a globular central region linked to extended amino-terminal and carboxyl-terminal domains. Histone H1 binds to the linker DNA of nucleosomes through its globular region and interacts with adjacent nucleosomes via its carboxyl-terminal domain thus inducing higher order chromatin structure (Allan et al., 1986). Histone H1 binding to chromatin is cooperative (Renz, 1975). This means that there is a strong tendency for a molecule of H1 to bind adjacent to another bound molecule of H1.

At least three models for the 30 nm fiber have been proposed (for reviews see Felsenfeld and McGhee, 1986; Pederson et al., 1986; Williams et al., 1986). In the model proposed by Thoma et al. (1979) using electron microscopic studies, the polynucleosome chain is

condensed by winding into a simple solenoidal structure (a one-start helix) with about six nucleosomes per helical turn and a pitch of about 11 nm (Figure 2A). The second model (Woodcock et al., 1984) shows a condensed ribbon containing two parallel rows of chromatosomes with the faces of alternate chromatosomes in contact, coiled to form a two-start helix (Figure 2B). In another model (McGhee et al., 1983), chromatosomes are oriented with faces roughly parallel to the fiber axis and positioned radially like the spokes of a wheel (Figure 2C). In these three models, the 30 nm fiber is a superhelical structure, containing a small number of nucleosomes (about 6) per turn. Within the superhelix, individual chromatosomes are oriented radially, with flat faces probably somewhat tilted relative to the long axis. Results from Russanova et al. (1987) on accessibility of the globular domains of histone H1 to antibodies indicate that antigenic determinants of H1 are masked probably by interaction with DNA or protein. This provides support for the three models shown for the 30 nm fiber as in each model histone H1 is situated at the interior of the solenoid where it is probably not accessible to the antibody.

The third level of hierarchical structure in both interphase nuclei and metaphase chromosomes is the folding of the 30 nm solenoid into loops (Benyajati and Worcel, 1976; Paulson and Laemmli, 1977) or domains (Igo-Kemenes and Zachau, 1978) that form the 300 nm fiber (Figure 1). Specific non-histone proteins at the base of chromatin loops are thought to anchor the loops to a supporting nuclear structure which has been called the nuclear matrix, scaffold or nucleoskeleton. Specific DNA regions, called scaffold attached regions (SAR) are found

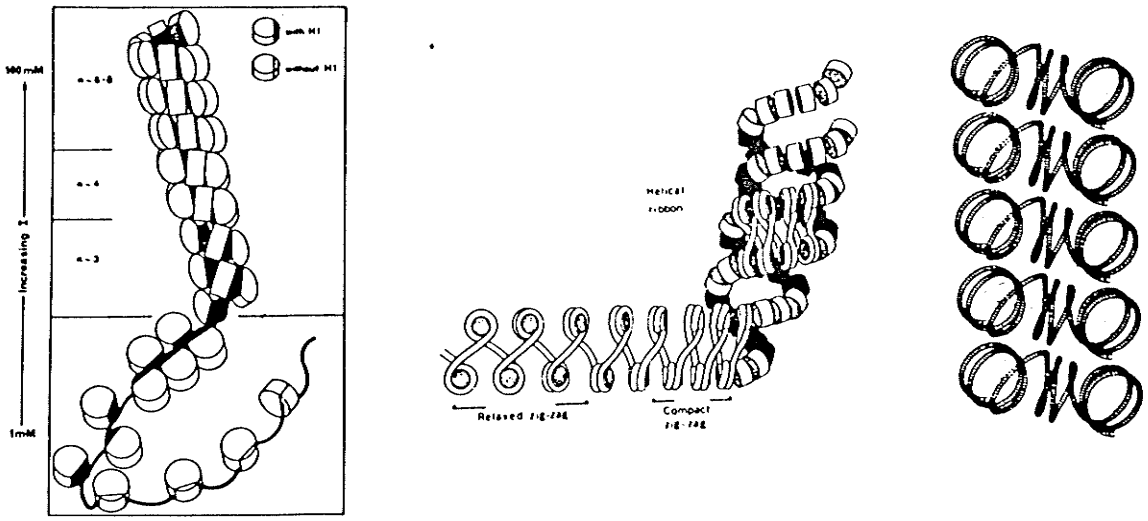

Figure 2. **Three models of the 30 nm fiber.**
In (A) and (B) the fully compacted structure is seen at the top of the figure. The bottom part of the figure illustrates proposed intermediate steps in the ionic strength-induced compaction. In (C), only the DNA path is shown and chromatosomes on the back side of the solenoid are omitted for clarity. (from Felsenfeld and McGhee, 1986)

at points where the scaffold interaction occurs (Gasser and Laemmli, 1987). The SARs are the same as the matrix association regions (MARs) described by Gross and Garrard (1987). Figure 3 shows an "artist's impression" of loop organization in a histone depleted nucleus. DNA in the loops varies in size between 5 and 100 kilobase pairs (kbp) and there appears to be a "loose" inverse relationship between loop size and level of transcription of genes contained within the loop.

Organization of chromatin into the loop structure of the 300 nm fiber may serve a functional role as well as a structural role as the nucleoskeleton has been implicated in DNA replication and transcription (for review see Jackson, 1986). Transcriptional regulation of eukaryotic genes is mediated by the interaction of cis-acting DNA sequences with trans-acting factors (Gross and Garrard, 1987). Compartmentalization of the DNA in the nucleus, such as seen in the organization of the loops in the 300 nm fiber reduces the volume that factors or polymerases will have to search to find specific DNA binding sites. SARs sometime reside close to transcriptional enhancers and contain consensus sequences for interaction with topoisomerase II, an enzyme localized at or near the bases of chromosomal loops in mitotic chromosomes (Gross and Garrard, 1987). With this arrangement one can imagine the DNA binding proteins confined within a subcompartment of the nucleus only having to scan the DNA at the base of the loops for potential binding sites. Once bound, to the base of a chromosomal loop, the level of DNA supercoiling and perhaps transcription in the entire loop could be regulated through interaction of topoisomerase II and other trans-acting factors with the SARs and enhancers.

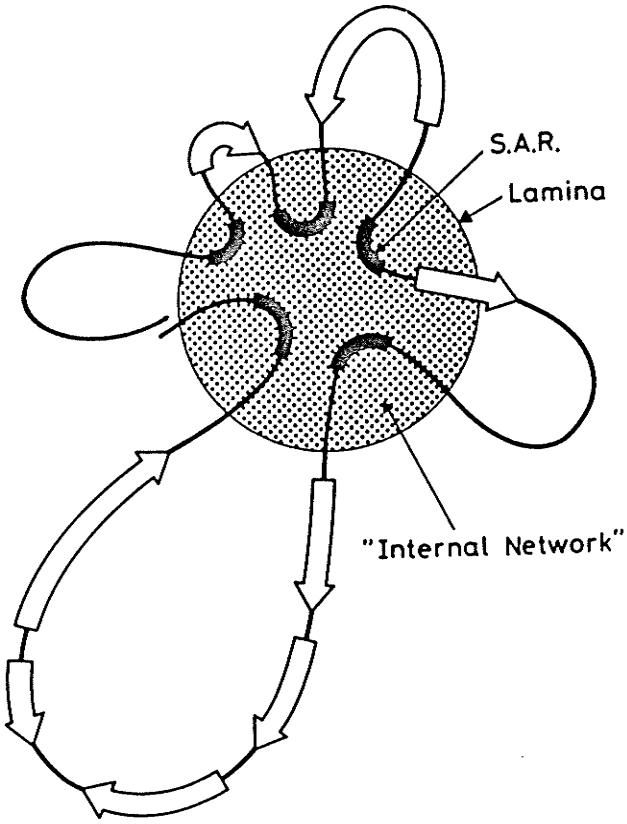

Figure 3. **Scheme of the loop organization in a histone-depleted nucleus.**
Genomic DNA is organized into loops varying in size between 5 and 100 kbp. The loops are anchored to the nuclear scaffold at highly specific DNA regions called SAR (scaffold associated region). Loops can have one or several transcription units, indicated by arrows. Available data are consistent with the notion that highly active or potentially highly active genes are organized into small loops of about 10 kbp, while genes with less transcriptional activity are arranged in much larger loops containing several transcription units. Observations of amounts of topoisomerase present are consistent with the possible role of this protein as a loop fastener. (from Gasser and Laemmli, 1987)

Topoisomerases and DNA supercoiling may be involved in the regulation of eukaryotic gene expression.

The exact arrangement of neighboring loops in the next level of organization is not known, but a helical arrangement progressing along the axis of the chromatid seems likely (Gasser and Laemmli, 1987).

B. Transcriptionally active chromatin

There is growing evidence that transcription elongation proceeds through the nucleosome core and that the position of the histone octamer remains unchanged during elongation (Losa and Brown, 1987; De Bernardin et al., 1986). However, the nucleosome structure of transcriptionally active chromatin may be modified.

The chromatin of transcriptionally active genes differs from the bulk of the genome in susceptibility to digestion by a variety of nucleases (for review see Reeves, 1984). The preferential sensitivity of active genes to DNase I has led to the concept that active genes are in a more "open" chromatin state which provides access to regulatory and transcription factors (Weintraub, 1985). This in turn has led to the view that the higher order structure of chromatin may be responsible for the repression of gene activity and a primary step in activating specific genes may involve local inactivation of this general repression system. Histone H1, which is extremely polymorphic and highly modified, is thought to play an important role in organizing the higher order structure of chromatin. The cooperative binding of histone H1 (Renz, 1975) is essential to the general repression of chromatin (Weintraub, 1985). Chemical modification of DNA, binding of non-histone proteins to DNA, as well as the presence of histone variants and modified histones may all play a role in maintaining the "open" structure of transcriptionally active chromatin.

Conversion of chromatin from an inactive to transcriptionally active state involves at least two steps. The first involves conversion of inactive chromatin to transcriptionally competent

chromatin. This is also known as gene activation or commitment. The second step involves the actual induction and regulation of expression of potentially active genes.

1. Nuclease digestion studies

A variety of endonucleases have been used to investigate chromatin structure. However, there are conflicting results or interpretations of chromatin digestion sensitivity which can have several causes (reviewed by Reeves, 1984). First, the structure and composition of chromatin as well as its nuclease digestibility depend on the way it has been isolated from cells or nuclei. Second, since chromatin is a dynamic structure unsuspected alterations may greatly influence experimental results. Third, the digestion characteristics of endonucleases are dependent on environmental factors as well as substrate concentrations, conditions and preferences, further complicating interpretation of data. Fourth, although the basic structure and architecture of chromatin appears the same in all eukaryotic cells there are compositional and functional differences between cell types which may influence nuclease sensitivity. Finally, nuclease sensitivity is probably the result of multiple and complex structural and/or compositional features.

As probes of chromatin structure the most widely used non-specific endonucleases are DNase I and micrococcal nuclease. These enzymes recognize different aspects of chromatin structure. DNase I recognizes internal structural features of nucleosomes and preferentially degrades active or competent genes. Micrococcal nuclease is capable of

distinguishing between exposed and non-accessible DNA regions as well as between nucleosome core DNA and linker DNA. It is used to detect chromatin features related to actual rates of transcription under some conditions. Micrococcal nuclease has a sequence specificity (preferring CTA or CATA sequences) and unless used properly can be a misleading probe for nucleosome phasing in chromatin.

Active genes, previously active or potentially active genes are selectively sensitive to DNase I. The establishment of a generalized DNase digestion sensitivity is thought to be one of the important steps in the commitment of genes or regions of chromatin to transcription (Reeves, 1984). The general DNase I sensitive region of most cellular genes is not restricted to the coding region, but extends for a considerable distance on either side of transcribed regions. In steroid hormone induced chicken oviduct cells the active ovalbumin gene and two related genes are found in a sensitive domain approximately 100 kbp long which includes non-transcribed sequences. The DNase I sensitive structure is not dependent on transcription. Withdrawal of hormone from estrogen-stimulated chicks eliminates transcription, but does not alter the DNase I sensitive conformation (Lawson et al, 1982). The human β-globin gene cluster also has an enhanced sensitivity to DNase I when erythroid cells are compared to non-erythroid cells (Arapinis et al., 1986). It is tempting to speculate that this DNA sensitive domain may correspond to a looped domain attached to the nuclear matrix as seen in the model of chromosome structure.

DNase I sensitivity may also be in the form of hypersensitivity. Hypersensitive sites are ten times more sensitive to digestion by DNase

I than the generalized sensitivity of transcriptionally active/competent genes. These sites are also hypersensitive to a variety of nucleases (endogenous nucleases, restriction endonucleases) and are also referred to as nuclease hypersensitive regions. Nuclease hypersensitivity in chromatin is most frequently confined to domains in the 3'- or 5'-flanking regions of genes (Elgin, 1981) and has been implicated in both tissue specificity and developmental control of gene expression in a number of systems (for review see Reeves, 1984). There is evidence to indicate that these sites are free of nucleosomes and contain sites for sequence specific, DNA binding proteins (Shimada et al., 1986; Emerson et al., 1985). Nucleosome free, nuclease hypersensitive sites may be important for initiation of transcription as initiation but not elongation is inhibited by the presence of nucleosomes (Losa and Brown, 1987; Lorch et al., 1987; De Bernardin et al., 1986).

Actively transcribed genes are also more sensitive to digestion with micrococcal nuclease than inactive genes or bulk chromatin (Bloom and Anderson, 1979; Levy and Noll, 1981; Senear and Palmiter, 1981; Bode et al., 1986). Micrococcal nuclease can be used as a probe to obtain information on the chromatin structure of specific genes. Digestion of bulk chromatin results in a regular nucleosomal array or canonical repeat of the resulting DNA fragments (Noll, 1974). Micrococcal nuclease digestion of some transcriptionally active genes has resulted in a disrupted nucleosomal pattern, such that the discrete DNA fragment pattern is smeared (Wu et al., 1979; Rose and Garrard, 1984; Cohen and Sheffery, 1985; Strätling et al., 1986; Einck et al.,

1986). These results are interpreted as being due to a loss of higher order chromatin structure in active genes such as a deficiency in histone H1 leading to an irregularity in extended nucleosomal spacing.

2. DNA modifications and structure

Potential mechanisms for altering transcriptional activity of genes include specific modifications of DNA, as well as changes in DNA structure and conformation. In vertebrates, 5-methylcytosine (5-mC) is the only modified base to be found in appreciable quantities (Ehrlich and Wang, 1981). The usual site for cytosine methylation is the dinucleotide CpG which, perhaps significantly, is statistically under-represented in most higher eukaryotes (Ford et al., 1980). There is a preferential localization of 5-mC in nucleosomes containing histone H1 (Ball et al., 1983a). Although there tends to be an inverse correlation between the expression of a gene and level of DNA methylation, the function of DNA methylation is unclear and does not appear to be the same in all genes or eukaryotic cells (Reeves, 1984). The importance of looking at this modification of DNA in relation to the sequences surrounding it has been shown by Bird (1986). There tends to be clustering of non-methylated CpG in G+C-rich "islands". Bird argues that these islands function to distinguish regions of the genome that are available for transcription by interaction with nuclear components in all cells. When the islands become methylated, evidence suggests the genes associated with the G+C "islands" become inactivated. Genes that are expressed in a highly tissue specific manner are not usually associated with these "islands" and

demethylation is not sufficient to activate these genes and may not be necessary either.

Structural changes in DNA, such as conversion of right-handed B-DNA to left-handed Z-DNA may play a role in the control (either positive or negative) of transcription. There are specific Z-DNA binding proteins (Kolata, 1983; Lipps et al., 1983) that can in some cases flip potential Z-DNA forming sequences from a B-form into a Z-form and stabilize it. Garner and Felsenfeld (1987) have shown that in plasmids, DNA in the Z-form cannot be incorporated within nucleosome core particles and this may result in a significantly altered local placement of nucleosomes. The transition of synthetic poly (dG.dC) oligonucleotides from B to Z form at near physiological salt concentration is facilitated by the methylation of cytosine residues in the polymers (Behe and Felsenfeld, 1981; Behe et al., 1981; Möller et al., 1981). Figure 4 shows how changing Z-DNA to B-DNA, or vice versa, can affect the level of supercoiling of an entire "domain" of DNA (Rich, 1983).

The role torsional stress may have in gene expression of eukaryotes is not well understood. Some observations made using DNA sequences injected into Xenopus oocytes indicate torsional stress is important. First, closed or nicked circular DNA was a much better template for transcription than a linear fragment (Wyllie et al., 1978; Probst et al., 1979). Second, DNA was assembled into chromatin prior to transcription (Wyllie et al., 1978; Gurdon and Brown, 1978). Villeponteau et al. (1984) have shown with chicken red blood cells that in the absence of the superhelical tension generated by supercoiling,

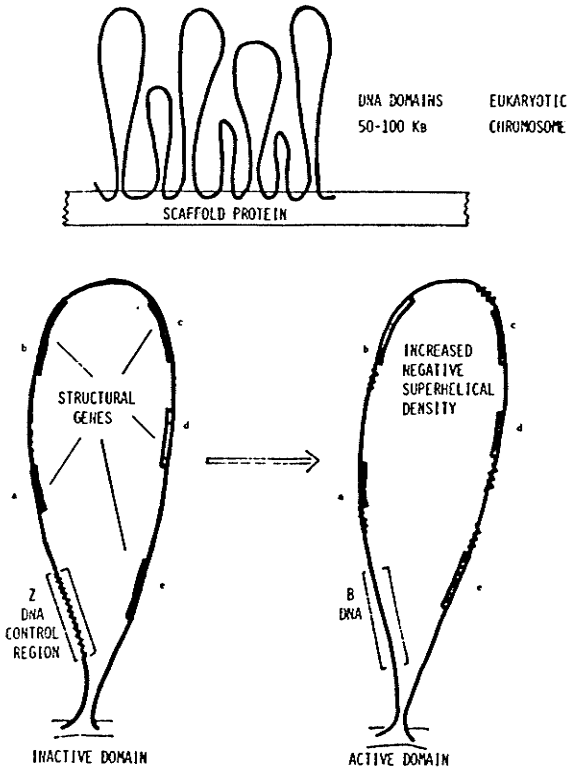

Figure 4. **A model of DNA-domain activation in chromatin.** (Top) DNA domains present in the eukaryotic chromosome as they are visualized by electron microscopy once histones have been removed. The DNA is found to loop out in segments from a nuclear matrix or scaffold protein, which isolates the DNA for supercoiling between the different domains. (Bottom) An individual domain that is inactive when the control region is in the form of Z-DNA but becomes activated when it turns to B-DNA. Activation is associated with a change in superhelical density, and it could uncoil the structure to make it available for polymerases. The loop in this case is a schematic representation of higher-order aggregation of nucleosomes. This loop is shown to have five different structural genes a-e. (from Rich, 1983)

active chromatin reverts to a less DNase I sensitive state. This seems to indicate that supercoiling is important in maintaining the features associated with active chromatin. However, the results of Petryniak and Lutter (1987) on the topological state of the DNA of the Simian Virus 40 transcription complex indicate that topological tension is not required for eukaryotic gene expression.

3. Chromatin fractionation

Chromatin fractionation has been used to differentiate between those proteins associated with active genes and those associated with inactive genes. Unfortunately, even the best fractionation provides only an enrichment, not purification of active chromatin. The conditions used for fractionation sometimes permit rearrangement or aggregation of chromatin leading to a misinterpretation of results. Conflicting data that are the result of fractionation of chromatin can be due to the particular conditions used by different investigators. Interpretation of any data that are the result of chromatin fractionation should be critically evaluated.

Most fractionation of chromatin involves digestion with nucleases and selection of nucleosomes on the basis of certain properties such as nuclease accessibility, solubility, electrophoretic mobility, reaction with specific antibodies (immunofractionation) or specific reagents.

Bloom and Anderson (1978) fractionated hen oviduct chromatin by micrococcal nuclease susceptibility and solubility properties. The active ovalbumin gene was sensitive to micrococcal nuclease and chromatin fragments generated by digestion were soluble in the

digestion buffer.

The Sanders (1978) procedure also fractionates chromatin by micrococcal nuclease accessibility and salt solubility. The importance of Mg^{2+} in maintaining higher order chromatin structure was recognized and this ion was included during isolation and digestion of nuclei. Increasing concentrations of NaCl were used to dissociate subpopulations of nucleosomes differing in their packing interactions. The low salt (0.2 M NaCl) eluted fraction was most sensitive to micrococcal nuclease, contained non-histone chromosomal proteins and was deficient in histone H1. Low salt eluted fractions of calf thymus and mature chicken erythrocyte chromatin are enriched in transcriptionally active or competent genes (Davie and Saunders, 1981; Rocha et al., 1984). These results indicate that active genes are found in the low salt soluble, histone H1 depleted fraction of chromatin. Transcriptionally active genes can also be associated with the insoluble nuclear material (Robinson et al., 1983; Ciejek et al., 1983; Rose and Garrard, 1984; Hentzen et al., 1984; Cohen and Sheffery, 1985; Einck et al., 1986; Strätling et al., 1986).

A fractionation method has been described that enriches for transcriptionally active chromatin from trout testis on the basis of micrococcal nuclease accessibility and solubility properties (Levy-Wilson and Dixon, 1979; Levy and Dixon, 1978). Limited digestion of nuclei was followed by solubilization of nucleohistone in 1 mM EDTA. The EDTA soluble fraction was divided into a fraction soluble in 100 mM NaCl, MN_1, and a fraction insoluble in 100 mM NaCl, MN_2. Both fractions were enriched in sequences that hybridized to cytoplasmic

polyadenylated RNA. The MN_2 fraction contained histone H1 (Hutcheon et al., 1980).

In mature chicken erythrocyte chromatin eluted at approximately physiological ionic strength following micrococcal nuclease digestion, the bulk of the DNA sequences, including the transcriptionally inactive vitellogenin gene, was in mononucleosomes. A small percentage of the solubilized chromatin fragments was polynucleosomes, including the transcriptionally competent β-globin gene (Ridsdale and Davie, 1987a). The β-globin polynucleosomes were complexed with lower levels of histones H1 and H5 than was unfractionated chromatin and this may account for differences in the solubility properties of β-globin gene chromatin (Ridsdale and Davie 1987b).

Nucleosomes have been fractionated by electrophoretic mobility (Levinger and Varshavsky, 1980) and the two-dimensional hybridization mapping technique used to correlate protein composition with specific genes (Levinger et al., 1981). Nucleosomes, DNA with associated proteins, are resolved by electrophoresis in the first dimension. Following DNA electrophoresis in the second dimension, DNA is transferred to filters and hybridized with specific gene probes. The protein composition of the nucleosomes is determined in parallel by using a gel system that resolves proteins in the second dimension. Finally, second dimension DNA and protein patterns are correlated.

Recently Walker and Sikorska (1986) demonstrated that the ionic composition of the buffers for nuclei isolation and subsequent digestion with micrococcal nuclease can influence the apparent nuclease sensitivity of chromatin. They report that physiological levels of

monovalent (150 mM KCl) and divalent (2-5 mM $MgCl_2$) cations are required to maintain the higher order structure of chromatin. The ionic composition of the buffer used for nuclease digestion can affect the amount of material digested by modulating higher order structure and by determining solubility of the released material. Magnesium ions at any concentration greater than 2 mM, and calcium ions at virtually any concentration can precipitate substantial amounts of the released fragments. Confirmation of their observations may lead to a reevaluation of some chromatin fractionation experiments and some of the properties associated with active chromatin.

4. Protein composition

a. Non-histone chromosomal proteins

The high mobility group (HMG) proteins are among the most extensively studied non-histone chromosomal proteins. This group of low molecular weight, salt extractable (0.35 M NaCl) and acid soluble proteins includes HMG-1, HMG-2, HMG-14 and HMG-17 (Cartwright et al., 1982). In trout testis HMG-6 is homologous to HMGs 14 and 17, and HMG-T is homologous to HMGs 1 and 2 from calf thymus (Cary et al., 1981). The role HMGs 14 and 17 may play in maintaining the DNase I sensitivity of active chromatin is uncertain. Many studies looking at association of HMGs 14 and 17 with transcriptionally active chromatin have yielded conflicting results (see Brotherton and Ginder, 1986).

Weisbrod and Weintraub (1979) found that nucleosome core particles depleted of HMGs 14 and 17 lost their preferential sensitivity to DNase I, but when an enriched fraction containing these proteins was added

back, DNase I sensitivity was restored. The same authors isolated actively transcribed nucleosomes with an HMG 14 and 17 affinity column (Weisbrod and Weintraub, 1981). Reeves and Chang (1983) were unable to use purified HMGs 14 and 17 to restore the preferential DNase I sensitivity of the β-globin gene in Friend erythroleukemia cells or the total complement of transcriptionally active genes in calf thymus. However, they found a preferential association between glycosylated HMGs 14 and 17 and the nuclear matrix that was mediated through the carbohydrate side chains. Since the nuclear matrix is thought to be involved in DNA replication and transcription this association may be significant.

Mathew et al. (1981) found HMGs 14 and 17 in fractions enriched for nontranscribed rat satellite DNA. The exact location of HMGs 14 and 17 containing nucleosomes within a gene may be important in conferring DNase I sensitivity. Originally, Varshavsky's group did not find a preferential association between HMGs 14 and 17 and the amplified, transcriptionally active dihydrofolate reductase gene in mouse cells (Barsoum et al., 1982; Varshavsky et al., 1983). In a later report the same authors allude to the association of HMGs 14 and 17 with the nucleosomes from the first exon of the same gene (Barsoum and Varshavsky, 1985). In a recent study, a transition from HMG-17 free to HMG-17 containing nucleosomes in the coding region of active genes was correlated with the high sensitivity to DNase I in the same region (Dorbic and Wittig, 1987). Therefore, it is possible that HMGs 14 and 17 found associated with transcriptionally inactive genes may be outside the coding region of a gene.

In vitro glycosylated and nonglycosylated HMGs 14 and 17 can partially inhibit histone deacetylase enzymes in nuclear assay systems (Reeves and Chang, 1983). If histone acetylation is responsible for maintaining the nuclease sensitivity of active chromatin then HMGs 14 and 17 may also be involved by inhibiting deacetylases.

b. Histone variants

Primary sequence analyses indicate that histones as a general class of proteins have highly conserved amino acid sequences (reviewed by Wu et al., 1986). Histone variants within the same species, may be responsible for making nucleosomes structurally and functionally heterogeneous, thus affecting nucleosome and chromatin organization. The patterns of histone variants associated with transcriptionally active chromatin have not been extensively investigated.

Histone H4 is the most evolutionarily conserved histone and generally has no variants. Histone H3 is also highly conserved between species and three variants have been observed (H3.1, H3.2 and H3.3). In quiescent lymphocytes histone H3.3 is the major H3 variant, but after stimulation with phytohemaglutinin, H3.1 and H3.2 are the major variants (Wu et al., 1983). It is not known if the change in histone H3 variants is related to changes in DNA replication or transcription. There was an increase in the level of histone H3.3 in the chromatin fraction enriched for transcriptionally competent sequences from mature chicken erythrocytes (Ridsdale and Davie, 1987b).

Histone H2B is evolutionarily more divergent than histones H3 and H4 and the number of variants differs from species to species. Histone

H2A is the most evolutionarily divergent of the core histones and has the most variant forms. In Tetrahymena the macronuclear specific histone, hv1, is a member of the H2A family and is enriched in transcriptionally active chromatin (Allis et al., 1986). The level of histone variant, H2A.Z is also elevated in the chromatin fraction enriched for transcriptionally competent DNA sequences (Ridsdale and Davie, 1987b). These variants of histone H2A may be one of the factors responsible for differentiating between active and inactive chromatin.

Histones H1, H1° and H5 belong to the linker histone family and play a major role in the organization of chromatin into higher order structures (Thoma et al., 1979). As higher order structure is thought to influence the transcriptional activity of chromatin, variation in linker histones may be an important factor in maintenance of the "open" structure associated with transcriptionally active chromatin. The number of histone H1 variants and the amount of each variant can differ from tissue to tissue, and for a given tissue can differ from one species to another (Wu et al., 1986). There are differences in the ability of the individual H1 subtypes to condense chromatin (Huang and Cole, 1984).

Histone H5, found in nucleated red blood cells, binds more tightly to chromatin than histone H1 and is thought to be responsible for the transcriptional inactivity of these terminally differentiated cells (Thomas and Rees, 1985; Thomas et al., 1985; Bates et al., 1981; Mazen et al., 1982). Histone H1° is similar in amino acid composition and immunochemical properties to histone H5 (see Mendelson et al., 1986). However, the exact role of histone H1° in chromatin is unclear.

Histone H1° is developmentally regulated (Gjerset et al., 1982) and may have a role in developmental gene regulation. Histone H1° was associated with the repressed α-fetoprotein gene, but not the expressed albumin gene in adult mouse liver (Roche et al., 1985). Immunofractionation of rat liver chromatin using an antibody that recognized histone H1° resulted in a slight enrichment of this histone in the coding region of the rat albumin gene (Mendelson et al., 1986). These authors have suggested that the histone H1° content of constitutively transcribed chromatin regions is not necessarily different from that of non-transcribed regions. They have also found a depletion of histone H1° in the 3'-region of the highly inducible cytochrome P-450 gene. Reduced levels of histones H1° and H1b have been found in rainbow trout hepatocellular carcinoma (Davie et al., 1987), but it is not known if these changes are related to an increase in DNA synthesis or transcription.

c. Histone modifications

There are many possible post-synthetic modifications of histones (reviewed by Wu et al., 1986), but how these modifications are involved in chromatin function is not known. The modifications most implicated in the control of gene transcription are poly(ADP)-ribosylation, acetylation and ubiquitination.

(i) Poly(ADP)-ribosylation

The chromatin associated enzyme, poly(ADP-ribose)polymerase, catalyzes transfer of the ADP-ribose moiety of NAD to a variety of

nuclear proteins including histones (Mandel et al., 1982). Electron microscopy has shown that in vitro ADP-ribosylation of histone H1 is associated with a relaxed chromatin structure (Frechette et al., 1985; de Murcia et al., 1986). This "open" structure may facilitate DNA replication, repair or transcription. Results of fractionation of HeLa cell chromatin using an anti-poly(ADP-ribose)-Sepharose column show that some, but not all, actively transcribed chromatin contains poly-ADP ribosylated proteins (Hough and Smulson, 1984)). Since these proteins are also associated with inactive genes, this modification may function in more than transcription. Kreimeyer et al. (1984) have shown that ADP-ribosylation of histone H1 in vivo was associated with DNA repair.

(ii) Acetylation

Core histones can be modified by the acetylation of the ε-amino groups of specific lysine residues within the amino-terminal regions. The involvement of histone acetylation in transcriptionally active chromatin has been controversial. Studies on hyperacetylation induced by treatment with sodium butyrate, an inhibitor of the enzyme deacetylase, can be misleading as butyrate can affect more than histone acetylation in the nucleus (Boffa et al., 1981). Some butyrate induced changes in the nuclease sensitivity of chromatin cannot be correlated with trancriptional activation (Birren et al., 1987). Currently it is believed that physiological hyperacetylation, in contrast to butyrate induced hyperactetylation, of histones H3 and H4 may have a significant function in transcriptionally active chromatin. When

chromatin in the presence of Mg^{2+} was digested with micrococcal nuclease and fractionated by solubility, an elevation in the level of hyperacetylated histone H4 was found in the fractions enriched for transcriptionally active/competent genes (Kuehl et al., 1980; Nelson et al., 1986; Rocha et al., 1984). The soluble polynucleosome fraction of chicken erythrocyte chromatin that was highly enriched in β-globin DNA was rendered insoluble by in vitro deacetylation (Alonso et al., 1987) demonstrating that the enhanced solubility of this active chromatin was a direct consequence of histone hyperacetylation.

Loidl and Gröbner (1987) have used a protamine competition assay as a probe of altered chromatin structure. In Physarum the facilitated release of histones by protamine was dependent on the physiologically correct acetylation state. Chromatin with butyrate induced hyperacetylation did not show the same property. This demonstrates that the mechanism by which acetylation changes nucleosome structure involves more than a decrease in the net positive charge of the N-terminal tails of the histones. The specific acetylation state seems to be a distinct signal and is missing in hyperacetylation induced by butyrate. Studies on Tetrahymena and Physarum have shown that the post-synthetic acetylation-deacetylation process associated with transcription is non-random and that functionally distinct acetylation sites exist (Chicoine et al., 1986; Pesis and Matthews, 1986). The sites used for butyrate induced acetylation may differ from those used naturally.

In contradiction to the results of Loidl and Gröbner (1987), two groups have shown that butyrate induced hyperacetylation can alter histone displacement by protamines (Oliva et al., 1987; Bode et al.,

1980). Histones displaced by protamine competition had a higher degree of hyperacetylation than residual histones which indicates that nucleosomes containing hyperacetylated histones were less stable (Oliva et al., 1987). Fractionation of chromatin by the increased accessibility of the sulfhydryl groups of histone H3 has shown a correlation between hyperacetylated histones H3 and H4 and transcriptionally active chromatin (Allegra et al., 1987; Sterner et al., 1987). This indicates that transcriptionally active nucleosomes may be conformationally different.

When Physarum ribosomal gene chromatin was actively transcribed, the sulfhydryl group of histone H3 became accessible to a sulfhydryl specific reagent (Prior et al., 1983). Electron microscopy of fragments with reactive sulfhydryl groups revealed extended nucleosomal subunits consisting of two roughly spherical bodies connected by a 50 bp nucleoprotein bridge. In the model proposed by these authors (shown in Figure 5) the unfolding of the nucleosome core particle is thought to be mediated by specific non-histone proteins, LP30 and LP32. These altered nucleosomes contained histone H3 that was predominantly tetra- and triacetylated (Johnson et al., 1987).

It is unclear exactly how the structure of nucleosomes containing hyperacetylated histones may be changed in other systems. Bode et al. (1983) using butyrate to induce hyperacetylation in lymphoblastoid cells found conformational changes correlated to the degree of acetylation. He proposed that there is a minimal level of acetylation required to "open" the nucleosome. In agreement with this model of

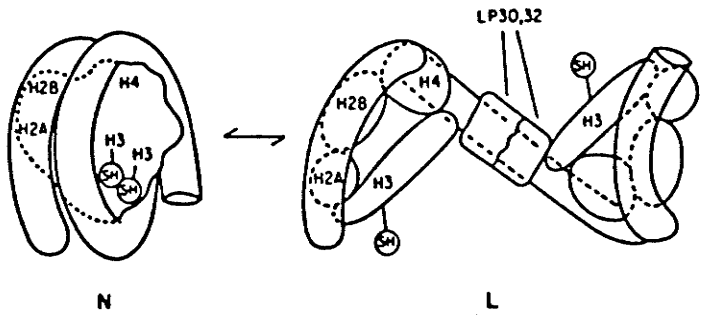

Figure 5. A model for the nucleosome (N) to lexosome (L) transition. (From Prior et al., 1983)

structural alteration by hyperacetylation Oliva et al. (1987) found by electron microscopy that hyperacetylated core particles from butyrate treated HeLa-S3 cells had an elongated shape compared to control nucleosomes. Other studies using butyrate induced hyperacetylation of the same strain of cells have shown no change in the structure of hyperacetylated chromatin as judged by light scattering, flow linear dichroism, hydrodynamic behaviour, circular dichroism and neutron scatter (Dimitrov et al., 1986; Ausio and van Holde, 1986; Imai et al., 1986).

Although physiological histone acetylation does appear to play an important role in modulating the "open" structure of transcriptionally active chromatin, the exact mechanism by which this is accomplished is not clear. Conflicting results may be due to the reasons discussed previously under nuclease digestion studies.

(iii) Ubiquitination

Ubiquitin, a small protein of 76 amino acids, is present in all cells. The amino acid sequence of ubiquitin is extremely conserved in evolution. Ubiquitin variants from animals, plants and yeast differ from each other in three or fewer residues (see Özkaynak et al., 1987). Ubiquitin has been implicated in a variety of cellular functions. In the cytoplasm, ubiquitin "tags" proteins for degradation via an ATP-dependent non-lysosomal pathway and the rate of proteolysis increases with the number of attached ubiquitins (Ciechanover et al., 1981). It has been suggested that an analogous system may operate in the nucleus with the proteolytic removal of nucleosomal proteins from activated

chromosomal regions being responsible for the striking changes in chromatin structure observed at very high rates of transcription (Varshavsky et al., 1983). In the nucleus ubiquitin can be found covalently joined via an isopeptide linkage to approximately 10% of the nucleosomal histone H2A (Busch and Goldknopf, 1981) and to approximately 1-2% of the histone H2B (West and Bonner, 1980). Ubiquitinated forms of histone H2A and histone H2B are known as uH2A and uH2B, respectively. In calf thymus uH2A the C-terminal glycine of ubiquitin is attached to the ε-amino group of lysine 119 in the C-terminal portion of the histone H2A molecule (Goldknopf and Busch, 1977). The site of ubiquitin attachment in uH2B from calf and pig is similar, with an isopeptide bond formed between the C-terminal glycine of ubiquitin and the ε-amino group of lysine 120 (Thorne et al., 1987). Modification of histones with ubiquitin is a dynamic process. The ubiquitin portion of uH2A turns over more rapidly than the H2A portion (Seale, 1981) and ubiquitin moieties of chromatin are in rapid equilibrium with the pool of free ubiquitin in dividing and nondividing cells (Wu et al., 1981). The function of ubiquitin in the nucleus is not clear. Ubiquitinated histones may be involved in preventing the formation of higher order chromosomal structures. During mitosis, condensation of chromatin into metaphase chromosomes has been correlated with a loss of ubiquitin from nucleosomes while decondensation has been correlated with the reappearance of ubiquitinated histones (Matsui et al., 1979; Wu et al., 1981; Mueller et al., 1985; Raboy et al., 1986). The association of ubiquitinated histones with transcriptionally active chromatin is more controversial.

Results of early studies on ubiquitinated histones are questionable because inhibitors of isopeptidase were not included. Isopeptidase, a cytoplasmic enzyme, is capable of cleaving the ubiquitin moiety from uH2A (Matsui et al., 1982). This enzyme requires free SH-groups for activity and can be inhibited by thiol blocking agents such as N-ethylmaleimide and iodoacetamide. In transcriptionally active erythroid cells from phenylhydrazine-treated chickens the level of uH2A was increased approximately 6-fold when compared to less transcriptionally active mature erythrocytes (Goldknopf et al., 1980). A significant increase in the levels of uH2A and uH2B has also been found in the chromatin fraction of mature chicken erythrocytes enriched for the transcriptionally competent β-globin gene (Ridsdale and Davie, 1987b). Inhibition of transcription did not affect the total amount of uH2A in chromatin which suggests that the overall level of ubiquitination was not directly coupled to transcription (Ericsson et al., 1986). Instead, the authors have proposed that the pattern of ubiquitination of H2A is established on a long-term basis and is related to overall organization of chromatin in the interphase nucleus. Levinger (1985) studied two simple satellite sequences in Drosophila by two-dimensional hybridization mapping of nucleosomes. The chromatin of one of these satellite sequences was enriched in uH2A and showed an increased sensitivity to micrococcal nuclease. These results indicate that uH2A may be involved in altering the higher order structure of chromatin as evidenced by an altered sensitivity to nuclease digestion.

Varshavsky's group has used the technique of two-dimensional hybridization mapping of nucleosomes to study the association of

ubiquitinated nucleosomes with some Drosophila and mammalian genes present in high copy number. Their results indicate that transcribed genes are associated with ubiquitinated nucleosomes while non-transcribed sequences, such as satellite DNA, are underubiquitinated (Levinger and Varshavsky, 1982; Varshavsky et al., 1983; Barsoum and Varshavsky, 1985).

One of the criticisms of these studies is that identification of ubiquitinated core mononucleosome DNA is based on a correlation of the second dimension DNA and protein patterns. Huang et al. (1986) have used isopeptidase to show that the altered electrophoretic mobility of nucleosomes containing the active immunoglobulin kappa chain gene was not due to the presence of ubiquitinated histones. Other histone variants or modifications of lower abundance, could be directly responsible for the alteration in nucleosomal mobility. Acetylation, for example, is known to alter the mobility of nucleosomes (Bode et al., 1983; Imai et al., 1986). For this reason, the results of the two-dimensional hybridization experiments are questionable and the role of ubiquitin in the nucleus is still not known.

C. Spermatogenesis in rainbow trout

Some of the characteristics associated with active chromatin have also been found associated with chromatin undergoing replacement of histones by protamines during spermatogenesis.

Spermatogenesis in vertebrates is a complex developmental process which sequentially generates five major germ cell types: the spermatogonium, the primary and secondary spermatocytes, the spermatid and the spermatozoan (Figure 6). With the onset of spermatogenesis, the spermatogonia undergo a series of mitotic divisions producing primary spermatocytes. Meiosis in these cells results in the production of secondary spermatocytes which in turn produce four haploid spermatids that differentiate into mature sperm. Terminal differentiation of germ cells to the highly specialized spermatozoa is accompanied by several morphological and biochemical changes. There is a decline in RNA synthesis from a high level in spermatogonia and spermatocytes to a low level in early spermatids and absence in late spermatids and mature spermatozoa (Gillam et al., 1979). However, the most striking change in morphology and molecular composition occurs during the final stages of sperm cell maturation with the synthesis of the highly arginine rich, sperm-specific nuclear proteins, the protamines.

The rainbow trout system has been useful for studying the biochemical changes that take place during spermatogenesis. Although rainbow trout testes do not contain completely synchronized cell populations, spermatogenesis proceeds in a semi-synchronous fashion. Cells at all developmental stages are present at all times, but one

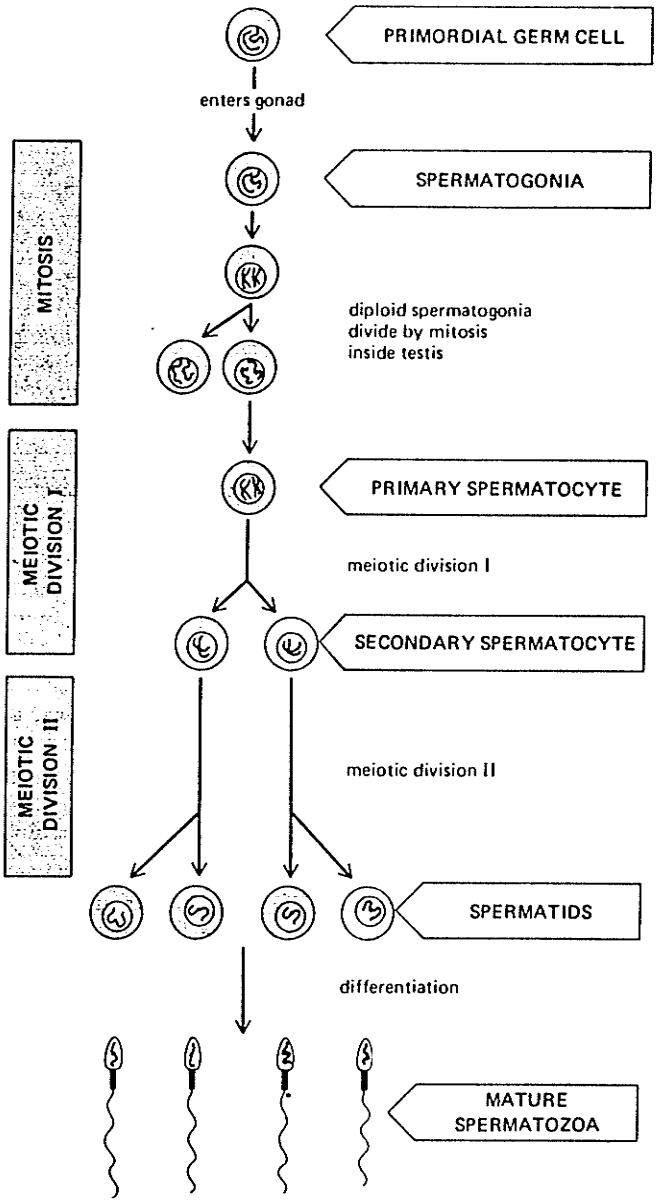

Figure 6. **The various stages of spermatogenesis.**
When the animal becomes sexually mature, the spermatogonia divide by mitosis to produce primary spermatocytes. The primary spermatocytes divide by meiosis producing secondary spermatocytes. Following completion of the second meiotic division, the secondary spermatocytes produce haploid spermatids that differentiate into mature sperm. (from Alberts et al., 1983)

particular cell type predominates depending on the stage of testis maturation (Iatrou et al., 1978). Injection of salmon pituitary gonadotropin accelerates spermatogenesis in trout and the development is more synchronous than in naturally maturing testis (Drance et al., 1976).

In trout, protamine mRNA synthesis occurs around the primary spermatocytes cell stage and newly transcribed protamine mRNA sequences are stored in the cytoplasm as messenger ribonucleoprotein (mRNP) particles until the spermatid cell stage at which time they are translated (Iatrou et al., 1978, Sinclair and Dixon, 1982). Replacement of histones by protamines begins at the middle spermatid stage and is complete by the late spermatid stage (Louie and Dixon, 1972). As a result of this replacement the chromatin of the spermatid nucleus becomes highly condensed and the nucleus itself undergoes a marked contraction. The replacement of histones by protamines allows the DNA to be packed very tightly into the limited volume of the sperm head and as a consequence of this the total repression of gene activity in the terminally differentiated sperm cell (Dixon, 1972).

The nucleohistone to nucleoprotamine transition may be explained as a simple process in which protamines compete with histones in binding to DNA. However, it is likely that there are several factors that could affect the transition such as changes in DNA topology, topoisomerase activity, nonhistone proteins, ionic environment and modifications of histones such as acetylation, ubiquitination or ADP-ribosylation. Histones, displaced in vitro from HeLa cell nuclei by protamine competition at physiological pH and salt concentrations show

a higher degree of histone acetylation than residual histones (Oliva et al., 1987). These authors have proposed that histone hyperacetylation facilitates in vitro nucleosome dissociation by protamines as well as decreasing the stability of nucleosomes. Histone hyperacetylation is thought to play a role in vivo in spermiogenesis as one of the mechanisms leading to nucleosomal disassembly. Acetylation of histones is found in spermatids and an increase in the level of hyperacetylated histone H4 has been observed in the late stages of trout testis development (Christensen and Dixon, 1982).

D. Research aims

1. To study the chromatin structure of the protamine genes in developing rainbow trout testis.

2. To determine if there are qualitative and/or quantitative changes in histone variants and particularly ubiquitinated histone species during spermatogenesis in rainbow trout.

3. To characterize the arrangement of the multiple ubiquitin moieties in polyubiquitinated histone H2A.

4. To examine the distribution of ubiquitinated and polyubiquitinated histones in fractionated chromatin.

II. GENERAL PROCEDURES

A. Tissues

1. Trout

Livers, erythrocytes and testes at various stages of development were obtained from rainbow trout (Salmo gairdnerii, Mount Shasta strain at the Oregon State University Food Toxicology and Nutrition Laboratory fish hatchery facility, Corvallis, Oregon and Nisqually strain at the Rockwood Fish Hatchery, Rockwood, Manitoba). The tissues were stored at -80°C.

The stage of the testis was evaluated by the following: (1) weight and size (Louie and Dixon, 1972; Gillam et al., 1979); (2) DNA to tissue wet weight ratio (early stage, 23 mg/g; intermediate stage, 44 mg/g; late stage, 80 mg/g) and (3) relative content of histones to protamines.

2. Calf

Calf liver and thymus were obtained from a local slaughterhouse. Tissues were stored at -80°C.

3. Chicken

Red blood cells from adult white leghorn chickens were collected in 75 mM NaCl, 25 mM EDTA, 30 mM Na butyrate, pH 7.5 and washed to remove the buffy coat (Rocha et al., 1984). Packed red blood cells were stored in 5 ml aliquots at -80°C.

B. Experimental Procedures

1. Preparation of nuclei

The following solutions were used in the preparation of nuclei:

Solution	Compositition
Buffer A:	1 M hexylene glycol 10 mM PIPES, pH 7.5 2 mM $MgCl_2$ 30 mM Na butyrate
Buffer B:	1 M hexylene glycol 10 mM PIPES, pH 7.0 2 mM $MgCl_2$ 30 mM Na butyrate 1% (v/v) thiodiglycol
Buffer C:	10 mM NaCl 10 mM Tris-HCl, pH 7.5 3 mM $MgCl_2$ 30 mM Na butyrate
0.54 M iodoacetamide	prepared fresh daily in buffer A or C, diluted in buffer just before using
25% (v/v) NP-40	
100 mM PMSF in isopropanol	

* 1 mM PMSF was included in all buffers used.

a. Trout testis nuclei

Trout testes were homogenized in 10 volumes of buffer A containing 10 mM iodoacetamide. Iodoacetamide is an inhibitor of the enzyme isopeptidase which will remove the ubiquitin moiety from a ubiquitinated protein (Matsui et al., 1982). The scissor minced tissue was homogenized in a Waring Blender cup at the lowest speed for 1 min. The homogenate was filtered through four layers of cheesecloth and centrifuged at 750 x g for 10 min. The pellet was resuspended in buffer A by trituration using a cut off Pasteur pipette, homogenized three times in a Potter-Elvehjem homogenizer and centrifuged at 750 x g for 10 min. The resulting pellet was resuspended in buffer B and centrifuged as before. This step was repeated once more.

b. Trout liver nuclei

Trout liver nuclei were isolated as described above except the liver was scissor-minced in buffer A containing 0.2 % (v/v) NP-40 and homogenized in a Potter-Elvehjem homogenizer. Following centrifugation, the pellet was resuspended in buffer A and the nuclei collected by centrifugation. The nuclei were resuspended in buffer B and centrifuged as before. This step was repeated once more.

c. Trout erythrocyte nuclei

Trout erythrocytes were thawed at 37°C in a portion of 10 volumes of buffer A containing 10 mM iodoacetamide. Once thawed, the remainder of the buffer was added and the erythrocytes were homogenized three times in a Potter-Elvehjem homogenizer. The nuclei were collected by

centrifugation at 750 x g for 10 min. The nuclei were resuspended in buffer A and centrifuged at 750 x g for 10 min. The resulting nuclei were resuspended in buffer B and collected by centrifugation at 750 x g for 10 min.

d. Calf thymus nuclei

Calf thymus nuclei were prepared as described for trout testis nuclei.

e. Calf liver nuclei

Calf liver nuclei were prepared as described for trout testis nuclei except that 0.2% (v/v) NP-40 was included in buffer A.

f. Chicken erythrocyte nuclei

Chicken erythrocytes were thawed at 37 °C in a portion of 10 volumes of buffer C containing 0.25% (v/v) NP-40 and 10 mM iodoacetamide. Once thawed the remainder of the buffer was added and the erythrocytes homogenized three times in a Potter-Elvehjem homogenizer. The nuclei were collected by centrifugation at 750 x g for 10 min. The nuclei were resuspended in buffer C containing 0.25% (v/v) NP-40 and 10 mM iodoacetamide and again centrifuged at 750 x g for 10 min. The nuclei were washed once more in buffer C containing 10 mM iodoacetamide and centrifuged at 750 x g for 10 min.

2. Micrococcal nuclease digestion of chromatin

Nuclei were resuspended at 40 A_{260} units/ml or 50 A_{260} units/ml for

chicken erythrocyte nuclei, in buffer B and made to 1 mM $CaCl_2$ using a 200 mM $CaCl_2$ solution. Nuclei were preincubated at 37°C for 10 min. Micrococcal nuclease (Pharmacia) was added and the nuclei incubated with mixing for the appropriate time. Enzyme units and digestion time are specified in the text. The reaction was terminated by the addition of 0.25 M EGTA to a final concentration of 10 mM and the mixture placed on ice. Nuclei were pelleted by centrifugation at 750 x g for 10 min and a supernatant, S0 collected. The fraction S0 contained nucleotides released by digestion with micrococcal nuclease and the amount of A_{260} absorbing material was indicative of the extent of digestion.

3. Sanders fractionation of chromatin

Chromatin was fractionated according to the Sanders (1978) procedure. The solutions required for this procedure are:

Solution	Composition
2 x buffer D:	100 mM Tris-HCl, pH 7.0 4 mM $MgCl_2$ 2% (v/v) thiodiglycol 50 mM KCl 20 mM EGTA 60 mM Na butyrate
2 M NaCl	

Micrococcal nuclease digested nuclei collected by centrifugation were sequentially incubated at 0°C in buffer D (same volume as that used for the micrococcal nuclease digestion) containing increasing concentrations of NaCl (between 50 and 500 mM NaCl) (Sanders, 1978). The incubations yielded supernatants designated as salt soluble (SS)

and a number corresponding to the molarity of NaCl used (i.e. SS0.05 = salt soluble in 0.05 M NaCl). Chromatin fractions were dialyzed against 1 mM EDTA, 0.1 mM PMSF overnight at 4°C to remove the salt prior to acid extraction of proteins and/or purification of DNA.

4. Acid extraction of proteins

Proteins could be acid extracted from chromatin directly or following lyophilization and resuspension in ddH_2O. The samples were made 0.4 N H_2SO_4 by the addition of 4 N H_2SO_4 while mixing. Samples were allowed to sit at least 30 min on ice before the insoluble nuclear material was removed by centrifugation at 12,000 x g for 10 min. The acid extracts were dialyzed against 0.1 N acetic acid overnight at 4°C and then against at least two changes of ddH_2O before being lyophilized. Proteins were redissolved in ddH_2O and the protein concentration measured using the TCA assay for protein. The amount of protein remaining in the sample was calculated and the sample relyophilized. Proteins were redissolved at a concentration of 10 mg/ml in ddH_2O.

5. Protamine release of histones

A portion of a chromatin sample of known A_{260} was lyophilized and resuspended to a concentration of 60 A_{260} units/ml in a sample buffer containing protamine (1% (w/v) protamine sulfate (grade X from salmon, Sigma), 100 mM Tris-acetate pH 8.8, 20% (v/v) glycerol, 8 M urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) thiodiglycol and 1% (w/v) cysteamine hydrochloride (modified from Richards and Shaw, 1982). Samples were

centrifuged approximately 1 min and the supernatant decanted into a clean tube. The supernatant was loaded directly onto an AUT 15% polyacrylamide gel.

6. Purification of DNA

Purification of DNA was done according to the method of Maniatis et al., 1982. The following solutions were required:

Solution	Composition
0.25 M EDTA, pH 7	
1.6 M NaCl	
22% (w/v) Na sarcosyl	
2 M Na acetate	
10% (w/v) 8-hydroxyquinoline in ethanol	
pronase: (Boehringer-Mannheim 165 921)	dissolve at 10 mg/ml in 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0, 0.01 M EDTA Heated at 70°C for 1 min to remove nucleases. Kept at room temperature while aliquoting. Stored at -20°C.
RNase:	bovine pancreatic RNase A (Sigma R5000) final conc. 5 mg/ml T_1 RNase (Sigma R8251) final conc. 5000 U/ml T_1 RNase dialyzed against ddH_2O 16 h at 4°C. Pancreatic RNase added and volume adjusted. Aliquoted on ice (0.5 ml) and then heated at 80°C for 10 min. Stored at -20°C.
phenol:	Redistilled phenol was thawed in hot water and 10% 8-hydroxyquinoline added to a final concentration of 0.1%. The phenol was then equilibrated with buffer by extracting with an equal volume of 1.0 M Tris-HCl, pH 8.0 followed by 0.1 M Tris-HCl, pH 8.0 containing 0.2% mercaptoethanol.
IAC:	24:1 (v/v) chloroform and isoamyl alcohol

DNA was purified from chromatin or directly from nuclei. Salt eluted fractions of chromatin were dialyzed against 1 mM EDTA, 0.1 mM PMSF overnight at 4°C and lyophilized. The DNA sample was adjusted to 10 mM EDTA, 0.16 M NaCl and 2.2% Na sarcosyl using stock solutions. Pronase (1/20 volume) was added and the sample incubated at 37°C for 16 h. RNase (1/50 volume) was added and the sample incubated at 37°C for 2-3 h. Pronase (1/20 volume) was added again and the sample incubated 2-16 h at 37°C. The DNA sample was extracted twice using an equal volume of a 1:1 mixture of phenol and IAC, and then twice using an equal volume of IAC alone. To increase the insolubility of small DNA fragments in ethanol, 1/10 volume of 2 M Na acetate was added. DNA was precipitated with ethanol or isopropanol. To precipitate DNA with ethanol, 3 volumes of ice cold ethanol were added and the tubes chilled at -20°C overnight. To collect the precipitated DNA, samples were centrifuged at 12,000 x g for 30 min. Pellets were washed with 70% ethanol and recentrifuged. After careful removal of the supernatant, the DNA pellets were dried by brief lyophilization. DNA was dissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and DNA content measured by absorbance at 260 nm.

Precipitation of DNA with isopropanol was done when it was necessary to limit the volume as only 1 volume of isopropanol is needed to precipitate DNA. Isopropanol was used routinely with small volume DNA preparations done in 1.5 ml microcentrifuge tubes.

7. Antibody to ubiquitin

a. Purification of ubiquitin

Ubiquitin was purified using the method of Haas and Wilkinson (1985) from out-dated packed human red blood cells obtained from the Red Cross. Typically 16 units of packed red blood cells yielded 140 mg of purified ubiquitin. Electrophoresis of purified ubiquitin on SDS polyacrylamide gels showed a single band migrating at the same mobility as a sample of purified ubiquitin obtained from Dr. Haas (apparent molecular weight between 8,250 and 9,500, $\bar{X}$=8,700, 5 determinations).

b. Antibody production

Affinity purified antibodies against SDS-denatured ubiquitin were used as the immunochemical stain for ubiquitin conjugates. To prepare antigen, ubiquitin was cross-linked to bovine γ-globulin and denatured with SDS according to the method of Hershko et al., 1982. Antiserum was produced by National Biological Laboratory Ltd., Dugald, Manitoba. Samples containing 0.1 mg of ubiquitin were mixed with an equal volume of Freund's complete adjuvant and injected into rabbits every two weeks.

c. Screening for antibody

The presence of antibody to ubiquitin was determined using a dot immunobinding assay and the Bio-Rad Immuno-blot (goat anti-rabbit IgG horseradish peroxidase conjugate) assay kit. The following solutions were required:

Solution	Composition
5 x Tris buffered saline: (5 x TBS)	100 mM Tris-HCl, pH 7.5 2.5 M NaCl
TBST:	20 mM Tris-HCl, pH7.5 500 mM NaCl 0.05% (v/v) Tween-20
Blocking solution:	3% (w/v) gelatin in TBS 0.01% (w/v) thimerosal
Antibody buffer:	1% (w/v) gelatin in TBS 0.01% (w/v) thimerosal

HRP color development solution (60 ml):

a. 30 mg HRP color development reagent (4-chloro-1-naphthol) was dissolved in 10 ml ice cold methanol and protected from light. This solution was made fresh daily.

b. Immediately prior to use, 30 ul of ice cold 30% H_2O_2 was added to 50 ml TBS. This was mixed with (a) at room temperature and used immediately.

To prepare the dots for the immunoassay, nitrocellulose membrane was washed 5 min in ddH_2O with gentle agitation, blotted between filter papers and air dried. One ul of each of four ubiquitin solutions (1 ug/ul, 0.1 ug/ul, 0.05 ug/ul and 0.01 ug/ul) was applied to the dry filter as a small dot. The dot arrays were allowed to dry thoroughly and the protein fixed to the nitrocellulose by heating at 65°C for 30 min. Nonspecific binding sites on the nitrocellulose were blocked by incubation of the membrane with blocking solution for 30 min at room temperature with shaking. This was followed by two washes of 5 min each in TBST. Sheets of dots could be prepared in advance and stored dry. Following blocking and washing, dot arrays were blotted between filter papers, allowed to air dry thoroughly, placed between fresh 3 MM filter papers and wrapped in foil.

To screen antisera for antibodies to ubiquitin a test strip was wet in TBS and incubated in antibody buffer containing a 1:100 dilution of antiserum overnight. All steps were done at room temperature with shaking. Following incubation with antiserum, nonspecifically adsorbed material was removed by two 5 min washes in TBST. The test strip was then transferred to the second antibody solution which contained a goat anti-rabbit IgG horse radish peroxidase conjugate (GAR HRP)(Bio-Rad) at a 1:3000 dilution and incubated for 1 h. Nonspecifically adsorbed antibody was removed by two 5 min washes in TBST followed by a 5 min wash in TBS. Specific binding of the GAR HRP to anti-ubiquitin IgG was detected by staining for horse radish peroxidase activity using the HRP color development solution. This solution was prepared immediately before use. Horse radish peroxidase activity was seen as purple spots. Color development typically took 10-15 min and the reaction was stopped by soaking the test strips in ddH_2O for 10 min with at least one change. The intensity of the purple spots was scored between very strong (+++) and negative (-).

d. Purification of antibody

Antibody directed against ubiquitin was purified by affinity chromatography on ubiquitin-Sepharose according to the method of Hershko et al., 1982. Solutions containing sodium azide were avoided as azide is a potent inhibitor of HRP and the antibody reactivity was checked using a dot immunobinding assay with the HRP detection system. Thimerosal (0.01%) was used as a bacteriostat. The protein concentration of purified anti-ubiquitin IgG was determined using the

Bio-Rad protein assay with bovine γ-globulin as a standard.

C. Analytical Procedures

1. Measurement of DNA concentration by absorbance at 260 nm

The absorbance at a wavelength of 260 nm could be used to estimate the DNA concentration of a sample. One A_{260} unit is equal to 50 ug of DNA.

a. Nuclei

Five ul of nuclei were added to 0.995 ml 5 M urea, 2 M NaCl and mixed using a vortex to release the DNA. Samples were done in quadruplicate. It was important to mix sample by trituration, avoiding bubbles, prior to adding to the cuvettes. The spectrophotometer was adjusted to zero using 5 M urea, 2 M NaCl.

b. Chromatin

Chromatin (10-100 ul) was diluted in 2 M NaCl (total volume 1 ml) and mixed using a vortex to dissociate the protein from the DNA. Samples were done in duplicate. The spectrophotometer was adjusted to zero using 2 M NaCl and the buffer that the sample was in. Chromatin contains an equal weight of histones and DNA. Therefore, absorbance at 260 nm could be used to estimate the amount of histone as well as DNA in a chromatin sample.

c. DNA

DNA (10-100 ul) was diluted in ddH_2O (total volume 1 ml) and mixed

using a vortex. The spectrophotometer was adjusted to zero using ddH_2O.

2. TCA assay for protein

To measure the protein concentration of acid extracted proteins the TCA assay was used. For protein samples < 1.2 mg protein/ml, 100 ul of sample was added to 0.7 ml ddH_2O and mixed. For protein samples > 0.5 mg/ml 10 ul of sample was added to 0.79 ml ddH_2O and mixed. To the diluted protein sample, 0.4 ml of 50% TCA was added and the sample immediately mixed using a vortex. A reference solution was prepared by mixing 1.6 ml ddH_2O and 0.8 ml 50% TCA. After approximately 15 min the absorbance at 400 nm was read spectrophotometrically. To calculate the protein concentration the following equations were used:

1. for 100 ul sample:

$$A_{400} \text{ reading} \times \frac{12}{0.0093} = x \text{ ug/ml protein}$$

2. for 10 ul sample:

$$A_{400} \text{ reading} \times \frac{120}{0.0093} = x \text{ ug/ml protein}$$

3. Bio-Rad assay for protein

The Bio-Rad protein assay (microassay procedure) was used to measure the protein concentration of non-histone protein samples. To 0.8 ml of diluted protein sample, 0.2 ml of Dye Reagent Concentrate was added. After 5-10 min, the absorbance at 595 nm was measured spectrophotometrically versus the reagent blank. Bovine γ-globulin was used as the protein standard (2-15 ug) and the absorbance of the reaction was linear to 10 ug of protein.

4. Liquid scintillation counting

Aqueous samples were placed in mini-scintillation vials to which 5 ml ACS (Amersham) was added.

To determine TCA insoluble counts two methods were used. In the first method the sample in 20% TCA was applied to a Whatman GF/C glass microfiber filter (prewet in 5% TCA) using a stainless steel filter holder and suction. The filter was washed three times with 5% TCA (approximately 5 ml each time) and once with 95% ethanol (approximately 10 ml), allowed to dry, put into a 15 ml scintillation vial and 10 ml ACS was added.

The second method for determining TCA insoluble counts gave more reproducible results than the first method. Typically, to 50 ul of sample in a 1.5 ml microcentrifuge tube, 10 ul bovine serum albumin (10 mg/ml) and 950 ul 21% TCA (20% final concentration) was added. This was left on ice for about 5 min to precipitate the TCA insoluble material. Samples were centrifuged 1 min at 4°C to pellet the TCA insoluble material and the supernatant was decanted. The insoluble material was resuspended, washed with ice cold 5% TCA and then recentrifuged. This was repeated for a total of four times. Following the last centrifugation, 100 ul NCS tissue solubilizer (Amersham) was added to each tube, mixed using a vortex and left about 1 h at room temperature with occasional mixing. The tubes were then opened, placed in 15 ml scintillation vials with 12 ml ACS and mixed. Vials were dark adapted overnight at 4°C prior to counting.

Radioactivity was measured by liquid scintillation counting in a Beckman scintillation counter.

5. SDS PAGE

SDS gel electrophoresis was performed in 15% polyacrylamide minislab gels using the discontinuous buffer system of Laemmli (1970). The following stock solutions were used:

Solution	Composition
Acrylamide:BIS (30:0.8)	30.0% (w/v) acrylamide 0.8% (w/v) BIS acrylamide
1.5 M Tris-HCl, pH 8.8	
0.5 M Tris-HCl, pH 6.8	
10% (w/v) SDS	
10% (w/v) ammonium persulfate (prepare fresh weekly)	
2 x SDS sample buffer:	125 mM Tris-HCl, pH 6.8 4% (w/v) SDS 20% (w/v) glycerol 10% (v/v) 2-mercaptoethanol 0.01% (w/v) bromophenol blue
4 x SDS running buffer:	200 mM Tris 1.52 M glycine 0.4% (w/v) SDS

The 15% polyacrylamide separating gel (6 cm x 9 cm x 1 mm) was prepared by mixing the following solutions:

3.5 ml acrylamide:BIS (30:0.8)
1.75 ml 1.5 M Tris-HCl, pH 8.8
1.65 ml ddH_2O
70 ul 10% SDS
3.5 ul TEMED
40 ul 10% ammonium persulfate

The 6% polyacrylamide stacking gel (approximately 2 cm x 9 cm x 1 mm) was prepared by mixing the following solutions:

0.6 ml acrylamide:BIS (30:0.8)
0.75 ml 0.5 M Tris-HCl, pH 6.8
1.5 ml ddH_2O
30 ul 10% SDS
3 ul TEMED
30 ul 10% ammonium persulfate

Protein samples were dissolved in ddH_2O usually at a concentration of 10 mg/ml. Prior to electrophoresis, samples were diluted with ddH_2O and 2 x SDS sample buffer added to give a protein concentration of 3 mg/ml. Samples were heated in a boiling water bath for 30 sec prior to loading on the gels. Electrophoresis was performed using a minislab apparatus (Idea Scientific, Corvallis, Oregon) at 170V constant voltage at 4°C until the dye front ran off the bottom of the gel and then an additional 20 min (about 1.5 h total).

Gels were stained in 0.04% (w/v) Serva Blue (Coomassie Blue) in aqueous 45% (v/v) methanol, 9% (v/v) acetic acid and destained 30-60 min by diffusion in aqueous 25% (v/v) methanol, 12.5% (v/v) acetic acid and then destained further in aqueous 5% (v/v) methanol , 7.5% (v/v) acetic acid.

6. AUT PAGE

Fifteen percent polyacrylamide 5.4% acetic acid-6.6 M urea-0.375% Triton X-100 minislab gels were prepared using the procedure of Davie (1982). The following stock solutions were required in addition to those for SDS PAGE:

Solution	Composition
4% TEMED, 43.1% acetic acid	
0.004% (w/v) riboflavin	
0.3 M Triton X-100	
3 M KAc, pH 4.0	17.2 ml 17.4 N acetic acid ddH_2O added to about 90 ml titrated to pH 4.0 with KOH ddH_2O added to 100 ml
2 x AUT sample buffer:	8 M urea 0.75 M K acetate, pH 4.0 30% (w/v) sucrose 0.1% (w/v) pyronin Y
running buffer:	0.9 N acetic acid

The 15% polyacrylamide AUT separating minislab gel (0.8 mm thick) was prepared by mixing the following:

3.2 g urea
4 ml acrylamide:BIS (30:0.8)
1 ml 4% TEMED, 43.1% acetic acid
0.8 ml 0.004% riboflavin
160 ul 0.3 M Triton X-100
80 ul thiodiglycol

The gel was photopolymerized.

The 6% polyacrylamide stacking gel was prepared by mixing the

following:

0.8 g urea
0.5 ml acrylamide:BIS (30:0.8)
0.25 ml 3 M K acetate, pH 4.0
0.2 ml 0.004% riboflavin
40 ul 0.3 M Triton X-100
20 ul thiodiglycol
20 ul TEMED

The gel was photopolymerized.

Protein samples were mixed with reducing solution and allowed to sit approximately 30 min at room temperature. Typically 6 ul of protein sample at 10 ug/ul was mixed with 4 ul of reducing solution (1 ul ddH_2O, 1 ul 1 M Tris-acetate, pH 8.8, 1 ul 2-mercaptoethanol, 1 ul 20% (w/v) cysteamine). An equal volume of 2 x AUT sample buffer was added and samples were loaded onto gels. Electrophoresis was performed at 200V at 5°C. To retain protamines and HMG proteins the dye front was allowed to reach the lower edge of the gel (about 2 h). For better resolution of histones the dye was allowed to run off the lower edge and more dye was added. The second dye front was allowed to run about 1/2 way through the separating gel (about 3.5 h total). Gels were stained and destained as described above for SDS PAGE.

7. Two-dimensional PAGE

To better resolve the histones two-dimensional PAGE was used. The first dimension was AUT PAGE and the second dimension was SDS PAGE. The following solution was required in addition to those used for AUT and SDS PAGE:

Solution	Composition
buffer O of O'Farrell:	10% (w/v) glycerol 5% (v/v) 2-mercaptoethanol 2.3% (w/v) SDS 62.5 mM Tris-HCl, pH 6.8

Acetic acid-urea-Triton X-100 minislab 15% polyacrylamide gels 0.8 mm thick were used for the first dimension. After electrophoresis the gel was stained and destained as described previously. The lane(s) of interest were cut out from the gel, put into 5 ml plastic tubes and equilibrated 30 min in buffer O of O'Farrell (1975). Equilibrated gels were used immediately or stored at -80°C until needed. The gel slice was applied horizontally to the top of the second dimension SDS polyacrylamide stacking gel (1.0 mm thick). Coomassie Blue from the stained proteins served as a tracking dye. Electrophoresis, staining and destaining were performed as described above for SDS PAGE.

To run molecular weight markers alongside a two dimensional gel a preparative comb was used when the stacking gel was cast. The preparative comb consists of one large well for the first dimension gel slice and a small well that is used for the molecular weight markers. Bio-Rad low molecular weight markers as well as ^{14}C-methylated molecular weight markers (Amersham) were used. Radioactive molecular weight markers used to estimate the size of proteins from two-dimensional gels used for immune detections, were mixed with a small amount of ubiquitin (4 ul ^{14}C markers to 0.1 ug ubiquitin), 5 x SDS sample buffer added and the mixture heated in a boiling water bath for 1 min. This mixture was then mixed with a small volume of separating gel solution and allowed to polymerize in the well prior to adding the first dimension gel.

8. Transfer of proteins to nitrocellulose

Proteins were electrophoretically transferred from second dimension SDS polyacrylamide gels to nitrocellulose. The following solution was required:

Solution	Composition
Transfer buffer:	25 mM Tris 192 mM glycine 0.01% (w/v) SDS

Two-dimensional PAGE (AUT → SDS) was used to resolve histone proteins and their variants. Eighteen ug of protein were routinely loaded per well on AUT gels. Following electrophoresis, the second dimension SDS polyacrylamide gels were equilibrated 30 min in transfer buffer before electroblotting. Nitrocellulose membrane (Bio-Rad) was hydrated overnight in ddH_2O and 30 min in transfer buffer prior to transfer. Transfers were performed with a Bio-Rad Transblot apparatus at 30V for 20 h with cooling (Lauda circulating bath, 5°C). Following transfer, the nitrocellulose was removed, blotted dry between filter papers and air dried. The nitrocellulose blots were placed between 3 MM filter papers, wrapped in foil and the proteins fixed to the membrane by heating at 65°C for 30 min.

9. India ink stain for proteins on nitrocellulose

Proteins on nitrocellulose could be stained with India ink using a modification of the method described by Hancock and Tsang (1983). The following solutions were used:

Solution	Composition
Tris-buffered saline (TBS)	see screening for antibody (p.45)
TBS-0.03% (v/v) Tween-20	

Nitrocellulose blots were washed four times for 10 min (40 min total) in TBS-0.03% Tween-20 at 37°C. Following the last wash the filters were rinsed with ddH_2O. The blots were stained in a solution of 1 ul India ink (Pelikan Fount India Drawing Ink) per ml TBS-0.03% Tween-20, using a minimum of 0.6 ml stain/ cm^2 nitrocellulose. Optimal staining occurred between 2 h and 18 h. Filters were destained by rinsing with ddH_2O for 5 min and then air dried. To store filters it was necessary to protect them from light as they faded.

10. Immunochemical staining for ubiquitin conjugates

Staining for ubiquitin conjugates was done as described by Haas and Bright (1985) with minor modifications. The following solutions were used:

Solution	Composition
10 x Tris/saline:	250 mM Tris-HCl, pH 7.5 1.5 M NaCl
Tris/saline/Triton X-100:	25 mM Tris-HCl, pH 7.5 150 mM NaCl 0.05% (v/v) Triton X-100
Buffer A*:	25 mM Tris-HCl, pH 7.5 150 mM NaCl 25 mg/ml BSA 0.02% (w/v) sodium azide filter using 0.45 um filter

The staining procedure was done at room temperature with constant shaking. Heat-fixed nitrocellulose filters were blocked by incubation

in buffer A* for 1 h. Filters were then incubated for 2 h in buffer A* containing 10 ug/ml affinity purified anti-ubiquitin IgG. To reduce the background staining of the nitrocellulose blots, the antibody solution was incubated with pieces of nitrocellulose membrane overnight at 4°C to remove any non-specific binding. Following incubation with antibody solution, nonspecifically adsorbed antibody was removed by successive 5 min washes: once in Tris/saline, twice in Tris/saline/Triton X-100 and once in Tris/saline. Specifically bound antibody was detected by incubation in buffer A* containing 2 x 10^5 cpm/ml affinity purified ^{125}I-protein A (Amersham, IM.144) for 1.25 h. Nonspecifically bound ^{125}I-protein A was removed by successive 5 min washes as above. Immunostained filters were blotted between filter paper, air dried 30 min and heat fixed at 65°C for 30 min prior to autoradiography. Solutions of antibody and ^{125}I-protein A could be used several times with no noticeable decline in signal.

Proteins could be stained using India ink following immune detection with a slight increase in background. However, staining filters prior to immune detection decreased the sensitivity of the immune detection.

11. Agarose gel electrophoresis, Southern transfer and hybridization.

Agarose gel electrophoresis, Southern transfer and hybridization were done according to the methods of Maniatis et al. (1982). The following solutions were used:

Solution	Composition
6 x TAE:	0.24 M Tris-acetate 0.006 M EDTA
10 x DNA sample buffer:	0.42% (w/v) bromophenol blue 0.42% (w/v) xylene cyanol 25% (w/v) Ficoll
20 x SSC:	3 M NaCl 0.3 M Na citrate
50 x Denhardt's:	1% (w/v) Ficoll 1% (w/v) BSA 1% (w/v) polyvinylpyrrolidone

Purified DNA was diluted in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 10 x DNA sample buffer added to final concentration of 1x. DNA samples were resolved on 1% agarose gels containing 1 ug/ml ethidium bromide, using TAE buffer at 35 V for 16 h. DNA was visualized with ultraviolet light and the gel photographed using a red filter.

Southern transfer of DNA to nitrocellulose (Bio-Rad) was done using 10 x SSC. Hybridizations to ^{32}P-labeled nick translated plasmid probes (prepared using an Amersham nick translation kit) were done in 50% formamide, 5 x SSC, 50 mM $NaPO_4$, pH 6.5, 1 x Denhardt's solution with 250 ug/ml yeast tRNA overnight at 42°C. Washes were as follows: 2 x SSC, 0.5% (w/v) SDS 5 min at room temperature, 2 x SSC, 0.5% (w/v) SDS 15 min at room temperature, 0.1 x SSC, 0.5% (w/v) SDS 2 h at 68°C and 0.1 x SSC, 0.5% (w/v) SDS 30 min at 68°C. The filters were air-dried and autoradiographed using intensifying screens and Kodak XAR film.

12. Slot blot analysis and hybridizations

Slot blots were done according to the method provided with the Bio-Dot microfiltration apparatus (Bio-Rad).

Briefly, DNA was diluted to 50 ug/ml in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, made 0.3 N NaOH with the addition of 1.0 N NaOH and denatured by heating at 37°C for 30 min. The samples were chilled on ice and an equal volume of ice cold 2 M ammonium acetate was added. The samples were applied to 0.45 um nitrocellulose (Schleicher and Schuell) using a Schleicher and Schuell slot blot apparatus. After binding the DNA, the nitrocellulose was briefly rinsed in 10 x SSC, air dried and heated for 2 h at 80°C.

Hybridizations to ^{32}P-labelled nick translated plasmid probes (prepared using an Amersham nick translation kit) were done in 6 x SSC, 1% SDS, 200 ug/ml heparin, 0.1% Na-pyrophosphate and 10 mM Na_2-EDTA overnight at 68°C (method adapted from Singh and Jones, 1984). Washes were as follows: 2 x SSC, 0.5% (w/v) SDS 5 min at room temperature, 2 x SSC, 0.5% (w/v) SDS 15 min at room temperature, 0.1 x SSC, 0.5% (w/v) SDS 30 min at 68°C and 0.1 x SSC, 0.5% (w/v) SDS 30 min at 68°C. The filters were air dried and autoradiographed as described previously.

III. EXPERIMENTAL RESULTS AND SPECIFIC PROCEDURES

A. The Protamine Gene in Developing Trout Testis

1. Introduction

The synthesis of protamines, which are germ line specific proteins, is controlled at both the transcriptional and translational level. Protamine mRNAs are synthesized during the primary spermatocyte cell stage in spermatogenesis and stored as inactive ribonucleoprotein particles in the cytoplasm until the middle spermatid stage when they are translated and protamines begin replacing the histones (Iatrou et al., 1978). Six distinct protamines have been purified and sequenced from a single rainbow trout testis indicating that there are at least six distinct alleles (McKay et al. 1986). The protamine genes do not have intervening sequences and show considerable sequence homology in their coding and immediate 5' and 3' flanking regions (States et al., 1982; Aiken et al., 1983).

A protamine gene, TPG-3, has been cloned and contains no abnormalities in the coding or regulatory sequences which would prevent its transcription in vivo (Gregory et al., 1982). This gene was capable of transcription when transfected into HeLa cells, and the mRNA start site was localized by S1-nuclease mapping and primer extension of in vivo synthesized trout testis poly A+-mRNA. A subclone of TPG-3, pPC23, has been constructed that recognizes the coding region as well as immediate 5' and 3' flanking regions of the protamine genes (Delcuve and Davie, 1987).

The chromatin structure of expressed genes is known to differ from that of repressed regions. Transcriptionally active as well as

transcriptionally competent genes are believed to be associated with domains of chromatin that are less condensed or in a more open conformation and show an increased susceptibility to digestion by DNase I. In contrast to DNase I, micrococcal nuclease can be used to discriminate between genes engaged in transcription and those in a state of transcriptional readiness (competent). Transcriptionally active chromatin is more sensitive to micrococcal nuclease digestion and there can be a disruption in the regular nucleosomal pattern obtained following micrococcal nuclease digestion (Wu et al., 1979; Rose and Garrard, 1984; Cohen and Sheffery, 1985; Strätling et al., 1986; Einck et al., 1986). Transcriptionally active chromatin can also be found associated with a specific subnuclear compartment such as the nuclear matrix (Robinson et al., 1983; Ciejek et al., 1983; Rose and Garrard, 1984; Hentzen et al., 1984; Cohen and Sheffery, 1985; Einck et al., 1986; Strätling et al., 1986).

In this study the protamine genes in trout erythrocytes and early stage testis were examined using micrococcal nuclease as a probe of chromatin structure. Hybridization to the protamine gene subclone, pPC23, revealed that in testes protamine genes were not present in the typical nucleosomal ladder. Fractionation of chromatin was also used to obtain information on the structure and nuclear location of the protamine genes. The low salt eluted fraction of testis chromatin was enriched in transcriptionally active/competent genes as labeled by the nuclear nick-translation method of Levitt et al. (1979). However, in testis protamine gene sequences were preferentially associated with the insoluble nuclear material remaining after fractionation.

2. Experimental procedures

a. Nick-translation of nuclei

Nuclei from early stage trout testes were isolated as described in Section II.B. except that buffer E (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 2mM $MgCl_2$, 30 mM Na butyrate, 1% thiodiglycol) was used. One mM PMSF was included in all buffers used. Nuclei were resuspended at a DNA concentration of 1 mg/ml in buffer F (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 ug bovine serum albumin per ml and 30 mM Na butyrate). Nuclei were digested by incubation with 0.3 units per ml of DNase I (Sigma D4527) for 10 min at 37°C. Polymerization was carried out in the presence of 4 uM each of dATP, dGTP, dCTP and [^{3}H] TTP (New England Nuclear, 80.1 Ci/mmol) for 5 min at 15°C after the addition of E. coli DNA polymerase I (Pharmacia) at 10 units per ml. The reaction was stopped by the addition of TCA to 20% final concentration or by transferring the nuclei to 0°C and adding cold buffer E which contained unlabeled DNase I digested nuclei. Hexylene glycol was then added to a final concentration of 1 M and the nuclei left on ice for 5 min. Nuclei were collected by centrifugation and then washed twice in buffer B (1 M hexylene glycol, 10 mM PIPES, pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol and 30 mM sodium butyrate). DNase I digested nuclei incorporated 4 to 8 fold more radioactivity than undigested nuclei.

Nick-translated nuclei were digested at 200 A_{260} enzyme units/ml of micrococcal nuclease at a DNA concentration of 2 mg/ml in buffer B containing 1 mM $CaCl_2$ at 15°C for 40 min. The reaction was terminated by the addition of 0.25 M EGTA to a final concentration of 10 mM and

placed on ice. Digested nuclei were collected by centrifugation which yielded the supernatant SO. The nuclei were then incubated at 0°C with buffer G (50 mM Tris-HCl, pH 7.0, 2mM $MgCl_2$, 1% thiodiglycol and 30 mM Na butyrate) containing 50 mM NaCl which yielded the supernatant SS 0.05 and a pellet, P. The pellet was redigested with micrococcal nuclease according to Davie and Saunders (1981). The fractions SO, SSO.05 and redigested pellet were applied to a Bio-Gel A-5m column (1.7 x 28 cm, 24 ml/hr). The column was equilibrated with 10 mM Tris-HCl, pH 7.5, 10 mM EDTA and 0.5 M NaCl.

b. Micrococcal nuclease digestion and fractionation

Testis and erythrocyte nuclei were isolated as described in Section II.B. To determine micrococcal nuclease sensitivity testis nuclei and erythrocyte nuclei were resuspended at 40 A_{260} units per ml in buffer B containing 1 mM $CaCl_2$. Testis nuclei were digested with 30 A_{260} enzyme units per ml and erythrocyte nuclei were digested with 50 A_{260} enzyme units per ml of micrococcal nuclease at 37°C for various times. The reaction was terminated by the addition of EGTA to a final concentration of 10 mM and the mixture placed on ice. Nuclei were collected by centrifugation and DNA purified.

For fractionation experiments testis and erythrocyte nuclei were digested as described above with 50 A_{260} units of micrococcal nuclease per ml for 20 and 15 min, respectively. Digested nuclei were collected by centrifugation, yielding the supernatant SO. For the Sanders (1978) fractionation procedure, the nuclei were sequentially incubated at 0°C in buffer D (50 mM Tris-HCl, pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol, 25 mM

KCl, 10 mM EGTA and 30 mM sodium butyrate) containing 50 mM NaCl and then buffer D containing 500 mM NaCl. Alternatively, following elution in buffer D containing 50 mM NaCl nuclei were resuspended in 10 mM EDTA. The incubations yielded supernatants SSO.05 and SSO.5 or SE, respectively. The remaining pellet was saved. Chromatin was also fractionated using an adaptation of the technique described by Levy and Dixon (1978). Briefly, nuclei at 40 A_{260} units per ml in buffer B were digested with 100 A_{260} units of micrococcal nuclease for 40 min at 25°C. Digested nuclei were collected by centrifugation and the chromatin solubilized by resuspension in 1 mM EDTA, pH 7.5. Centrifugation yields a soluble fraction (S2) and an insoluble pellet (P2). The soluble fraction (S2) is made 0.1 M NaCl by the dropwise addition of 2 M NaCl and centrifuged. This results in a soluble fraction (VI soluble) and an insoluble fraction (VI insoluble). In addition to these two fractionation techniques, chromatin was fractionated on the basis of solubility in EDTA. Digested nuclei were incubated at 0°C with 10 mM EDTA and the soluble chromatin collected by centrifugation at 12,000 x g for 20 min with the insoluble nuclear material, the pellet, also saved.

c. DNA analysis

DNA was purified, resolved on 1% agarose gels, transferred to nitrocellulose and hybridized to ^{32}P-labeled plasmid probes as described in Section II. The probes used were pPC23, which recognizes the coding region of the trout protamine genes (Delcuve and Davie, 1987) and pSgVg225 which recognizes the trout vitellogenin gene (a gift

from Dr. M. Tenniswood).

Enrichment of protamine sequences in the various fractions was determined by slot blot analysis. DNA from total digested nuclei (T) and from SE and P chromatin fractions was applied to nitrocellulose (0.45 um) using a Schleicher and Schuell manifold and hybridized to ^{32}P-labeled plasmid probes (pPC23 and pSgVg225) as described in Section II.

Calculation of enrichment of protamine sequences was done by analyzing the densitometric scans of autoradiograms from slot blots. Scans were chosen that fell within the linear response of the film. For each fraction the ratio of protamine sequences to protamine sequences in total unfractionated DNA was calculated. To compensate for differences in the sizes of DNA between fractions, the same ratio was determined for vitellogenin sequences. The enrichment of protamine sequences in a particular fraction was then calculated as a ratio of enrichment of protamine sequences to enrichment of vitellogenin sequences in that fraction.

d. Protein analysis

Proteins from chromatin fractions were acid extracted and analyzed by AUT-15% PAGE as described in Section II.

3. Results and discussion

a. Altered nucleosomal conformation of the protamine gene in early stage testis

The nucleosomal conformation of the protamine gene and the

vitellogenin gene in testis and erythrocytes was examined by digesting nuclei from these tissues to increasing extents with micrococcal nuclease. Total digested DNA was purified from each time point and the fragments resolved by 1% agarose gel electrophoresis. The ethidium bromide stained pattern represents the bulk of the DNA. DNA from the gels was transferred to nitrocellulose by Southern blotting and specific gene sequences detected by hybridization to ^{32}P-labeled probes.

The results presented in Figure 7 show that the bulk DNA from testis and erythrocytes exhibits a typical nucleosomal ladder with approximately the same repeat length. The inactive vitellogenin genes in both testis and erythrocytes, as well as the protamine genes in erythrocytes, mirror the pattern seen in the bulk DNA. However, the protamine genes from testis show a nondiscrete continuum of DNA lengths (Fig. 7, lane d) that with more extensive digestion yields mono-, di- and trinucleosomal fragments (Fig. 7, lane f). This disrupted chromatin structure has also been described for the immunoglobulin light chain genes during B cell development (Rose and Garrard, 1984), the cytochrome P-450c gene of liver following induction (Einck et al., 1986), the hormonally stimulated ovalbumin gene of chick oviduct (Bloom and Anderson, 1982), the lysozyme gene from hen oviduct (Strätling et al., 1986), the globin gene of induced murine erythroleukemia cells (Cohen and Sheffery, 1985) and the heat shock gene of Drosophila following heat shock (Wu et al., 1979). The disrupted structure is largely limited to DNA sequences lying within the coding region of the gene (Strätling et al., 1986; Cohen and Sheffery, 1985). The reason

Figure 7. **Chromatin structure of bulk DNA, transcriptionally active and inactive genes.**
Nuclei (2 mg/ml) isolated from erythrocytes or early stage testes were digested with 50 A_{260} units and 30 A_{260} units, respectively, of micrococcal nuclease at 37°C for 0, 1, 2, 5, 10, 15 and 20 min (lanes a to g, respectively). The digested nuclear DNA was resolved on a 1% agarose gel, stained with ethidium bromide and photographed. DNA was denatured, transferred to nitrocellulose and hybridized with the indicated ^{32}P-labeled probes, and complementary sequences were visualized by autoradiography.

for the disrupted chromatin structure of active genes is thought to be an irregularity in extended nucleosomal spacing due to a deficiency of histone H1 (Rose and Garrard, 1984). The variability in linker length results in the generation of a continuum of DNA fragments rather than discrete bands. The smeary pattern could also be the result of an altered nucleosomal structure, such that micrococcal nuclease is able to cleave within the nucleosomal DNA. Allegra et al. (1987) studying the accessibility of the SH-group of histone H3 in active chromatin have postulated that there is some unfolding at the middle of the nucleosome. This change in conformation may make the nucleosomal DNA more accessible to nucleases. However, Rose and Garrard (1984) found the unevenness in nucleosomal spacing was not due to transcription per se as the non-transcribed allelic partner of the active immunoglobulin light chain gene also showed unevenness in nucleosomal spacing and H1 deficiency. This is however in contrast to the other genes where the smeary pattern was only found when the gene was transcriptionally active.

b. Distribution of transcriptionally active genes among chromatin fractions

Chromatin fractionation methods can be used to obtain information on the chromatin structure of specific genes. The fractionation technique of Sanders (1978) which is based on micrococcal nuclease accessibility and solubility properties has been shown to enrich for H1 deficient chromatin in the low salt eluted fraction. It has been shown that the low salt eluted fractions of calf thymus and chicken

erythrocyte chromatin are enriched in transcriptionally active or competent genes (Davie and Saunders; 1981, Rocha et al., 1984).

Transcriptionally competent regions of trout testis chromatin were specifically labeled using nuclear nick-translation (Levitt et al., 1979) which is reported to incorporate over 85% of the label into transcribed sequences. This method labels a subset of genes that are highly expressed and preferentially localized at the nuclear periphery (Hutchinson and Weintraub, 1985). The cartoon shown in Figure 8 illustrates the principles of this technique. Nuclei are digested with low concentrations of DNase I which preferentially nicks the DNA of transcriptionally competent chromatin both within the linker region and within the nucleosomal region (Figure 8a). Label is incorporated into these sites using E. coli DNA polymerase and a radioactive nucleotide triphosphate (Figure 8b). Labeled nuclei are then digested with micrococcal nuclease which has a preference for cleaving linker DNA of transcriptionally active/competent genes (Figure 8c). To determine if the low salt eluted fraction of trout testis chromatin obtained by the Sanders fractionation method is enriched in transcriptionally competent sequences, following digestion with micrococcal nuclease chromatin was fractionated into the SO, SSO.05 and pellet fractions. Results from a single experiment are shown in Table I. Similar results were obtained with four separate experiments. As a control, chromatin from nick-translated nuclei not digested with micrococcal nuclease was fractionated. In this case, the majority of both A_{260}-absorbing material and label was present in the pellet (Table I). However, a small amount of A_{260}-absorbing material and about 15% of the total

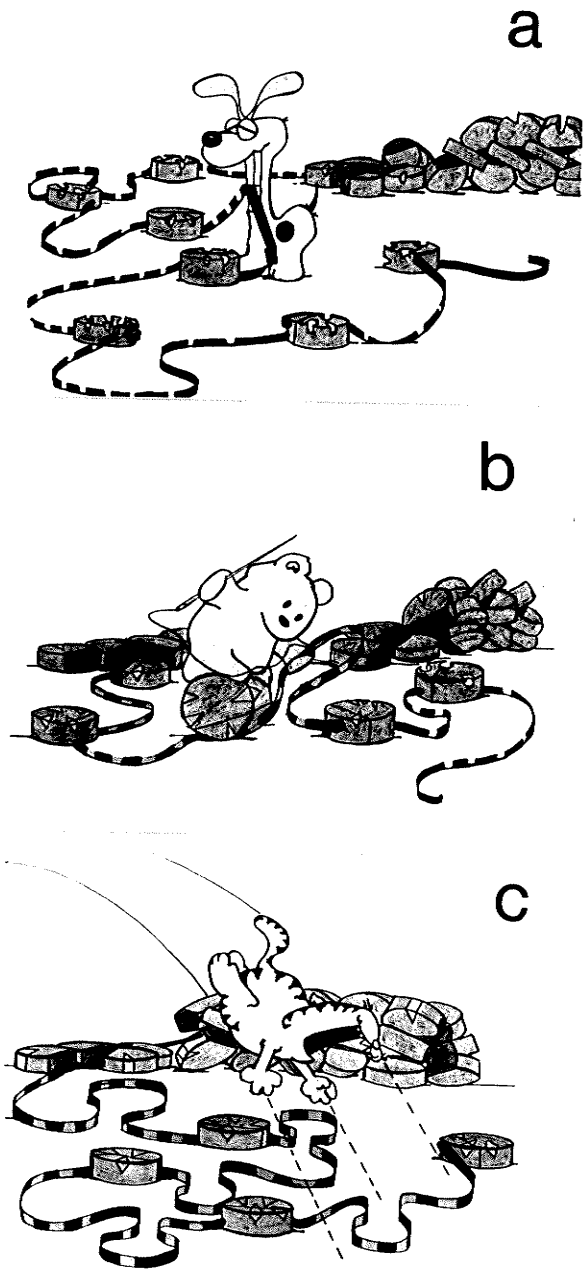

Figure 8. **Cartoon depicting the principle of labeling transcriptionally active chromatin by nuclear nick-translation prior to micrococcal nuclease digestion and fractionation.**
Nuclei are digested with low concentrations of DNase I (a). Radioactive label is incorporated into "nicks" using E. coli DNA polymerase (b). Labeled nuclei are digested with micrococcal nuclease (c). The discs represent nucleosomes and the ribbons joining the discs represent linker DNA.

TABLE I. **Quantification of A_{260}-absorbing material, total cpm and TCA precipitable cpm in the various fractions obtained from undigested and MNase digested nick-translated nuclei with the Sanders (1978) procedure.**

Fraction	% A_{260}	% total cpm	% TCA cpm
A. Undigested nick-translated nuclei			
S0	1.1	14.6	4.0
SS0.05	0.1	10.5	22.4
Pellet	98.9	74.9	73.6
B. MNase digested nick-translated nuclei			
S0	8.2	58.6	4.5
SS0.05	5.9	11.2	42.2
Pellet	85.9	30.2	53.3

label were released into the low salt soluble fraction (SSO.05). This is explained by a previous report which showed that mild DNase I digestion of trout testis nuclei followed by incubation in a solution containing 0.1 M NaCl released nucleosomes into the supernatant (Davie and Candido, 1980). Following micrococcal nuclease digestion of labeled nuclei, there was a shift in amount of labeled material among the various fractions (Table I). The SO fraction contained approximately 60% of the total labeled material with less than 5% of this being TCA insoluble. This material is probably from the linker DNA region (see Davie and Saunders, 1981). The pellet which contained the bulk of the A_{260}-absorbing material was depleted in labeled material suggesting that transcriptionally competent chromatin was susceptible to micrococcal nuclease digestion and was present mainly in fractions SO and SSO.05.

The labeled material associated with each fraction was characterized by Bio-Gel A-5m gel exclusion chromatography (Figures 9 and 10). The results were similar to those seen using calf thymus chromatin (Davie and Saunders, 1981). In the SO fraction both the A_{260}-absorbing material and label eluted with the nucleotide fraction (approximately fraction number 40). The majority of A_{260}-absorbing material in the SSO.05 (digested nuclei) fraction corresponded to mononucleosomes (approximately fraction number 30) with the TCA insoluble counts peaking in the same fractions (Figure 9). To determine the content of labeled material associated with the linker and with nucleosomes for the pellet fractions, the chromatin of these fractions was redigested with micrococcal nuclease prior to gel

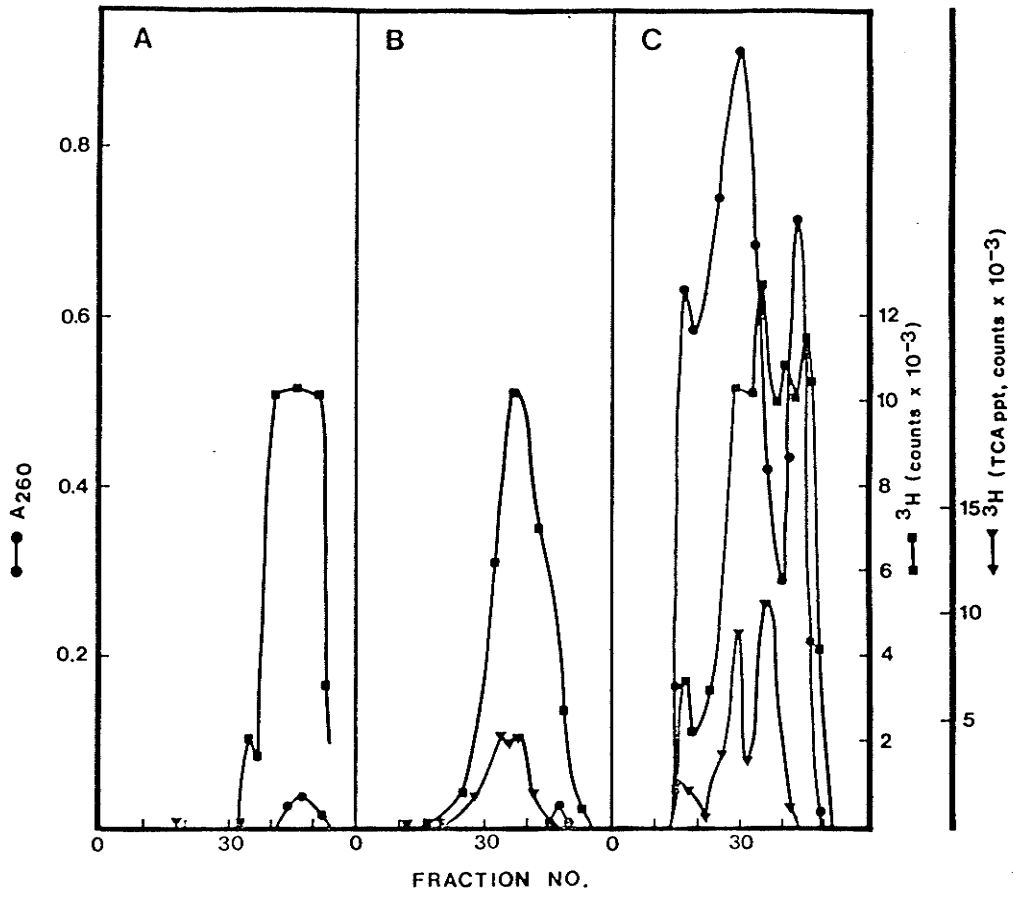

Figure 9. **Gel exclusion chromatography of various fractions obtained from nick-translated nuclei.**
Fractions were obtained from nick-translated nuclei as described in "Experimental Procedures". Approximately 1 ml of each fraction SO (A), SSO.05 (B) and redigested pellet (C) were applied to a Bio-Gel A-5m column which was equilibrated with 10 mM Tris-HCl, pH 7.5, 10 mM EDTA and 0.4 M NaCl. One ml fractions were collected and assayed for A_{260}; 0.8 ml samples were assayed for total counts and TCA (20%)-precipitable (TCA ppt) counts.

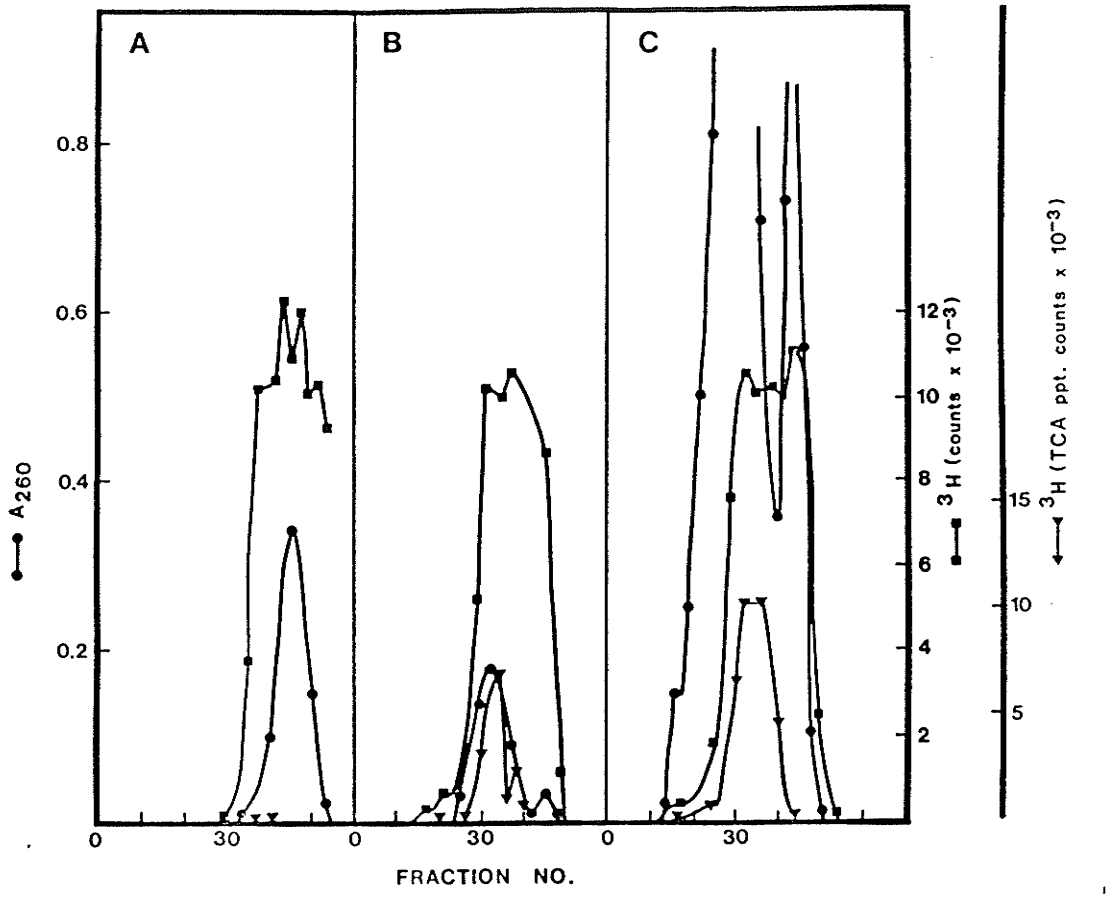

Figure 10. **Gel exclusion chromatography of various fractions obtained from micrococcal nuclease digested nick-translated nuclei.** Nick-translated nuclei (2 mg/ml) were digested with micrococcal nuclease (200 A_{260} enzyme units/ml) at 15°C for 40 min. The reaction was terminated by the addition of EGTA to a final concentration of 10 mM and placed on ice. The fractions were obtained from the digested nuclei as described in "Experimental Procedures". Approximately 1 ml of each fraction SO (A), SSO.05 (B) and redigested pellet (C) were applied to a Bio-Gel A-5m column which was equilibrated with 10 mM Tris-HCl, pH 7.5, 10 mM EDTA and 0.4 M NaCl. One ml fractions were collected and assayed for A_{260}; 0.8 ml samples were assayed for total counts and TCA (20%)-precipitable (TCA ppt)counts.

exclusion chromatography. The specific activity (TCA insoluble cpm/A_{260}) of the mononucleosome peak for the redigested pellet (undigested nuclei), SSO.05 (digested nuclei) and redigested pellet (digested nuclei) were compared (Figures 9 and 10, Table II). The results shown are from a single experiment. The experiment was done twice with similar results. There is a depletion of the TCA insoluble label in the redigested pellet (digested nuclei) of about 0.72 fold when compared to the redigested pellet from undigested nuclei. The mononucleosome fraction from the SSO.05 (digested nuclei) shows a 5.3 fold enrichment in specific activity when compared to the same fraction from the redigested pellet (digested nuclei) and a 3.8 fold enrichment when compared to the redigested pellet (undigested nuclei). This demonstrates that the low salt eluted fraction (SSO.05) is enriched in transcriptionally competent chromatin.

Chromatin from labeled nuclei was also fractionated using an adaptation of the Levy and Dixon technique (1978). The low salt eluted fraction (VI soluble) was enriched in transcriptionally active/competent sequences (Table III) confirming the previous results of Levy and Dixon (1978) obtained by hybridization to cytoplasmic RNA. The results obtained are similar to those seen using the Sanders procedure. In the control, nick-translated nuclei that were not digested with micrococcal nuclease prior to fractionation, the majority of both the A_{260}-absorbing material and label were present in the pellet (P2) (Table III). A small amount of A_{260}-absorbing material and about 14% of the total label were released into the low salt fraction (VI soluble) resulting in a high specific activity (TCA cpm/A_{260}) for

TABLE II. Distribution of TCA precipitable cpm in the mononucleosome size DNA from fractions obtained from undigested and MNase digested nick-translated nuclei.

Fraction	Peak Fraction (mononucleosome)
	TCA cpm/A_{260}
Redigested pellet (undigested nuclei)	10,055
SS 0.05 (digested nuclei)	38,333
redigested pellet (digested nuclei)	7,214

TABLE III. Quantification of A_{260}-absorbing material, total cpm and TCA precipitable cpm in the various fractions obtained from undigested and MNase digested nick-translated nuclei with the Levy and Dixon (1978) procedure.

Fraction	%A_{260}	%total cpm	%TCA cpm	total cpm/A_{260}	TCA cpm/A_{260}
A. Undigested nick-translated nuclei					
S0	1.1	9.5	2.2	22,416	1,870
P2	94.5	73.4	68.0	687	682
VI soluble	2.1	13.7	23.0	17,133	10,524
VI insoluble	2.3	3.4	6.8	4,170	2,801
B. MNase digested nick-translated nuclei					
S0	3.4	47.9	7.6	36,305	2,351
P2	55.2	17.4	34.3	708	655
VI soluble	7.4	22.5	34.1	7,848	4,863
VI insoluble	34.0	12.2	24.0	1,092	743

this fraction. This is similar to the finding for the control SS0.05 fraction of the Sanders fractionation. Following micrococcal nuclease digestion of labeled nuclei, there was a shift in the amount of labeled material among the fractions (Table III). The SO fraction contained approximately 48% of the total labeled material with the majority coming from linker DNA as only 8% was TCA insoluble. The two fractions, P2 and VI insoluble contained the bulk of the A_{260} absorbing material, but were depleted in labeled material. This suggests that transcriptionally competent chromatin was more susceptible to micrococcal nuclease digestion and was present mainly in fraction SO and the VI soluble fraction.

c. Protamine genes and insoluble nuclear material

Following fractionation of testis chromatin using the Sanders procedure we had anticipated that protamine gene sequences would be found in the low salt soluble fraction as protamine genes are sensitive to DNase I (Levy-Wilson et al., 1980) and using the Sanders fractionation the low salt eluted fraction of trout testis chromatin was the most DNase I sensitive (Davie and Candido, 1980). However, protamine gene sequences were enriched in the pellet fraction, which contains insoluble nuclear material remaining after fractionation, and not in the low salt eluted fraction of trout testis chromatin (Figure 11). Analysis of the proteins associated with the chromatin fractions agreed with the findings of Sanders (1978). The low salt eluted fraction (SS0.05) showed a depletion of histone H1 as well as an increase in the level of hyperacetylated histone H4 species (Figure

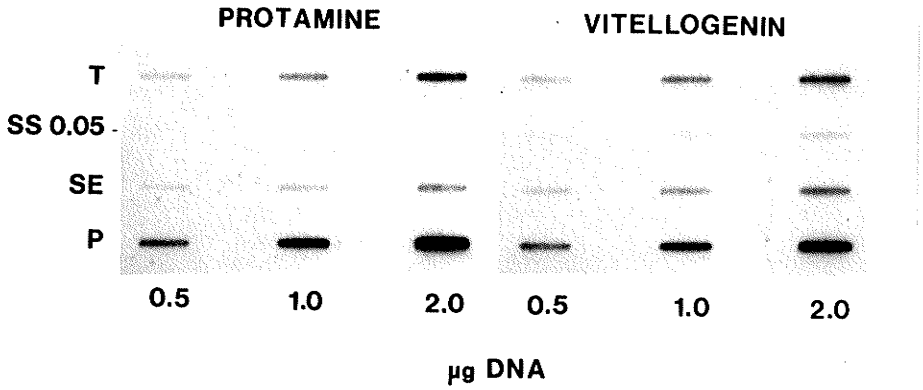

Figure 11. **Distribution of protamine and vitellogenin sequences among chromatin fractions following Sanders fractionation.**
Nuclei (2 mg/ml) isolated from early stage trout testes were digested with micrococcal nuclease (50 A_{260} enzyme units/ml) at 37°C for 20 min and total chromatin (T) as well as chromatin fractions SS0.05, SE and P were prepared. Purified DNA from these fractions was denatured and slot-blotted at the indicated amounts onto nitrocellulose filters. The filters were hybridized with the indicated ^{32}P-labeled probes and subjected to autoradiography.

12). Although an equivalent amount of protein was used for each lane, the pellet shows a decrease in the amount of histones. This is due to the large number of non-histone proteins present in this fraction.

More extensive micrococcal nuclease digestion did not change the partitioning of protamine sequences as had been the case of the active immunoglobulin gene (Rose and Garrard, 1984). The partitioning of protamine gene sequences was also studied using the fractionation method of Levy and Dixon (1978) where the low salt soluble fraction (VI soluble) of trout testis chromatin is enriched in sequences complementary to cytoplasmic polyadenylated RNA. In accord with the previous results, protamine gene sequences were enriched (approximately 1.28 fold) over total in the EDTA insoluble material (P2) while nuclear nick-translated DNA sequences were enriched in the low salt soluble fraction, VI soluble (Tables III and IV). The partitioning of the protamine DNA sequences is in contrast to a number of active genes such as the β-globin gene of chicken erythrocytes (Rocha et al., 1984), the ovalbumin gene of hen oviduct (Bloom and Anderson, 1978), and the immunoglobulin light chain gene of B cells on more extensive micrococcal nuclease digestion (Rose and Garrard, 1984) which were all enriched in the low salt soluble chromatin fraction. However, the protamine genes differ from these genes. The protamine genes are much smaller genes, containing no intervening sequences and they are germ line specific genes.

Knowing that the EDTA insoluble material was enriched in protamine sequences, the remainder of the experiments were performed by separating chromatin soluble in 10 mM EDTA (SE) from the insoluble

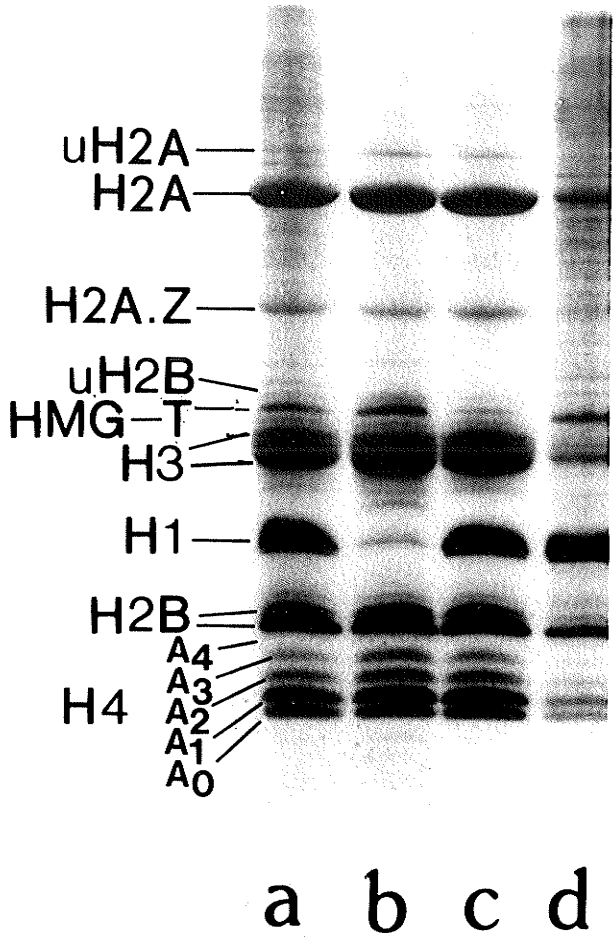

Figure 12. **Acid soluble proteins from chromatin fractions of early stage trout testis.**
Acid extracted proteins obtained from micrococcal nuclease digested, fractionated chromatin were resolved by AUT-15% PAGE. The lanes correspond to total unfractionated chromatin (a), SSO.05 (b), SE (c) and the pellet (d).

TABLE IV. Distribution of protamine sequences in trout testis chromatin fractions obtained using the Levy and Dixon (1978) procedure.

Fraction	Enrichment over Total*
Total	1.00
P2	1.28
VI soluble	0.73
VI insoluble	0.83

*Calculation of enrichment of protamine sequences:

$$\frac{\frac{\text{protamine sequences P2}}{\text{protamine sequences total}}}{\frac{\text{vitellogenin sequences P2}}{\text{vitellogenin sequences total}}}$$

nuclear material (pellet (P)). The pellet fraction would include chromatin possibly associated with the nuclear matrix. DNA was extracted from the fractions and the distribution of protamine and vitellogenin sequences assayed by slot blot analysis. Figure 13 shows the results of a typical experiment. The enrichment of protamine sequences in the fractions from testis and erythrocyte has been calculated as shown in Table V. Data presented in Table V and Figure 13 are from separate experiments. Typically, the pellet fraction of testis was enriched in protamine sequences compared to vitellogenin sequences (1.81 fold) with a range of 1.41 to 2.47 found in four separate experiments. In erythrocytes the ratio of protamine sequences to vitellogenin sequences in the pellet fraction was 1.01 (two experiments, 1.21/0.81).

These results suggest an association between the active protamine sequences and the insoluble nuclear matrix. This type of organization could have an effect on gene expression. If transcription proceeds by passage of DNA through a complex which is associated with the nuclear matrix, then genes associated with this substructure will be transcribed in preference to those remote from it (Jackson, 1986). Ciejek et al. (1983) have shown that during hormone stimulation the actively transcribing ovalbumin gene is associated with the nuclear matrix and this association is reversed when hormone is withdrawn. It is also possible that transcriptionally active genes may be associated with the nuclear matrix through the association of mRNA with a component of the matrix that is required for transport of mRNA to the cytoplasm (Bag and Pramanik, 1987). Similar associations with the

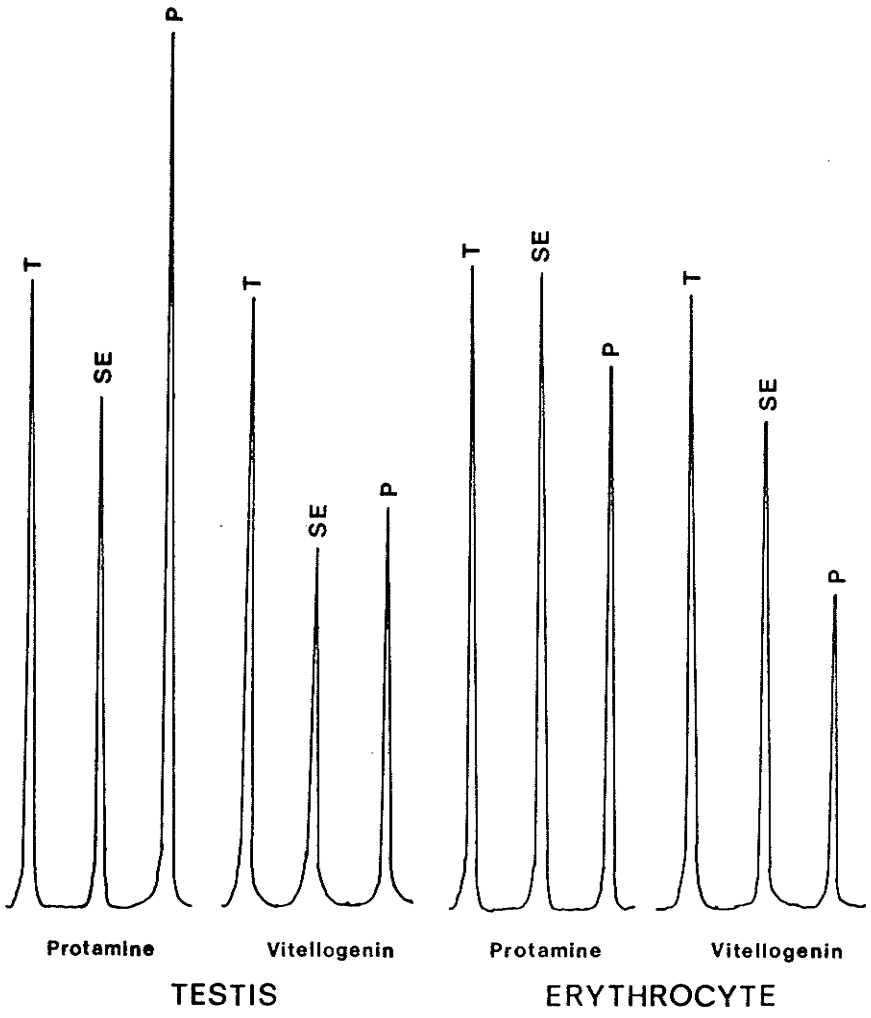

Figure 13. **Distribution of specific sequences among different chromatin fractions.**
Nuclei (2 mg/ml) isolated from either erythrocytes or early stage testes were digested with micrococcal nuclease (50 A_{260} enzyme units/ml) at 37°C for 15 min and 20 min, respectively. The digested nuclei were incubated in 10 mM EDTA, pH 7.0, 30 min at 0°C, yielding a supernatant (SE) and insoluble nuclear material in the pellet (P). T is total digested nuclear DNA. The DNA was purified, denatured and dot blotted onto nitrocellulose. Filters were hybridized with the indicated ^{32}P-labeled probes and complementary sequences quantitated by densitometric scanning of the autoradiograms.

TABLE V. **Distribution of protamine sequences in chromatin fractions obtained using the Sanders (1978) procedure.**

Tissue	Fraction	Enrichment over Total*
Testis	Total	1.00
	SE	1.17
	P	2.47
Erythrocyte	Total	1.00
	SE	0.94
	P	1.21

*Calculation of enrichment of protamine sequences:

$$\frac{\frac{\text{protamine sequences SE}}{\text{protamine sequences total}}}{\frac{\text{vitellogenin sequences SE}}{\text{vitellogenin sequences total}}}$$

insoluble chromatin have been observed with the active immunoglobulin genes of a plasmacytoma cell line (Rose and Garrard, 1984), following induction of the cytochrome P-450c gene in rat liver (Einck et al., 1986), with the chicken lysozyme gene of oviduct (Strätling et al., 1986) and with the induced globin gene in murine erythroleukemia cells (Cohen and Sheffery, 1985). The enrichment of protamine sequences seen in the pellet fraction of testis is not as great as the six fold enrichment of immunoglobulin light chain sequences (Rose and Garrard, 1984). This may be partly explained by the fact that more than one protamine gene is recognized by our protamine probe, pPC23, and the data shown may actually be a composite from transcriptionally active, competent and inactive protamine genes. Also, although spermatogenesis in trout is fairly synchronous with a large number of cells being at the same stage of development at any one time, there will be cells present where the protamine genes are not expressed (e.g. spermatids).

d. Chromatin structure of fractionated sequences from testis and erythrocytes

DNA fragments from fractionated testis and erythrocyte chromatin were resolved by 1% agarose gel electrophoresis. The ethidium bromide stained pattern which is indicative of the bulk of the DNA shows a typical nucleosomal ladder in the three fractions from both testis and erythrocyte (Figure 14). In testis the protamine sequences appear to be slightly more sensitive to micrococcal nuclease than the vitellogenin sequences (compare protamine lane a to vitellogenin lane

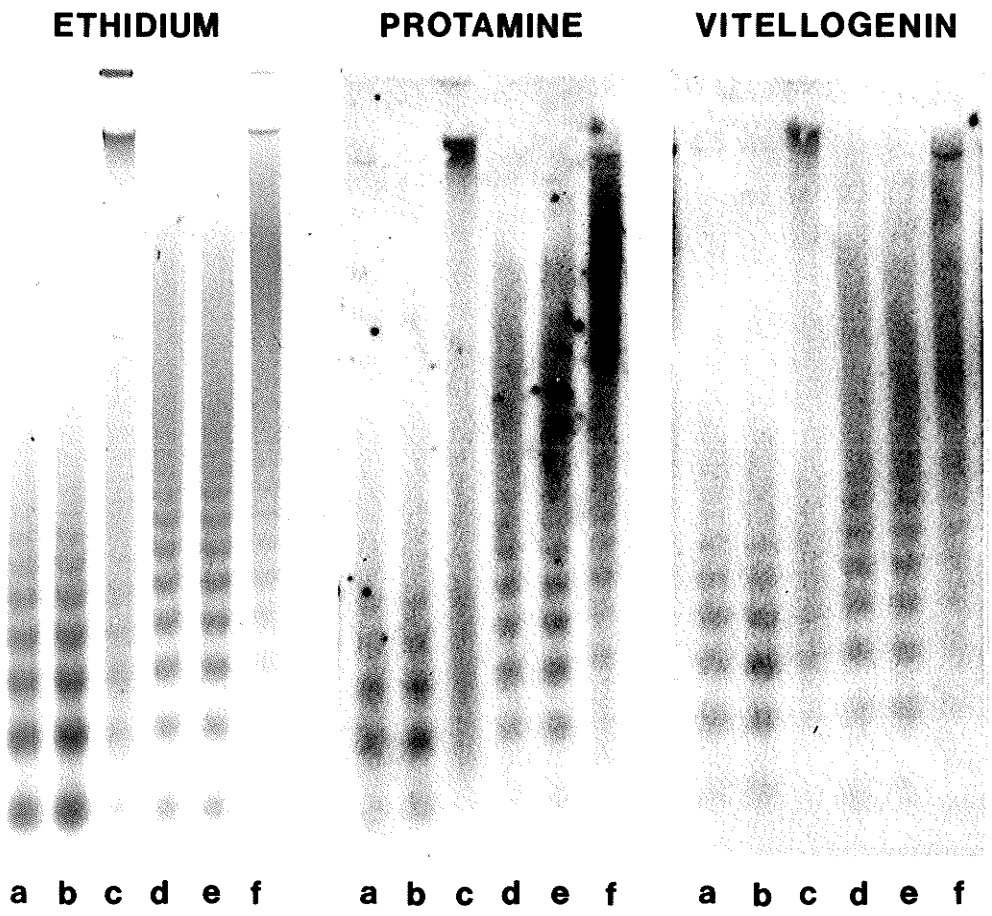

Figure 14. **Chromatin structure of fractionated sequences from testis and erythrocytes.**
Nuclei (2 mg/ml) isolated from testis and erythrocytes were digested with micrococcal nuclease, 50 A_{260} enzyme units/ml at 37°C for 20 min and 15 min, respectively. After chromatin fractionation DNA was purified from fractions SE, P and unfractionated digested nuclei (total). Samples (10 ug/lane) were analyzed as described in Figure 7. The lanes correspond to testis total (a), SE (b), pellet (c), erythrocyte total (d), SE (e) and pellet (f).

a). In erythrocytes there does not appear to be much difference in the size of the fragments (protamine lane d and vitellogenin lane d). The protamine sequences in testis are found in a less distinct nucleosomal ladder in all three fractions when compared to the vitellogenin sequences. However, in the pellet fraction of testis the protamine sequences are more smeary than either the total or SE fraction. Since more than one protamine gene is recognized by our protamine probe, pPC23, the data shown may actually be a composite of transcriptionally active, competent and inactive genes. The expressed protamine genes may fractionate in the pellet fraction and their active chromatin conformation may result in the pattern being more smeary in this fraction than in the total or SE fraction. This would agree with the results from a number of investigators where there was a disruption of nucleosomes associated with the coding region of transcriptionally active chromatin and the transcriptionally active genes were associated with the nuclear matrix (Cohen and Sheffery, 1985; Strätling et al., 1986; Einck et al., 1986).

A high molecular weight band can be seen in the pellet fraction of testis by ethidium bromide staining as well as with both gene probes. This band probably represents that portion of chromatin which has undergone protamine replacement. Replacement of histones with protamines renders the DNA insensitive to digestion with micrococcal nuclease as well as insoluble in EDTA. For these reasons chromatin that has undergone protamine replacement is found as high molecular weight DNA in the pellet fraction. The fact that the protamine sequences in the pellet fraction are not found exclusively as high

molecular weight DNA, as well as their lack of a distinct nucleosomal pattern due to micrococcal nuclease susceptibility shows that the association of these sequences with the pellet is not due to their preferential association with protamines.

In summary, chromatin of the germ-line expressed genes, the protamine genes, in early stage trout testis shows the altered nucleosomal structure of an actively transcribed gene when probed with micrococcal nuclease and an association with insoluble nuclear material which may be important in the developmental expression of this gene. The enrichment of transcriptionally competent/active sequences labeled by nuclear nick translation with the low salt eluted material and finding the transcriptionally active protamine sequences associated with the insoluble nuclear material can be reconciled. It is possible that transcriptionally competent genes not in the process of being actively transcribed are eluted in the low salt fraction by virtue of their nuclease accessibility and solubility properties. However, genes that are actively transcribed may be associated with the nuclear matrix through an interaction involving regulatory proteins or mRNA. It is also possible that due to the small size of the protamine genes the association with the nuclear matrix will not be as easily disrupted as with a larger gene.

B. Changes in H2A.Z and polyubiquitinated histone species in developing trout testis

1. Introduction

In the final stages of spermatogenesis in rat and rooster, the histones are also replaced by low molecular weight proteins. In these systems several alterations occur in chromatin composition prior to the replacement process. There is an increase in the level of hyperacetylated histone H4 species (Grimes and Henderson, 1983; Oliva and Mezquita, 1982), the appearance of germ-line specific histone variants (Trostle-Weige et al., 1982) and an increase in the level of ubiquitinated histone H2A species (uH2A) (Agell et al., 1983). It is presently thought that these components promote decondensation of the chromatin fiber and facilitate displacement of histones.

In this study the trout histone variant, H2A.Z, was identified by electrophoretic mobility on two-dimensional polyacrylamide gels and by its N-terminal amino acid sequence. At the various stages of testis development the levels of histone H2A.Z as well as ubiquitinated species of histones H2A, H2A.Z and H2B were studied by polyacrylamide gel electrophoresis. Ubiquitinated histone species were detected immunochemically with an anti-ubiquitin IgG and ^{125}I-labeled protein A. In late stage testis chromatin the levels of H2A.Z and uH2B were reduced, uH2A.Z was absent and u_2H2B was increased.

2. Experimental procedures

a. Histone isolation

Livers and testes at various stages of development were used to

prepare nuclei (Section II). Nuclei at 40 A_{260} units/ml were digested with 50 A_{260} enzyme units /ml of micrococcal nuclease at 37°C for 20 min. The reaction was terminated by the addition of EGTA to a final concentration of 10 mM and the mixture placed on ice. Digested nuclei were collected by centrifugation at 750 x g for 10 min, yielding a supernatant SO. To extract nucleohistone, the digested nuclei were then incubated at 0°C in buffer D containing 0.5 M NaCl for 30 min. The supernatant SSO.5, which contained the majority of the nucleohistone, was collected by centrifugation at 12,000 x g for 10 min and then dialyzed against 1 mM EDTA, 0.1 mM PMSF overnight at 4°C. A portion of the sample was lyophilized and prepared for AUT 15% PAGE by protamine release (Section II.B). Alternatively, the supernatant SSO.5 was acid extracted and the proteins redissolved in ddH_2O.

b. Isolation of histone H2A.Z

Total trout testis histones were fractionated by gel exclusion chromatography on a Bio-Gel P30 column (110 x 2.5 cm, Bio-Rad) eluted at room temperature with 50 mM NaCl, 10 mM HCl (flow rate 40 ml/h). Typically 75 mg of histones was dissolved in 10 mM Tris-acetate, pH 8.8 containing 10% (v/v) 2-mercaptoethanol and loaded onto the column. Elution of the histones was monitored at 230 nm. The pooled fractions were dialyzed against 0.1 N acetic acid, followed by water and lyophilized. Trout histone H2A.Z was isolated from fraction c (see Figure 15).

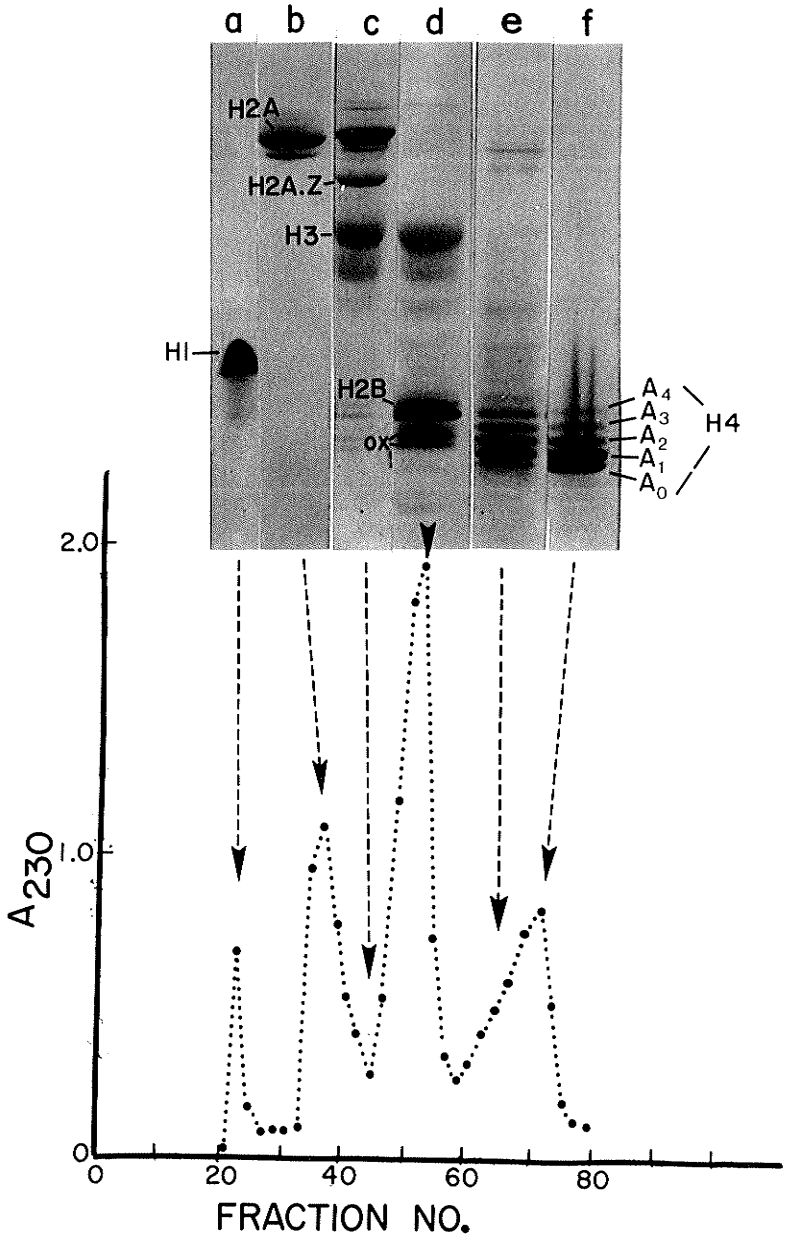

Figure 15. **Gel exclusion chromatography of the trout testis histones.** Trout testis histones were fractionated on a Bio-Gel P30 column. The histones were pooled into six fractions (a-f). The histones in each fraction were electrophoretically resolved by AUT 15% PAGE. ox denotes oxidized H2B. A_0, A_1, A_2, A_3, and A_4 represent the un-, mono-, di-, tri-, and tetraacetylated species of H4, respectively.

c. Amino acid sequence determination of trout histone H2A.Z

Trout testis H2A.Z was isolated by preparative AUT 15% PAGE of fraction c. After briefly staining the gel with Coomassie Blue and briefly destaining, the gel slice containing H2A.Z was cut out, wrapped in plastic wrap and sent to Drs. Allis and Cook at the Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, for sequencing.

d. Peptide mapping with cyanogen bromide

Cleavage of proteins with cyanogen bromide was done as described by Tung et al., 1984. Briefly, 60 ug of acid extracted protein was resuspended in 100 ul of 44% formic acid. A small piece of solid CNBr (approx. 1 mg) was added to the tube. A control sample in 44% formic acid, but without CNBr was also prepared. Samples were incubated 16 h at 4°C and lyophilized. Proteins were resuspended in 6 ul ddH_2O and 4 ul reducing solution (see AUT PAGE), mixed with 10 ul 2X AUT sample buffer and analyzed by AUT 15% PAGE.

3. Results and Discussion

a. Identification and characterization of H2A.Z

Histones isolated from early stage testis (a germ line tissue) and from trout liver (a somatic tissue) were electrophoretically resolved by two-dimensional PAGE (AUT→SDS) (Figure 16). The putative trout H2A.Z was designated as such because it comigrated with calf thymus H2A.Z on two dimensional gels (not shown). Three variants of histone H3 (H3.1, H3.2 and H3.3) were observed. It is unlikely that

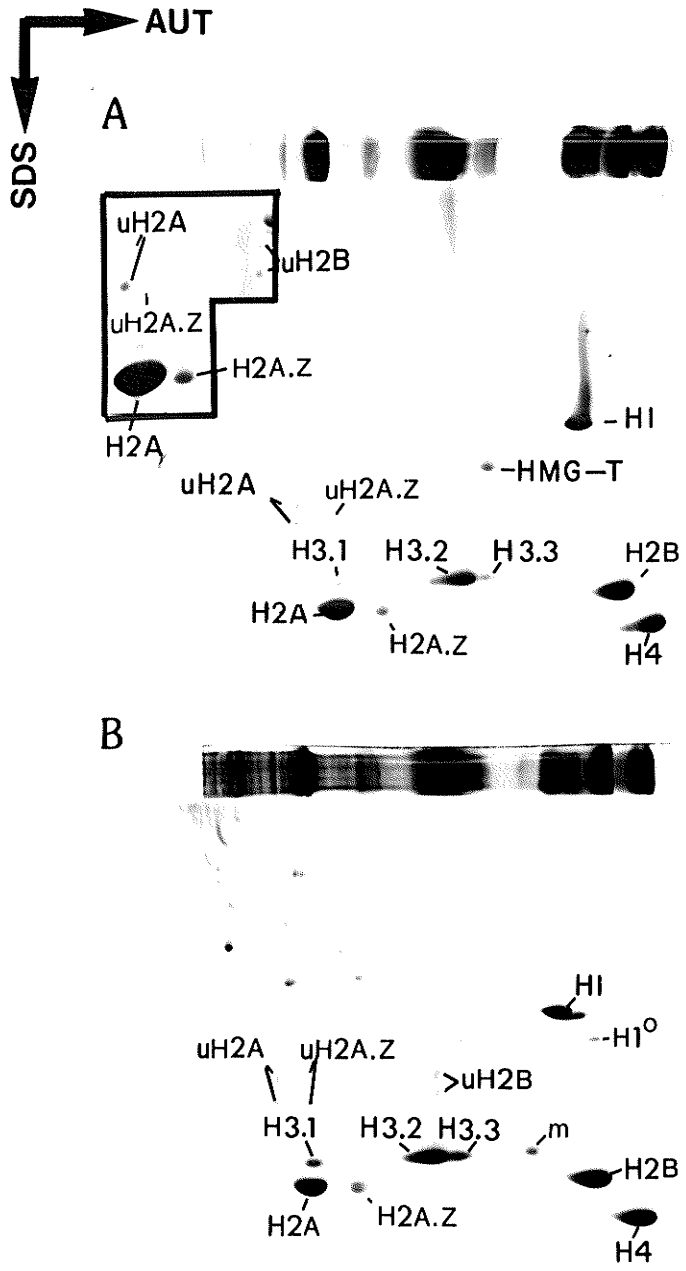

Figure 16. **Two-dimensional electrophoretic patterns of protamine released proteins from early stage testis nuclei and trout liver nuclei.**
Histones (10 ug) from fraction S0.50 from either early stage trout testis (A) or trout liver (B) nuclei were obtained by protamine release. The proteins were resolved by two-dimensional PAGE (AUT→SDS). m is micrococcal nuclease. (A) Inset: A portion of a two-dimensional gel pattern containing 40 ug of early stage trout testis histones. The ubiquitin adducts of H2A, H2A.Z and H2B are denoted as uH2A, uH2A.Z and uH2B, respectively.

the H3 variants are artifacts of oxidation. Relative levels of the H3 variants did not change when iodoacetamide was excluded from nuclei isolation buffers. Also, histones were isolated by the protamine release method of Richards and Shaw (1982) which was modified to minimize protein oxidation. Thirdly, thiodiglycol which is an efficient scavenger of oxidizing agents was included in the isolation of nuclei to prevent partial oxidation of methionine residues (Urban et al., 1979).

Visual inspection of the protein patterns showed there were no qualitative differences in the histone variants associated with liver and testis chromatin. However, there were several quantitative differences in the relative levels of histone variants H3.1 and H3.3 in liver and testis chromatin, with these variants being more abundant in liver than in testis (Figure 16). Thus, trout testis cells do not display germ cell specific histone variants like those observed during mammalian spermatogenesis (Trostle-Weige et al., 1982).

The putative H2A.Z was enriched by gel-exclusion chromatography on a Bio-Gel P30 column. Fraction c contained the putative trout H2A.Z (Figure 15) and this fraction was used to provide H2A.Z for amino acid sequence determination.

Neither bovine H2A.Z nor chicken H2A.Z (also called H2A.F and M1) contains methionine residues (Ball et al., 1983b); Harvey et al., 1983: Urban et al., 1979). To determine whether the putative trout H2A.Z had methionine residues, trout testis histones were treated with cyanogen bromide which will cleave on the carboxyl side of methionine residues. Putative trout H2A.Z and H2A, which does not have methionine residues

(Connor et al., 1984), were not cleaved while histones that do contain methionine residues (e.g., histones H3, H2B and H4) were processed (Figure 17). These results suggest the putative trout H2A.Z does not contain internal methionine residues as would be expected if this protein were similar to other H2A.Z proteins.

Further support that we have identified the trout histone H2A variant H2A.Z comes from sequence analysis of the 32 amino-terminal residues. Similar to bovine H2A.Z, trout H2A.Z had an unblocked amino terminus. Comparisons of the first 30 amino acid residues of trout H2A.Z with those of trout H2A, bovine H2A.Z and chicken H2A.F shown in Figure 18 demonstrated the following points. First, trout H2A.Z had the conserved H2A sequence AGLQFPV (Wu et al., 1986). Second, 18 of the first 27 residues in trout H2A are identical with those in trout H2A.Z. Third, the first 29 amino acids of trout H2A.Z were identical with those of chicken H2A.F and differed from those of bovine H2A.Z at only one position. Thus, the amino-terminal part of histone H2A.Z appears to be highly conserved.

b. The level of histone H2A.Z is reduced in chromatin from late stage trout testis

Histones were isolated from testes at different stages of development. On the basis of the work of Louie and Dixon (1972), we expect the different testis stages to contain a preponderance of the following cell types: early stage, primary and secondary spermatocytes; intermediate stage, early and middle spermatids; and late stage, late spermatids and spermatozoa. The percentage of DNA

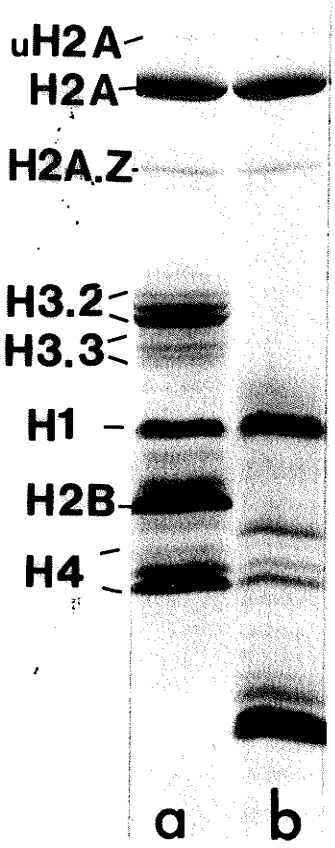

Figure 17. **Cyanogen bromide cleavage of trout testis histones.** Trout testis histones were treated with cyanogen bromide as described in Experimental procedures. The peptides were resolved by AUT 15% PAGE and stained with Coomassie Blue. The lanes correspond to untreated histones (a) and cyanogen bromide treated histones (b).

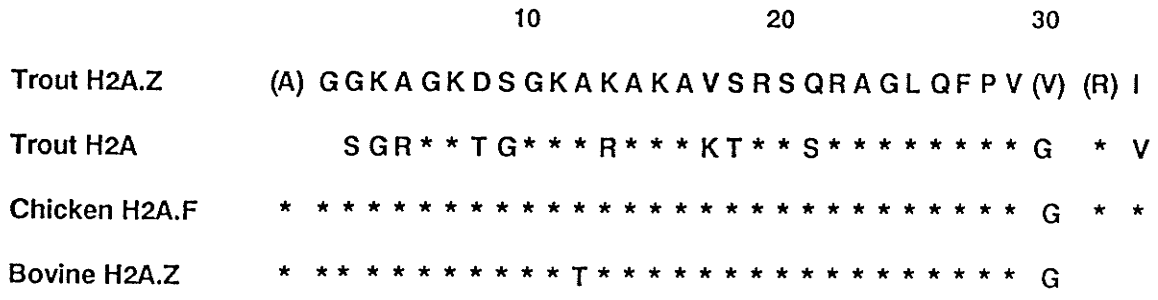

Figure 18. **Amino acid sequences of the amino-terminal residues of trout testis histones H2A.Z and H2A and other H2A.Z proteins.** The N-terminal amino acid sequences of trout histone H2A.Z along with trout H2A (Connor et al., 1984) are shown (one letter code). For comparison, the sequences of chicken H2A.F (Harvey, et al., 1983) and bovine H2A.Z (Ball et al., 1983b) are also shown. Parantheses in the trout histone H2A.Z sequence indicate that only a tentative identification of these amino acids has been made. Amino acids which are identical with those in trout histone H2A.Z have been given a asterisk, and only those amino acids which are different are indicated.

complexed as nucleohistone was approximately 70, 30 and 7 in early, intermediate and late stage testis respectively. Christensen and Dixon (1982) demonstrated that testis maturation was accompanied by an increase in levels of hyperacetylated histone H4 species and a reduction in the content of HMG-T. We have confirmed their observations (Figure 19). Moreover, there was a marked reduction in the level of H2A.Z in the late stage nucleohistone. This suggests that chromatin regions containing nucleosomes with H2A.Z were selectively converted to nucleoprotamine or that H2A.Z was associated with the transcriptionally active chromatin of early stage testis. H2A.Z may be involved in decondensation of chromatin which would facilitate the replacement of histones with protamines. Alternatively, since H2A.Z can be ubiquitinated, it is conceivable that in late stage testis chromatin H2A.Z was converted to its ubiquitinated form. Thus, the levels of ubiquitinated histone species at various stages of trout testis development were ascertained.

c. Identification of trout ubiquitinated histone species

Trout testis histones were resolved by two-dimensional PAGE (AUT→SDS), transferred to nitrocellulose and ubiquitin conjugated proteins detected with anti-ubiquitin IgG and ^{125}I-labeled protein A (Figure 20). The ubiquitinated species of histones H2A, H2A.Z and H2B were detected.

The existence of polyubiquitinated species of histone H2A *in vitro* has recently been reported (Kanda *et al.*, 1986). However, naturally occurring polyubiquitinated histones have not been described.

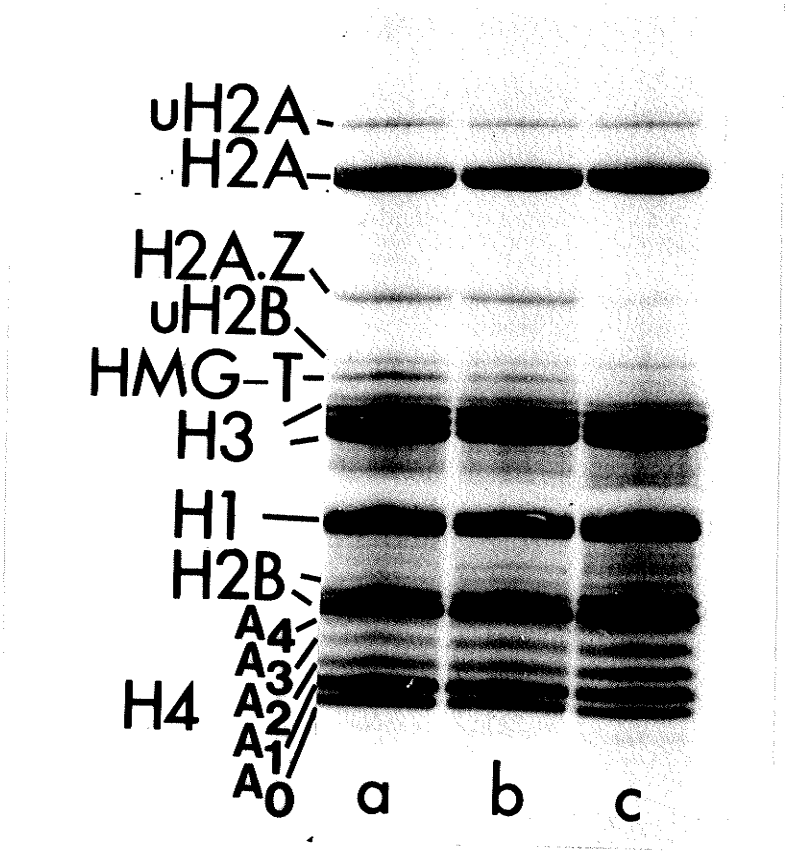

Figure 19. **Histone composition in developing trout testis.** Acid-extracted histones isolated from early (a), intermediate (b), and late (c) stage trout testis were separated by AUT 15% PAGE. The gel was stained with Coomassie Blue.

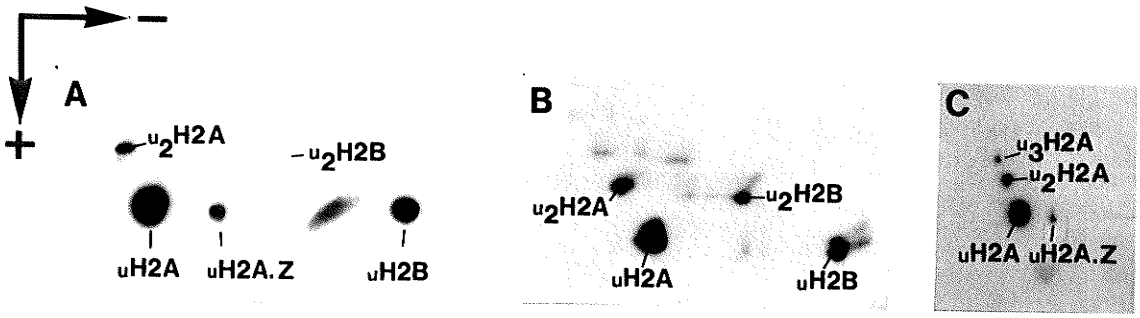

Figure 20. **Identification of trout testis ubiquitinated histone species.**
Histones isolated from early stage trout testis (A), late stage trout testis (B), and Bio-Gel P30 column fraction b (shown in Figure 15) (C) were resolved by two-dimensional PAGE (AUT→SDS). The proteins were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with anti-ubiquitin IgG and ^{125}I-labeled protein A. The autoradiograms are shown. The ubiquitin adducts of histones H2A, H2A.Z and H2B are denoted as uH2A, uH2A.Z and uH2B, respectively. The polyubiquitinated histone species are labeled as u_2 and u_3 representing the attachment of two and three ubiquitins, respectively.

Assignment of the polyubiquitinated species of histone H2A was based on the following observations: (1) several ubiquitinated species coeluted with histone H2A in the Bio-Gel P30 column fraction b (Figure 20C); (2) when the protein pattern visualized by India ink staining of the nitrocellulose was superimposed on the autoradiogram shown in Figure 20C, it was evident that some of the ubiquitinated species and histone H2A were on a diagonal line on the two-dimensional gel pattern; (3) when relative mobilities on SDS gels of histone H2A and these ubiquitinated forms were plotted vs the log polypeptide molecular weight, ubiquitinated species labeled uH2A, u_2H2A and u_3H2A increased by increments of 8.5 kDa, corresponding to the addition of one ubiquitin molecule (Watson et al., 1978); and (4) treatment of the protein sample (Bio-Gel column fraction b) with cyanogen bromide did not alter the pattern shown in Figure 20C. (Note neither histone H2A, histone H2A.Z nor ubiquitin contain internal methionine residues.) Histone H2B, uH2B and the ubiquitinated species identified as diubiquitinated H2B were also on a diagonal line on the two-dimensional gel pattern and were cleaved by cyanogen bromide treatment.

The levels of the ubiquitinated histone species in early and late stage trout testis chromatin (nucleohistone) were compared (Figure 20). For both sources, uH2A was the predominant ubiquitinated histone species, and levels of ubiquitinated and diubiquitinated H2A species were similar. Qualitative and quantitative differences in levels of other ubiquitinated histone species were noted. Ubiquitinated H2A.Z was not detected in the late stage trout testis. The content of uH2B was reduced slightly while the amount of diubiquitinated histone H2B

increased in the late stage testis chromatin.

Reduced levels of H2A.Z in the late stage trout testis chromatin were not due to conversion of H2A.Z to uH2A.Z. Nucleosomes with histone H2A.Z may be selectively disassembled in late stage testis or H2A.Z may be associated with transcriptionally active chromatin. The level of transcription in late stage trout testis is low to absent (Gillam et al., 1979). We did not observe an increase in levels of ubiquitinated histones species in the late stages of trout spermatogenesis such as that seen in rooster. However, it is conceivable that the presence of ubiquitinated histone species in late stage testis nucleohistone may aid in the replacement process, perhaps synergistically with histone hyperacetylation. Increased levels of uH2B have been found associated with active chromatin (see Section III. C). However, in late stage testis the rate of mRNA synthesis is very low, so the persistence of uH2B and u_2H2B may serve a different function. Rao and Rao (1987) have found DNA cleavage sites that are more accessible in rat pachytene spermatocyte core particles than in rat liver core particles. These cleavage sites correspond to the region where H2B interacts with nucleosomal core DNA. This suggests that the histone-DNA interaction is weaker in these particles possibly due to the presence of the germ cell specific histone variant, TH2B. Perhaps the ubiquitinated species of H2B in trout serves a similar function, i.e. maintaining the more open structure of chromatin to aid in the replacement of histones with protamines. It is also possible that polyubiquitination of the histones may prepare or tag the histones for degradation such as that seen in cytoplasmic proteins.

C. Ubiquitinated and polyubiquitinated histones in chromatin

1. Introduction

The role of histone ubiquitination in chromatin function is not clear. In the cytoplasm, ubiquitin is used to "tag" proteins for protease digestion with the rate of proteolysis increasing with the number of attached ubiquitins (Ciechanover et al., 1981). It has been suggested that an analogous system may operate in the nucleus with the proteolytic removal of nucleosomal proteins from activated chromosomal regions being responsible for the striking changes in chromatin structure observed at very high rates of transcription (Varshavsky et al., 1983).

Our discovery of polyubiquitinated histones in trout testis chromatin was the first report of naturally occurring histones with more than one ubiquitin attached. It is not known whether this modification prepares histones for degradation. In the cytoplasm the attachment of additional ubiquitin molecules is believed to occur at multiple sites along the molecule tagged for degradation (Hershko et al., 1980; Bachmair et al., 1986; Kanda et al., 1986).

In this study the arrangement of ubiquitin molecules in polyubiquitinated H2A from trout liver and calf thymus has been characterized by peptide mapping. Figure 21 illustrates the features of the monoubiquitinated H2A molecule necessary to understand the strategy used in the following experiments. In bovine uH2A ubiquitin is attached at lysine 119 of the H2A molecule. The enzyme H2A specific protease, which acts exclusively on histone H2A and its ubiquitinated forms, catalyzes the removal of 15 amino acids from the carboxyl end of

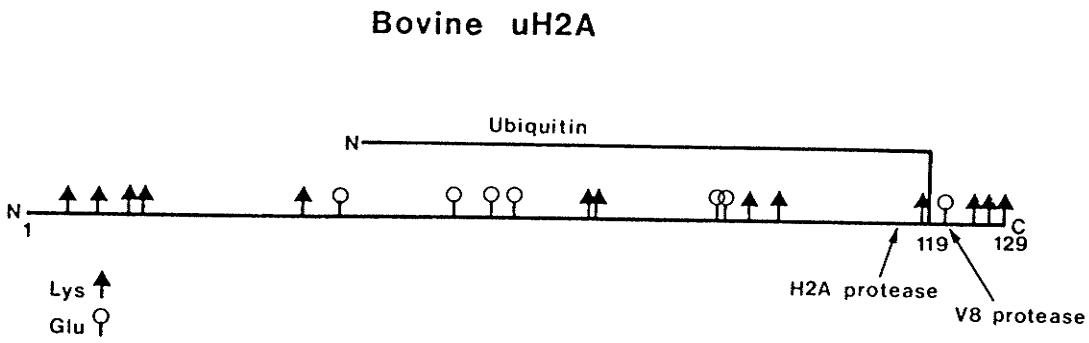

Figure 21. **Schematic illustration of the bovine uH2A molecule.** The attachment of ubiquitin to histone H2A at lysine 119 is shown as well as the location of additional lysine residues (↑) and glutamic acid residues (♀). The sites for cleavage by H2A specific protease and V8 protease are also indicated.

the bovine H2A molecule (13 amino acids in trout H2A) by specific cleavage between valine 114 and leucine 115 (Eickbush et al., 1976; Watson and Moudrianakis, 1982; Davie et al., 1986). The use of this enzyme has allowed us to determine that up to three ubiquitin molecules can be attached to H2A between residue 115 and the C-terminal. V8 protease (Staphylococcus aureus protease, V8 strain) has been used as a probe of exposed nonbasic residues in nucleosomes (Rill and Oosterhof, 1981). Digestion of chromatin results in cleavage at the carboxyl side of glutamic acid 121 of histone H2A and its ubiquitinated species. This enzyme has allowed us to determine that ubiquitin is not attached to H2A between residue 122 and the C-terminal. Ubiquitin itself is not digested by these enzymes.

Nothing is known about the distribution of polyubiquitinated histone species in chromatin. In this study the distribution of ubiquitinated and polyubiquitinated histones in chromatin was examined by analyzing proteins from fractionated chromatin by two-dimensional PAGE (AUT→SDS) followed by immunochemical detection of ubiquitin. We found that the chromosomal proteins from the transcriptionally active macronuclei of Tetrahymena and the low salt soluble fractions of chromatin previously shown to be enriched in transcriptionally active/competent gene sequences were enriched in polyubiquitinated and ubiquitinated histone species, especially uH2B.

2. Experimental procedures

a. Preparation of trout liver histones and chromatin

Liver nuclei isolated as described previously (Section II.B) were

digested with micrococcal nuclease (nuclei at 40 A_{260} units/ml, 50 A_{260} enzyme units/ml MNase, 10 min at 37°C). Digestion was terminated by placing the suspension on ice and adding 0.25 M EGTA to 10 mM. Digested nuclei collected by centrifugation at 2000 x g for 10 min, were resuspended in 1 mM EDTA, pH 7.5, 1 mM PMSF. The EDTA released chromatin was collected by centrifugation at 12,000 x g for 10 min. Acid soluble proteins were extracted from the supernatant directly with 0.4 N H_2SO_4. In addition, H1 stripped chromatin was prepared. The supernatant was adjusted to 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EDTA and CM-Sephadex was added as described by Libertini and Small (1980). The suspension was filtered and dialyzed against 10 mM EDTA pH 7.5 prior to concentration against polyethylene glycol (PEG). This procedure removes histone H1 and the majority of the nonhistone chromosomal proteins.

b. Preparation of calf thymus histones and chromatin

Calf thymus nuclei isolated as described previously (Section II.B) were digested with micrococcal nuclease (nuclei at 50 A_{260} units/ml, 50 A_{260} enzyme units/ml, 5 min at 37°C). The digestion was terminated by placing the mixture on ice and adding 0.25 M EGTA to 10 mM. Digested nuclei collected by centrifugation at 2000 x g for 10 min were resuspended in 1 mM EDTA, pH 7.5, 1 mM PMSF and the released chromatin collected by centrifugation at 12,000 x g for 10 min. A portion of this supernatant (SE) was used to prepare total calf thymus histones by acid extraction using 0.4 N H_2SO_4. Alternatively, a low salt soluble chromatin fraction was prepared by adjusting the SE to 0.15 M NaCl

using 4 M NaCl and collecting the soluble material (S0.15) by centrifugation at 12,000 x g for 10 min. The S0.15 chromatin fraction was used for H2A specific protease studies as both the source of the enzyme and the substrate. In addition, a portion of the S0.15 fraction was treated with CM-Sephadex to remove histone H1 as described above.

c. Isolation of calf thymus histone H2A

Total calf thymus histones were fractionated by gel exclusion chromatography on a Bio-Gel P30 column (110 x 2.5 cm) eluted at room temperature with 50 mM NaCl, 10 mM HCl (flow rate 40 ml/h). Typically 75 mg of histones was dissolved in 10 mM Tris-acetate, pH 8.8 containing 10% (v/v) 2-mercaptoethanol and loaded onto the column. Elution of the histones was monitored at 230 nm. The pooled fractions were dialyzed against 0.1 N acetic acid, followed by water and lyophilized. Calf thymus histone H2A and its ubiquitinated species were enriched in the second fraction (elution profile similar to Figure 15).

d. V8 protease digestion of chromatin

Chromatin (stripped of histone H1) at 1 mg/ml in 50 mM NH_4HCO_3, pH 8.0, 0.2 mM EDTA was incubated with or without S. aureus protease (V8 strain) (Miles Scientific) at 0.25 mg/ml (protease/chromatin = 25 ug/100 ug) at 37°C for 1, 2 or 3 h. The reaction was stopped by placing the samples on ice, adding 1/10th volume 8.3 N NH_4OH, heating the sample at 100°C for 1 min, cooling on ice and adding PMSF to 10 mM. The sample was lyophilized and prepared for AUT 15% PAGE by protamine

release. Proteins were resolved by two dimensional PAGE (AUT→SDS), transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG and ^{125}I-labeled protein A (Section II). The extent of H2A digestion was determined by removing a portion of the sample following incubation, adding it directly to an equal volume of 2 x SDS sample buffer, heating to 100°C for 1 min and analyzing the proteins by SDS PAGE.

e. H2A specific protease digestion of histones

An enriched preparation of H2A specific protease was obtained from calf liver chromatin. Calf liver nuclei were isolated as described previously (Section II.B) and digested with micrococcal nuclease (nuclei at 40 A_{260} units/ml, 50 A_{260} enzyme units/ml, 20 min at 37°C). The reaction was terminated by placing the suspension on ice and adding 0.25 M EGTA to a final concentration of 10 mM. The digested nuclei were collected by centrifugation at 750 x g for 10 min and then resuspended in 10 mM EDTA. The insoluble nuclear material was removed by centrifugation at 12,000 x g for 10 min. The resulting supernatant was adjusted to 150 mM NaCl by the dropwise addition of 2 M NaCl, centrifuged at 12,000 x g for 10 min and the pellet material saved. H2A specific protease was separated from the bulk of the chromatin by gel exclusion chromatography on a Bio-Gel A5m column (30 x 1.5 cm). The pellet was redissolved at approximately 50-70 A_{260} units/ml in the column elution buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 4 M NaCl) and approximately 0.5 ml loaded onto the column. Two ml fractions were collected and the elution of chromatin monitored at 260 nm. The

presence of H2A specific protease in the column fractions was determined according to the method of Davie et al. (1986). Briefly, 20 ul of calf thymus histone H2A (10 mg/ml) was mixed with 230 ul of a column fraction and 250 ul of 20 mM Tris pH 10.0 was added, resulting in a pH of 8.5. Samples were incubated at 37°C for about 2 h. Following incubation the proteins were acid extracted using 0.4 N H_2SO_4. The percentage of H2A molecules cleaved was determined as described by Eickbush et al. (1976) by scanning Coomassie Blue stained 15% polyacrylamide SDS gels. Figure 22 shows the distribution of chromatin and H2A specific protease following gel exclusion chromatography.

Typically, seven fractions (about 14 ml) on the right hand side of the A_{260} elution profile (A_{260} about 0.5) were pooled, concentrated against PEG and used as the source of H2A specific protease for further studies. One mg of BSA was added for each ml of enzyme preparation and aliquots were stored at -80°C. It was possible to store the concentrated enzyme fraction for at least two weeks without significant loss of activity.

To digest trout liver histones, 200 ug of histones (10 mg/ml) were mixed with ddH_2O and the H2A specific protease preparation to give a final volume of 250 ul. An equal volume of 20 mM Tris pH 10.0 was added and the samples incubated at 37°C for 2 and 4 h. Following digestion the proteins were acid extracted using 0.4 N H_2SO_4.

A slightly different approach was used for the digestion of calf thymus histone H2A species. The SO.15 fraction of calf thymus chromatin, isolated as described above, contained both the H2A specific

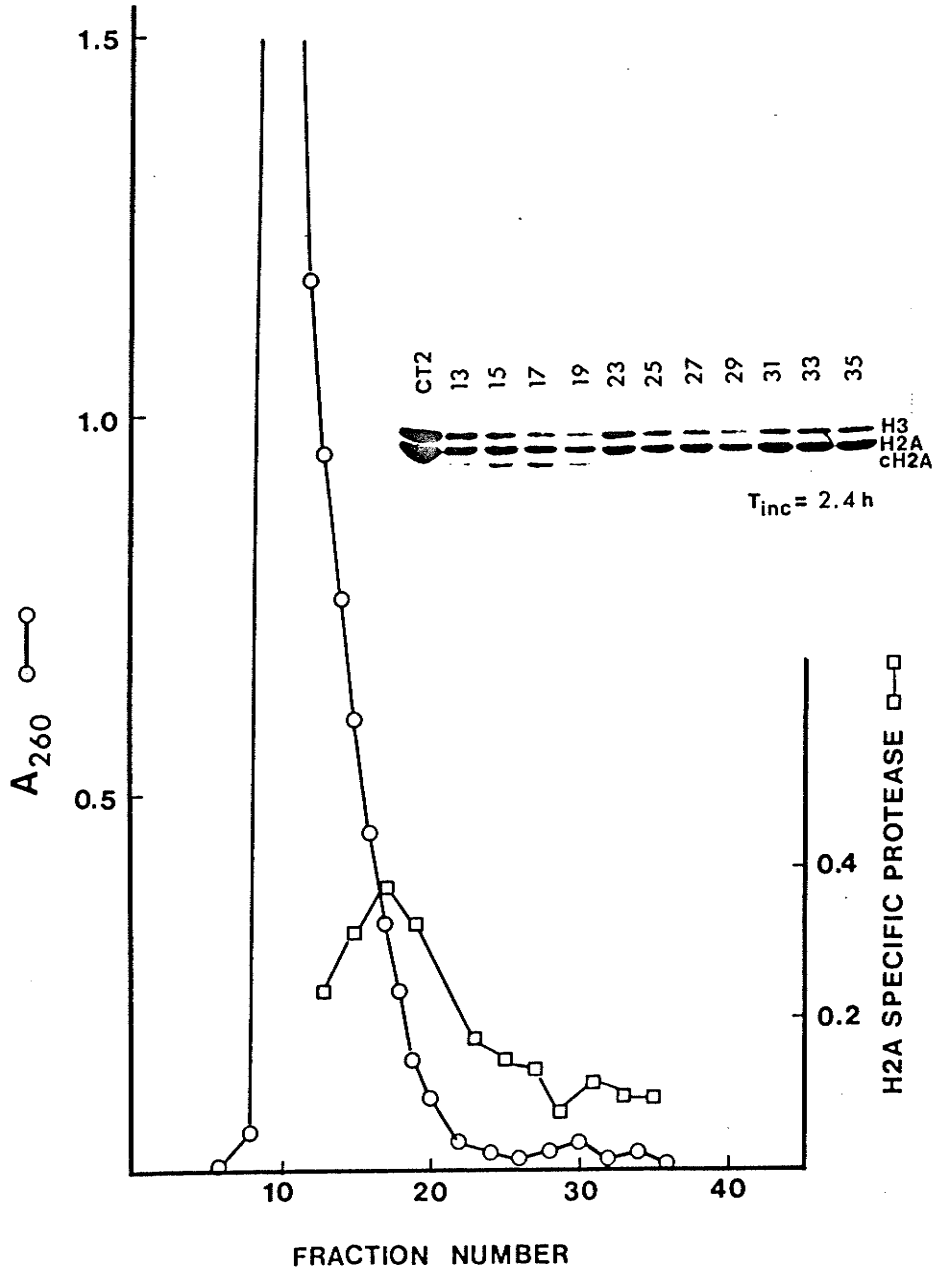

Figure 22. Distribution of chromatin and H2A specific protease following gel exclusion chromatography.
The elution of chromatin was monitored at 260 nm. Fractions containing H2A specific protease were determined by digestion of calf thymus H2A and analysis by SDS 15% PAGE (inset). The relative amounts of H2A specific protease were calculated as a ratio of H2A converted to cH2A (cH2A: cH2A + H2A) in a 2.4 hour incubation.

protease as well as its substrates. To the S0.15 fraction was added 1/20th volume 1 M Tris-HCl, pH 7.0 and the sample was preincubated at 37°C. An equal volume of prewarmed 100 mM Tris, pH 10.0, 4 M NaCl was added and the mixture incubated at 37°C for 0, 2, 4 and 6 h. At each time point a sample was removed and the proteins acid extracted using 0.4 N H_2SO_4. Proteins were resolved by two dimensional PAGE (AUT→SDS), transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG and ^{125}I-labeled protein A.

f. Cleavage of histones with CNBr

Trout liver histones and a calf thymus histone fraction enriched for histone H2A and its ubiquitinated species were cleaved with CNBr (Section III.B). The peptides were resolved by two-dimensional PAGE (AUT→SDS), transferred to nitrocellulose and immunochemically stained for ubiquitin using an anti-ubiquitin IgG and ^{125}I-protein A.

g. Fractionation of chromatin

Trout testis chromatin was fractionated using the Sanders' (1978) method (Section II.B) to yield fractions SS0.05, SS0.10, SS0.20, SS0.50 and pellet. Alteratively, fractionation was done using the modification described in Section III.A. Following removal of the low salt eluted fraction (SS0.05), chromatin was released using EDTA to yield the SE fraction and the EDTA insoluble pellet (P). Histones were prepared by acid extraction, resolved by two-dimensional PAGE (AUT→SDS) and electrophoretically transferred to nitrocellulose. Ubiquitin conjugated proteins were detected with an anti-ubiquitin IgG and ^{125}I-

labeled protein A.

To facilitate comparison of the levels of ubiquitinated histones associated with different fractions several precautions were taken. AUT 15% PAGE was used to determine the amount of sample required for equivalent histone levels. Typically, 9 ug of protein was used. Following Coomassie Blue staining and destaining the lanes were scanned with a densitometer. The protein load was then calculated to give equal densities of histones H2B and H3. Samples were reanalyzed by two dimensional PAGE, transferred to nitrocellulose and immunochemically stained for ubiquitin. Protein blots from the same fractionation experiment were analyzed together, or as close in time to each other as possible. Following immune detection, blots from the same experiment were exposed to film for autoradiography together.

Calf thymus nuclei at 40-50 A_{260} units/ml were digested with 50 A_{260} enzyme units of micrococcal nuclease at 37°C for 10 min. Chromatin was fractionated using the Sanders' (1978) method (Section II.B) to yield fractions SSO.05, SSO.10, SSO.20, SSO.50 and pellet. Alternatively, fractionation was done as described above in preparation of calf thymus histones. The EDTA released chromatin corresponds to total chromatin (T) and this is divided into the chromatin soluble in 0.15 M NaCl (SO.15) and that insoluble in 0.15 M NaCl (P). Proteins from each fraction were acid extracted and analyzed as described above.

Mature chicken erythrocyte nuclei at 50 A_{260} units/ml were digested with 25 A_{260} enzyme units/ml of micrococcal nuclease at 37°C for 40 min. Chromatin was fractionated using the Sanders' (1978) method (Section II.B) to yield four fractions: total unfractionated chromatin

(T); a low salt eluted fraction (SSO.1); a high salt solubilized fraction (SSO.6) and an insoluble pellet (P). Histones were prepared by acid extraction and analyzed as described above.

Mature chicken erythrocyte chromatin was also fractionated by the method of Ridsdale and Davie (1987b). Briefly, micrococcal nuclease digested nuclei were resuspended in 10 mM EDTA, 1 mM PMSF and incubated on ice for 2 h to release chromatin fragments into solution. Insoluble nuclear material was removed by centrifugation at 12,000 x g for 20 min. The EDTA soluble material (SE) was diluted to 30 A_{260} units/ml by the addition of 10 mM EDTA and made 0.15 M NaCl by the addition of 4 M NaCl while mixing. Centrifugation at 12,000 x g for 20 min yielded a supernatant (SO.15) and a pellet. The SO.15 fraction was concentrated against PEG and the chromatin fragments resolved by gel exclusion chromatography on a Bio-Gel A5m column (110 x 2.5 cm) equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.15 M NaCl. Histones were isolated by acid extraction prior to analysis as described above.

Tetrahymena macronuclei and micronuclei chromosomal proteins were prepared by Dr. C.D. Allis of Verna and Marrs McLean Department of Biochemistry Baylor College of Medicine, Houston, Texas. Proteins were analyzed as described above.

3. Results and discussion

a. Characterization of polyubiquitinated H2A

The attachment of several ubiquitin molecules to one protein molecule can theoretically occur in more than one way. Figure 23 illustrates four possible models for the arrangement of more than one

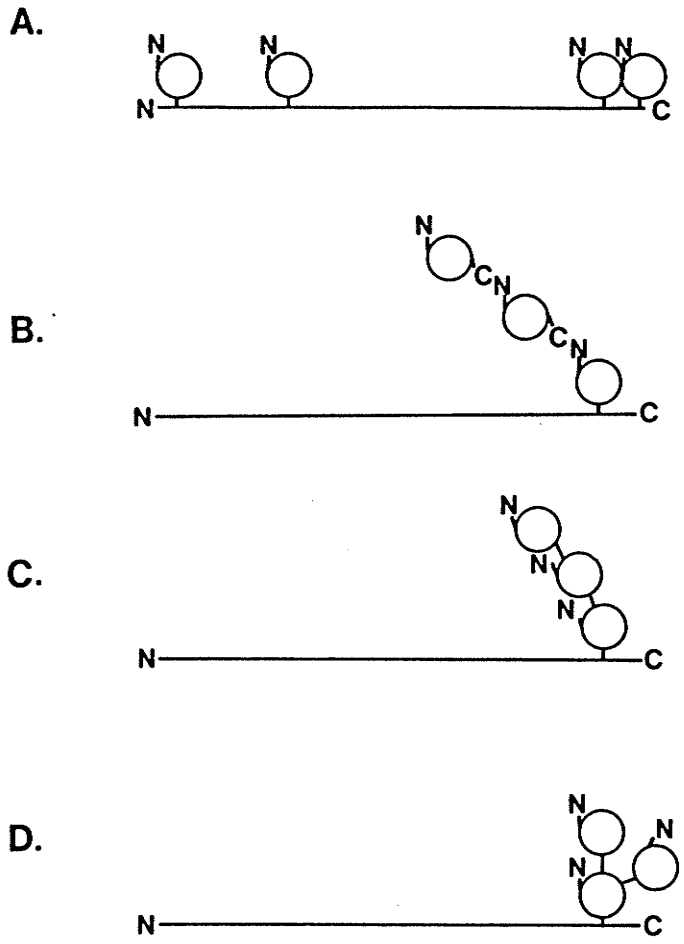

Figure 23. Models for the arrangement of more than one ubiquitin molecule on the histone H2A molecule.
Histone H2A is shown as a straight line and ubiquitin molecules are shown as circles. Models are described in the text.

ubiquitin molecule on the histone H2A molecule. A combination of these models may also be possible. In the first model additional ubiquitin molecules are linked through their C-terminal glycine residues to the ε-amino groups of lysine residues distributed along the histone H2A molecule. Figure 21 shows the positions of lysine residues in the histone H2A molecule. The remaining models show additional ubiquitins linked to ubiquitin molecules already joined to histone H2A.

(i) Ubiquitin is not attached to H2A between residue 122 and the C-terminal

Removal of the six residue C-terminal fragment from trout liver and the eight residue C-terminal fragment from calf thymus histones H2A and their ubiquitinated species by V8 protease causes a slight shift in the mobility of the larger fragment (cH2A) (Figures 24 and 25). This is seen by comparing the mobility of the digested and undigested ubiquitinated H2A species to the ubiquitinated H2B species which are not digested by V8 protease under these conditions. The larger fragment can contain one or more ubiquitin moieties. Ubiquitin is not attached to the C-terminal fragment of trout liver or calf thymus histone H2A, as an additional ubiquitinated peptide at a molecular weight of approximately 9.1 or 9.4 kDa, respectively, is not seen in either the trout or the calf sample. Digestion of calf thymus chromatin with V8 protease did result in the appearance of additional ubiquitinated peptides of about 26 kDa molecular weight (marked with arrows in Figure 25). These may be the result of other proteases that remain associated with H1 stripped chromatin.

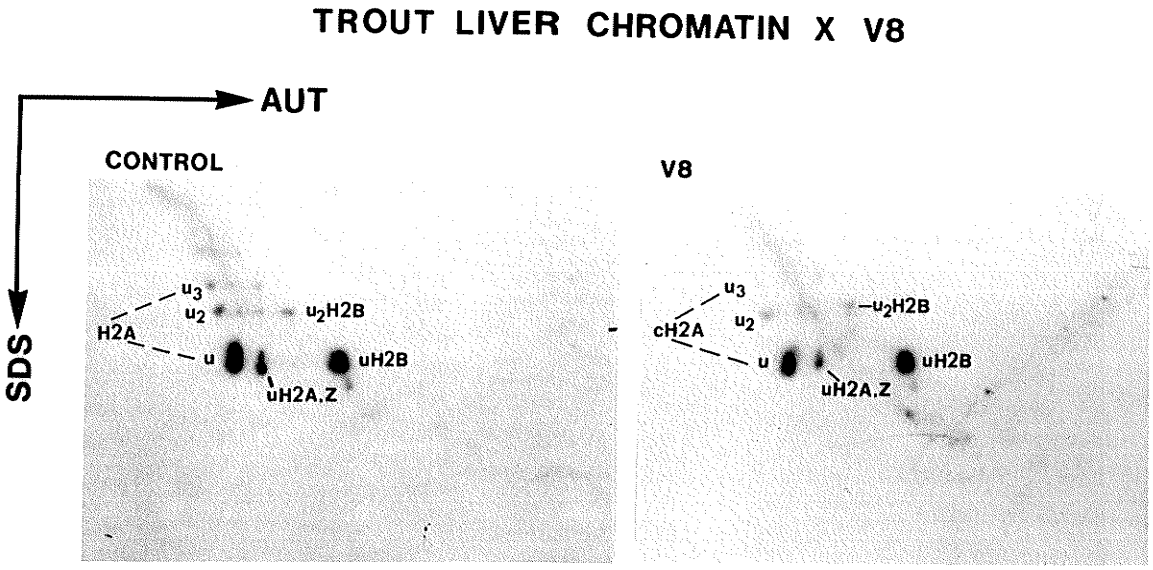

Figure 24. **Identification of ubiquitinated peptides following digestion of trout liver chromatin by V8 protease.** Trout liver chromatin with histone H1 removed was digested with V8 protease and the resulting peptides resolved by two-dimensional PAGE (AUT→SDS). Peptides were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with anti-ubiquitin IgG and ^{125}I-labeled protein A. The autoradiograms are shown. The ubiquitin adducts of histones H2A, H2A.Z and H2B are denoted as uH2A, uH2A.Z and uH2B, respectively. The polyubiquitinated histone species are labeled as u_2 and u_3 representing the attachment of two and three ubiquitins, respectively.

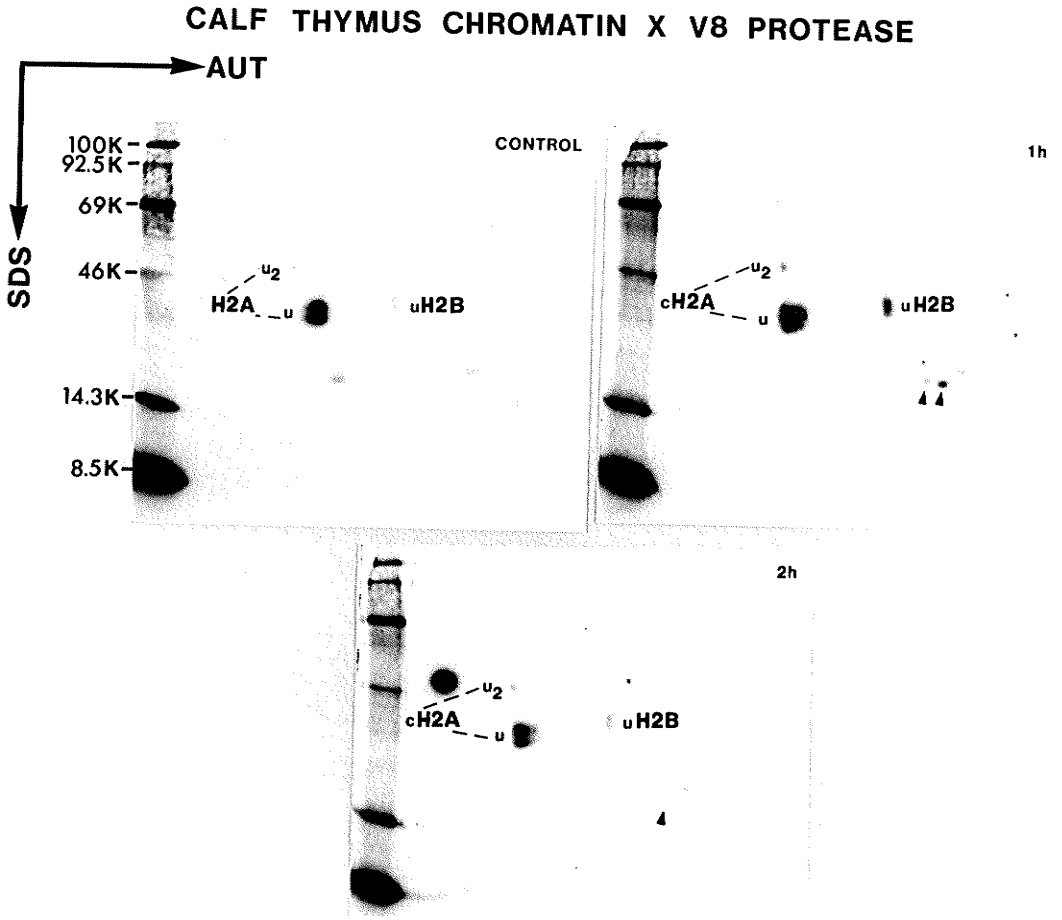

Figure 25. **Identification of ubiquitinated peptides following digestion of calf thymus chromatin with V8 protease.**
The low salt soluble fraction (S0.15) of calf thymus chromatin with histone H1 removed was digested with V8 protease for the indicated times and the resulting peptides analyzed as described in Figure 24. Additional ubiquitinated peptides are indicated by arrows.

(ii) Ubiquitin is attached to H2A between residues 115 and 121

The C-terminal fragment (13 residues, m.w.=1438) generated by digestion of trout liver histones with an enriched preparation of H2A specific protease contains up to three ubiquitin moieties designated uC, u_2C and u_3C (Figure 26). Digestion of calf thymus H2A with H2A specific protease results in a C-terminal fragment (15 residues, m.w.=1686) which can contain two ubiquitin moieties (Figure 27). The low levels of more highly ubiquitinated H2A species may preclude detection of C-terminal fragments containing more than three ubiquitins in trout liver or two ubiquitins in calf thymus. At more extensive digestion of trout liver H2A species (Figure 26D) there appears to be a minor population of ubiquitinated H2A molecules (labeled uP, u_2P etc.) with slightly greater mobility when compared to mobility of ubiquitinated H2B species. These may correspond to ubiquitin attached to the N-terminal portion of H2A (residues 1-114) or these fragments may be the result of other proteolytic activities due to the prolonged time required for digestion and the greater amount of enzyme preparation added. It should be noted that the signal intensity for all ubiquitinated H2A species as well as uH2A.Z, seems decreased at the most extensive digestion, a finding which supports the idea of other proteolytic activities.

Similar results are seen with the digestion of calf thymus histones with H2A specific protease (Figure 27). Between two and four hours digestion time there is a decrease in the level of uH2A, but a corresponding increase in the level of uC is not observed. At the six hour time point the level of uC has not changed noticeably and the

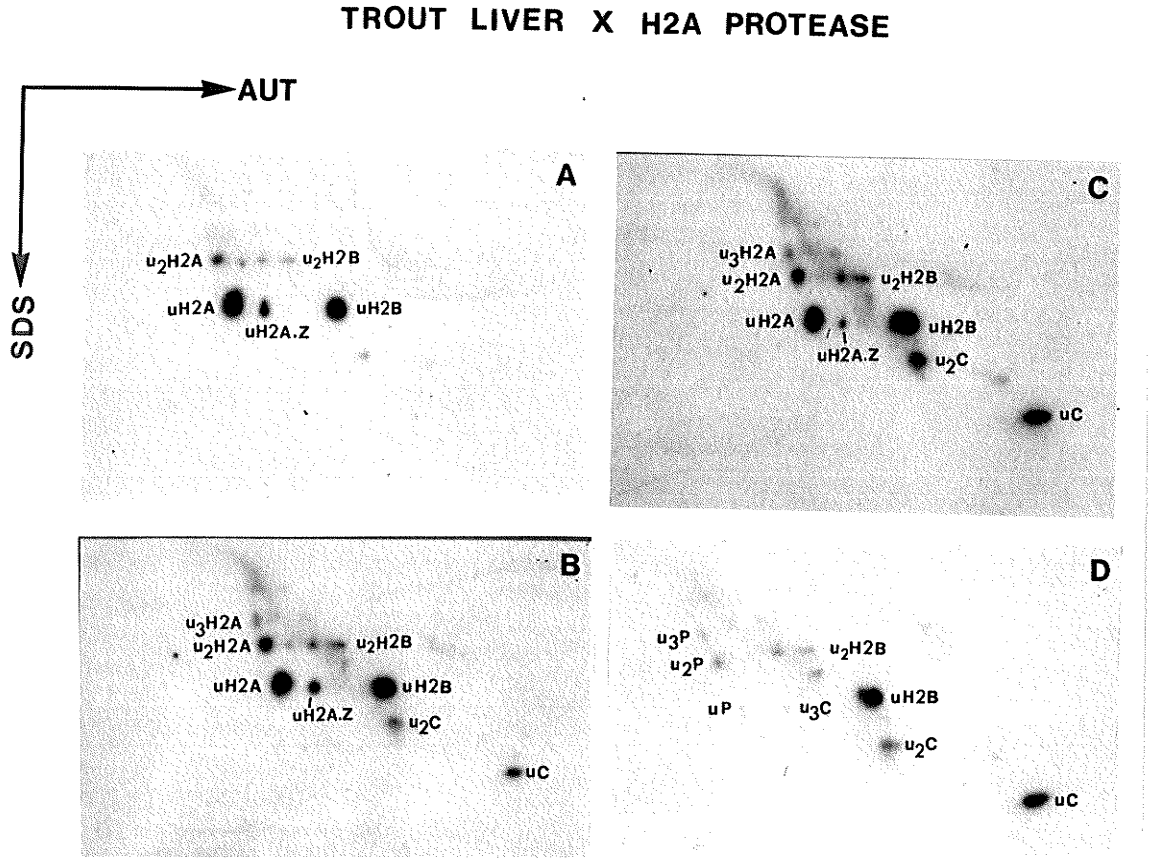

Figure 26. **Identification of ubiquitinated peptides following digestion of trout liver histones by H2A specific protease.** Trout liver histones were digested with an enriched preparation of calf liver H2A specific protease. The resulting peptides were analyzed as described in Figure 24. Ubiquitinated fragments of the H2A molecules are designated as uC, u_2C and u_3C if they contain the C terminal portion of the H2A molecule and uP, u_2P and u_3P if they contain a larger fragment of the H2A molecule. A, undigested trout liver histones, B, trout liver histones digested 2h with 130 ul of H2A protease preparation, C, trout liver histones digested 4h with 130 ul of H2A protease preparation and D, trout liver histones digested 4h with 180 ul of H2A protease preparation.

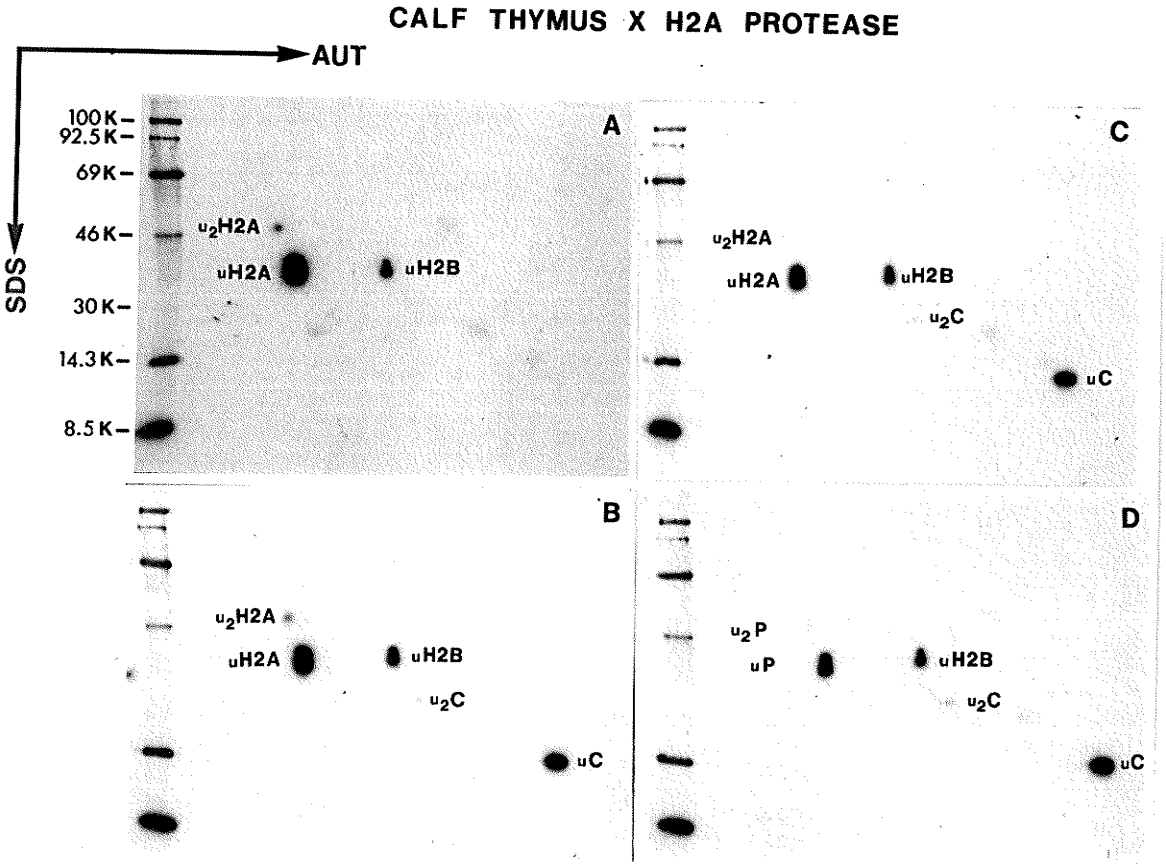

Figure 27. **Identification of ubiquitinated peptides following digestion of calf thymus histones with H2A specific protease.** The low salt soluble fraction of calf thymus chromatin (S0.15) was isolated and digested with its associated H2A specific protease for 0h (A), 2h (B), 4h (C) and 6h (D). The resulting peptides were acid extracted and analyzed as described in Figure 24. The ubiquitin containing C-terminal portion of histone H2A molecules are designated as uC and u_2C while ubiquitinated species containing a larger fragment of H2A are designated as uP and u_2P.

mobility of uH2A and u_2H2A seems to be slightly greater when compared to uH2B. It is possible that the H2A specific protease is no longer active and another proteolytic activity is responsible for the slight decrease in size of the ubiquitinated H2A species.

Therefore, digestion of polyubiquitinated H2A by V8 protease and H2A specific protease shows that a seven residue fragment of H2A (residues 115-121) with lysine residues at positions 118 and 119, can contain three ubiquitin molecules. It is unlikely, due to steric hindrance that both lysine residues can be modified by ubiquitination. This implies that additional ubiquitin must be conjugated to histone H2A through ubiquitin already linked to histone H2A (Figure 23, B, C and D). It is not entirely clear whether additional ubiquitin molecules can be attached to the N-terminal portion of the histone H2A molecule between residues 1 and 114. The results from experiments where trout liver histones were digested with H2A specific protease indicate that the adduct resulting from such a linkage would be a minor component if found at all. The data from experiments using calf thymus histones are more difficult to interpret due to low levels of polyubiquitinated histone H2A as well as an apparently low level of H2A specific protease activity.

(iii) Ubiquitin to ubiquitin attachment

In model B, (Figure 23) ubiquitin molecules are found in a "head-to-tail" arrangement with the C-terminal glycine of one ubiquitin linked to the N-terminal methionine of the next ubiquitin. The last ubiquitin moiety is linked via its C-terminal glycine to the ε-amino

group of lysine 119 of H2A. This arrangement is the same as that found in the polyubiquitin precursor molecule. Polyubiquitin genes have been identified when cloning ubiquitin-coding DNA sequences (Dworkin-Rastl et al., 1984; Özkaynak et al., 1984, Bond and Schlesinger, 1985; Wiborg et al., 1985; Gausing and Barkardottir, 1986; St. John et al., 1986) and these genes are involved in the heat shock response (Bond and Schlesinger, 1985,1986; Finley et al., 1987). It is unlikely that the intact polyubiquitin molecule can be conjugated to other proteins. The 3' final ubiquitin sequence does not code for glycine, which is the residue involved in the isopeptide linkage to histone H2A, and there is a protease activity with a specificity for cleaving the glycine-methionine bond between ubiquitin subunits (Bond and Schlesinger, 1985). Cyanogen bromide, which cleaves on the carboxyl side of methionine residues, was used as a probe for the linkage found in the polyubiquitin precursor. Neither ubiquitin, trout histone H2A nor the major calf thymus histone H2A (H2A.1) contain internal methionine residues (Watson et al., 1978; Connor et al., 1984; Wu et al., 1986). The calf histone H2A.2 does contain a methionine residue at position 51 (Wu et al., 1986). CNBr cleavage of trout liver histones does not result in removal of ubiquitin moieties from the polyubiquitinated H2A (Figure 28). Trout histone H2B contains methionine residues at positions 57 and 60 (Kootstra and Bailey, 1978) and is cleaved by CNBr. The ubiquitin containing fragment, cuH2B, corresponds to the C-terminal peptide of H2B with one ubiquitin molecule attached.

Calf thymus polyubiquitinated H2A also persists following CNBr cleavage (Figure 29). There is one additional ubiquitinated peptide

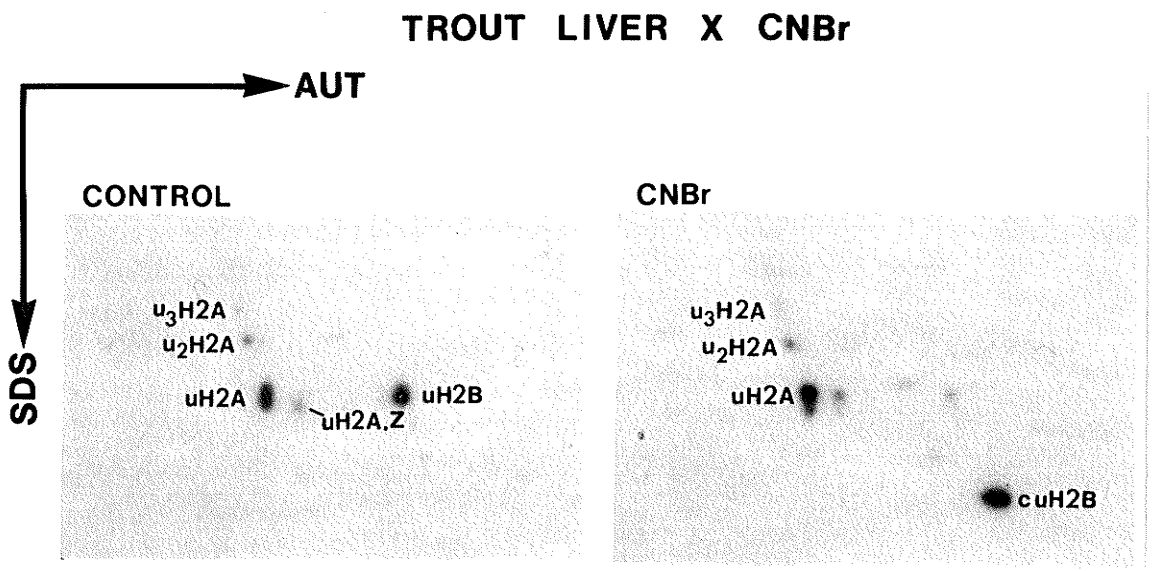

Figure 28. **Identification of ubiquitinated peptides following cleavage of trout liver histones by CNBr.**
Histones isolated from trout liver were cleaved with CNBr and the resulting peptides analyzed as described in Figure 24. The ubiquitin containing portion of the cleaved uH2B and u_2H2B is designated as cuH2B and cu_2H2B, respectively.

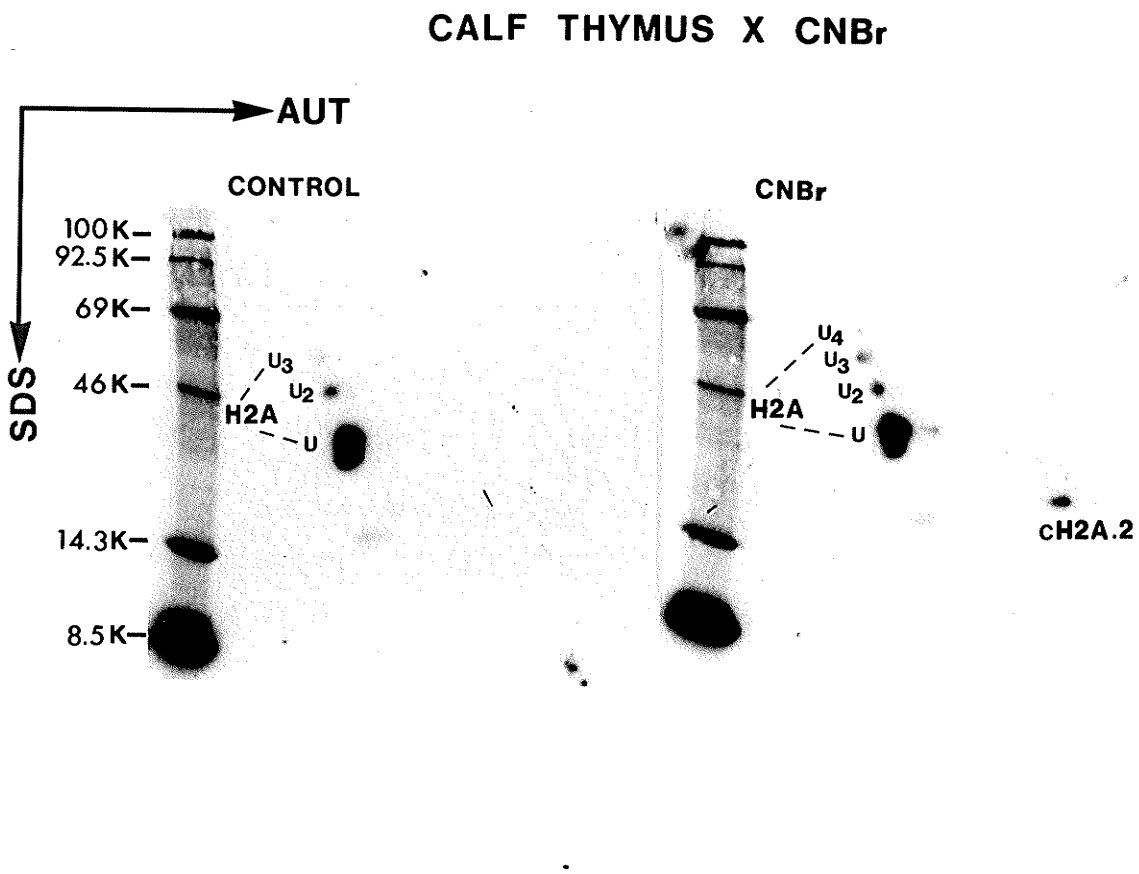

Figure 29. **Identification of ubiquitinated peptides following cleavage of calf thymus histones by CNBr.**
The Bio-Gel P30 fraction of calf thymus histones enriched for histone H2A and its ubiquitinated species was cleaved with CNBr and the resulting peptides analyzed as described in Figure 24. Molecular weights of radioactive standards (in kDa) are indicated on the left side of the figure.

that probably corresponds to the ubiquitinated fragment generated by the cleavage of the methionine containing variant, uH2A.2, with CNBr.

Therefore, the "head to tail" arrangement of ubiquitins in polyubiquitinated H2A does not occur and polyubiquitination of histone H2A is unlikely to be the result of the attachment of a polyubiquitin precursor molecule.

In porcine ubiquitin, lysine residues at positions 6, 11 and 33 can be fully modified by acetylation, while lysines at positions 27, 29, 48 and probably 63 are only partially modified (Zhu et al., 1986). It is possible that the same lysine residues available for acetylation may be available for modification by ubiquitination. Our studies could not distinguish between models C and D in Figure 23.

b. Ubiquitinated histone species are associated with transcriptionally active chromatin

Fractionation of chromatin by micrococcal nuclease sensitivity and solubility properties has shown that transcriptionally active sequences are associated with the low salt soluble, nuclease sensitive fraction (Davie and Saunders, 1981; Rocha et al., 1984). The present studies demonstrate that there is an increase in the levels of uH2A, polyubiquitinated H2A, uH2A.Z and most significantly uH2B in the low salt soluble fractions when compared to total chromatin.

Chromatin from bovine thymus and trout testis was fractionated by nuclease accessibility and solubility properties according to the Sanders (1978) procedure. Table VI shows the distribution of A_{260} absorbing material in each fraction. Histones from each fraction were

TABLE VI. Distribution of A_{260}-absorbing material following fractionation of trout testis and bovine thymus chromatin with the Sanders procedure.

Chromatin fraction	% of total A_{260}-absorbing material	
	trout testis*	bovine thymus
SS0.05	2.8	9.7
SS0.10	1.7	5.2
SS0.20	4.8	12.1
SS0.50	15.4	36.8

* nucleoprotamine pellet contained 68.8% of the total A_{260}-absorbing material

analyzed by AUT 15% PAGE (Figure 30). For both sources of chromatin, the low salt-soluble nucleosomes (SSO.05) were enriched in the HMG proteins and depleted in histone H1. Elevated levels of HMG proteins in the low salt eluted fractions are partly due to the extraction of these proteins from chromatin under these conditions (Davie and Saunders, 1981). Ubiquitinated species of H2A were enriched in the low salt soluble, nuclease sensitive fraction of both trout testis and bovine thymus chromatin (Figure 30 and Table VII). The relative enrichment of uH2B in bovine thymus chromatin fraction SSO.05 was greater than that of uH2A (1.36 vs 1.69).

Analysis of chromatin fraction proteins by two-dimensional PAGE and immunochemical staining for ubiquitin allows better resolution and increased sensitivity in the detection of ubiquitinated histone species. Analysis of total EDTA solubilized chromatin (total), 150 mM NaCl soluble (soluble) and 150 mM salt insoluble (pellet) fractions of calf thymus chromatin shows that more than one ubiquitin can be attached to histones H2A, H2A.Z and H2B (Figure 31). In the soluble fraction there is an increase in the levels of uH2A, polyubiquitinated H2A, uH2A.Z and most strikingly, uH2B when compared to the levels seen in total chromatin. The levels of ubiquitinated histones in the insoluble (pellet) fraction were similar to those seen in total chromatin.

Chromatin fractions from early stage trout testis obtained by the Sanders (1978) technique were also analyzed using these methods (Figure 32). The levels of uH2A, polyubiquitinated H2A and uH2B in the low salt soluble fraction (SSO.05) are slightly greater than the levels

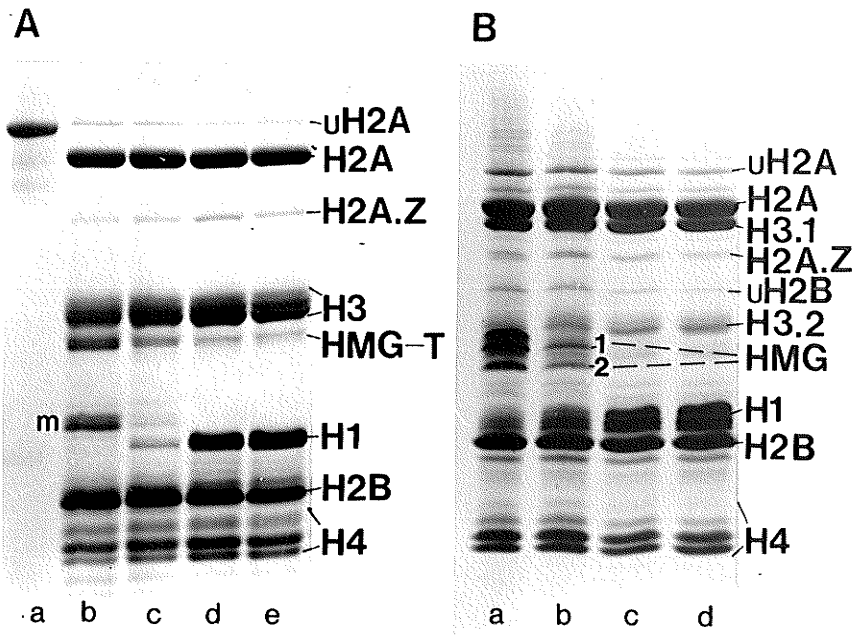

Figure 30. **Low salt soluble, nuclease sensitive chromatin regions are enriched in ubiquitinated histone species.**
A. Acid soluble proteins from trout testis chromatin fractions SSO.05 (b), SSO.10 (c), SSO.20 (d) and SSO.50 (e) and calf thymus uH2A (a) were resolved by AUT 15% PAGE. M is micrococcal nuclease.
B. Acid-extracted histones from bovine thymus chromatin fractions SSO.05 (a), SSO.10 (b), SSO.20 (c), and SS 0.50 (d) were resolved by AUT 15% PAGE. The gels were stained with Coomassie Blue. uH2A and uH2B represent the ubiquitin adducts of H2A and H2B, respectively.

TABLE VII. **The ubiquitinated species of histones H2A and H2B are enriched in the low salt soluble, nuclease sensitive chromatin fractions.**
The content of the ubiquitinated histone species was determined by densitometric scanning of Coomassie Blue stained AUT polyacrylamide gel patterns shown in Figure 29. For each sample the proportion of the histone which was ubiquitinated was calculated as uH2A/(uH2A + H2A). In trout testis chromatin 3.6 ± 0.5% of histone H2A was ubiquitinated and in bovine thymus chromatin, 17.3 ± 1.0% of histone H2A and 5.0 ± 1.0% of histone H2B was ubiquitinated. The levels of the ubiquitinated histone species in each fraction are shown as a ratio of those in total chromatin (e.g. H2A ubiquitinated in SSO.05/ H2A ubiquitinated in total). Data represent mean ± S.E. for at least 3 determinations.

Chromatin fraction	Relative levels of ubiquitinated histones		
	Trout testis	Bovine thymus	
	uH2A	uH2A	uH2B
SSO.05	1.40 ± 0.13	1.36 ± 0.03	1.69 ± 0.18
SSO.10	1.14 ± 0.15	1.05 ± 0.09	1.36 ± 0.03
SSO.20	0.66 ± 0.28	1.05 ± 0.05	1.00 ± 0.34
SSO.50	0.62 ± 0.22	0.80 ± 0.07	0.65 ± 0.24

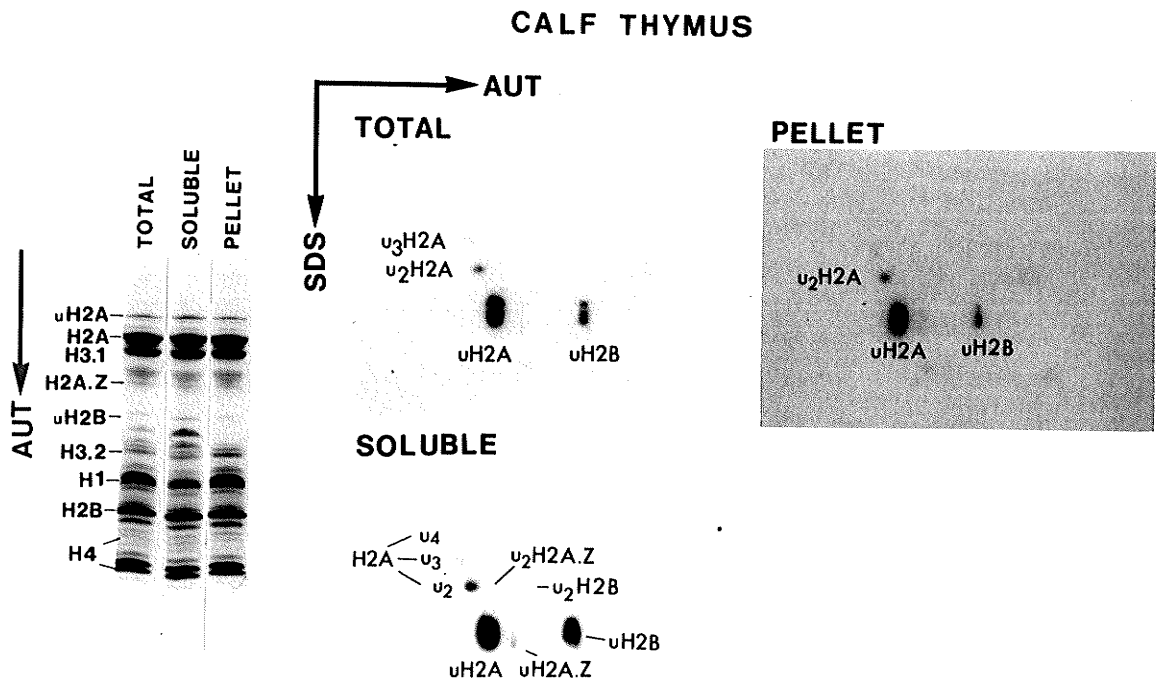

Figure 31. **Ubiquitinated histone species are enriched in the salt soluble fraction of calf thymus chromatin.**
Chromosomal proteins from EDTA solubilized chromatin (total), 150 mM NaCl soluble (soluble) and 150 mM NaCl insoluble (pellet) fractions were analyzed by AUT 15% PAGE and as described in Figure 24. The distribution of A_{260}-absorbing material was 77.0% in the EDTA solubilized fraction (total), 26.7% in the soluble fraction and 50.4% in the pellet.

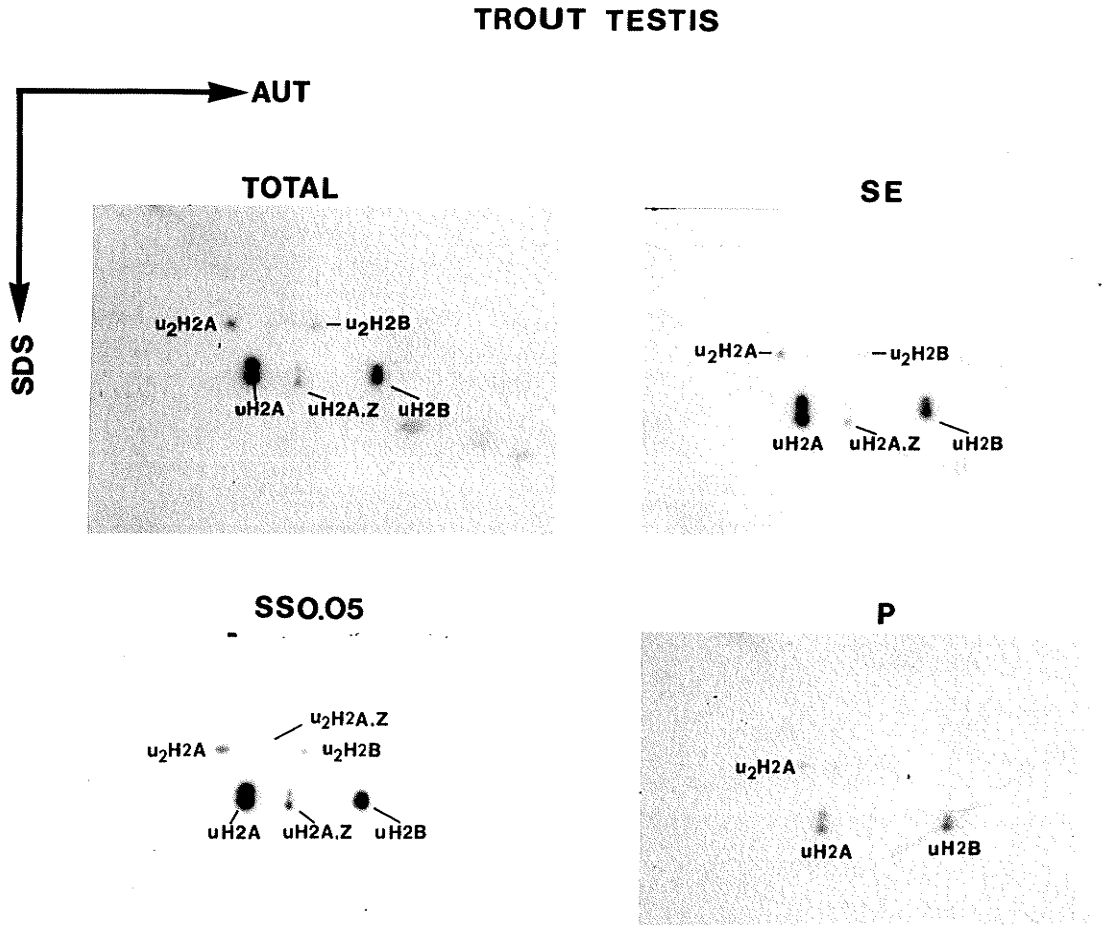

Figure 32. **Ubiquitinated histone species are enriched in the low salt soluble fraction of trout testis chromatin.**
Chromosomal proteins from total, low salt soluble (SSO.05), EDTA soluble (SE) and EDTA insoluble (P) fractions were analyzed as described in Figure 24. Figure 12 shows the same samples following AUT 15% PAGE. Protein loads were adjusted as described in "Experimental procedures" prior to two-dimensional PAGE. The distribution of A_{260}-absorbing material was 10.7% in SSO.05, 41.4% in SE and 35.1% in P.

seen in total chromatin, SE and pellet fractions. The level of uH2A.Z is increased in the SSO.05 fraction compared to the total, SE and pellet fractions. Differences in levels of ubiquitinated histone species between chromatin fractions of trout testis are not as great as those seen in calf thymus chromatin fractions. This may be due to the low level of histone ubiquitination seen in trout testis. In trout testis chromatin 3.6 ± 0.5% of H2A was ubiquitinated compared to bovine thymus chromatin where 17.3 ± 1.0% of H2A and 5.0 ± 1.0% of H2B was ubiquitinated (Table VII).

Fractionation of mature chicken erythrocyte chromatin using the Sanders (1978) technique enriches for the transcriptionally competent β-globin gene in the low salt eluted fraction, SSO.1 (similar to Ridsdale and Davie, 1987a). Analysis of the ubiquitinated proteins associated with the different fractions showed an increase in the levels of ubiquitinated histone species (uH2A, uH2B and poly-ubiquitinated H2A and H2B) in the SSO.1 fraction (Figure 33). Mature chicken erythrocyte chromatin fractionated by the procedure of Ridsdale and Davie (1987b) results in a salt soluble polynucleosome fraction that contains less than 1% of the total genomic DNA, yet is enriched 50 fold in transcriptionally competent β-globin sequences. Analysis of ubiquitinated histones from total EDTA solubilized chromatin, salt soluble polynucleosomes and salt soluble mononucleosomes shows that the polynucleosomes are highly enriched in uH2A, polyubiquitinated H2A, uH2A.Z and uH2B when compared to the mononucleosomes (Figure 34). Comparison of ubiquitinated histones in polynucleosomes to total chromatin revealed an increase in the levels of uH2A and poly-

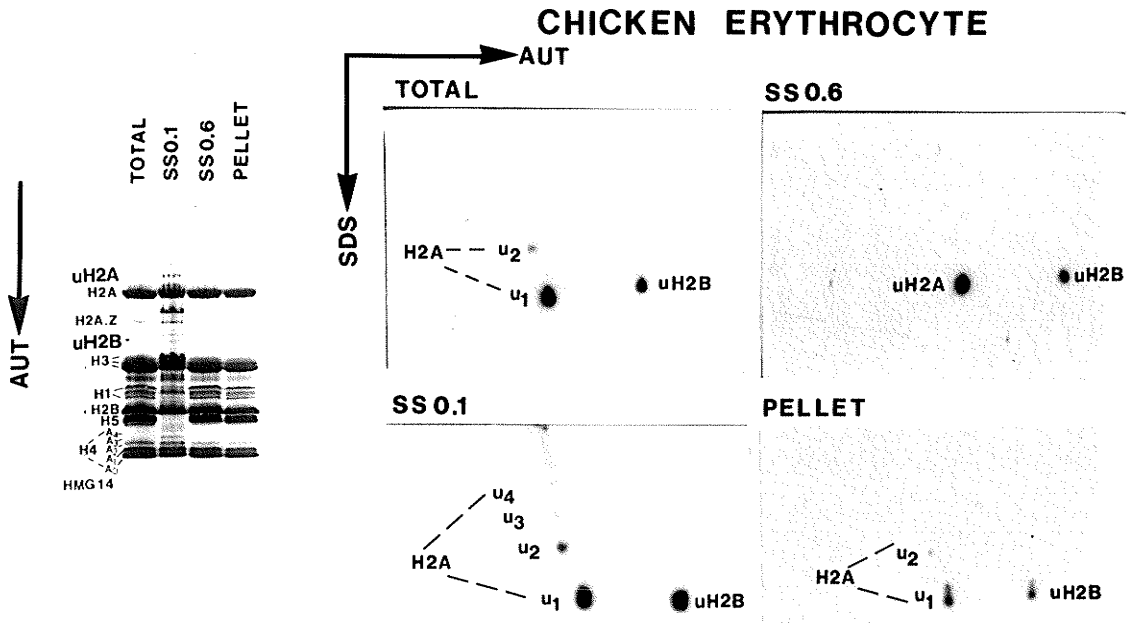

Figure 33. **Ubiquitinated histone species are enriched in the low salt soluble fraction of chicken erythrocytes.**
Chromosomal proteins from total, low salt soluble (SS0.1), high salt soluble (SS0.6) and insoluble (P) fractions were analyzed by AUT 15% PAGE and as described in Figure 24. The distribution of A_{260}-absorbing material was 5.0% in SS0.1, 71.2% in SS0.6 and 18.9% in P.

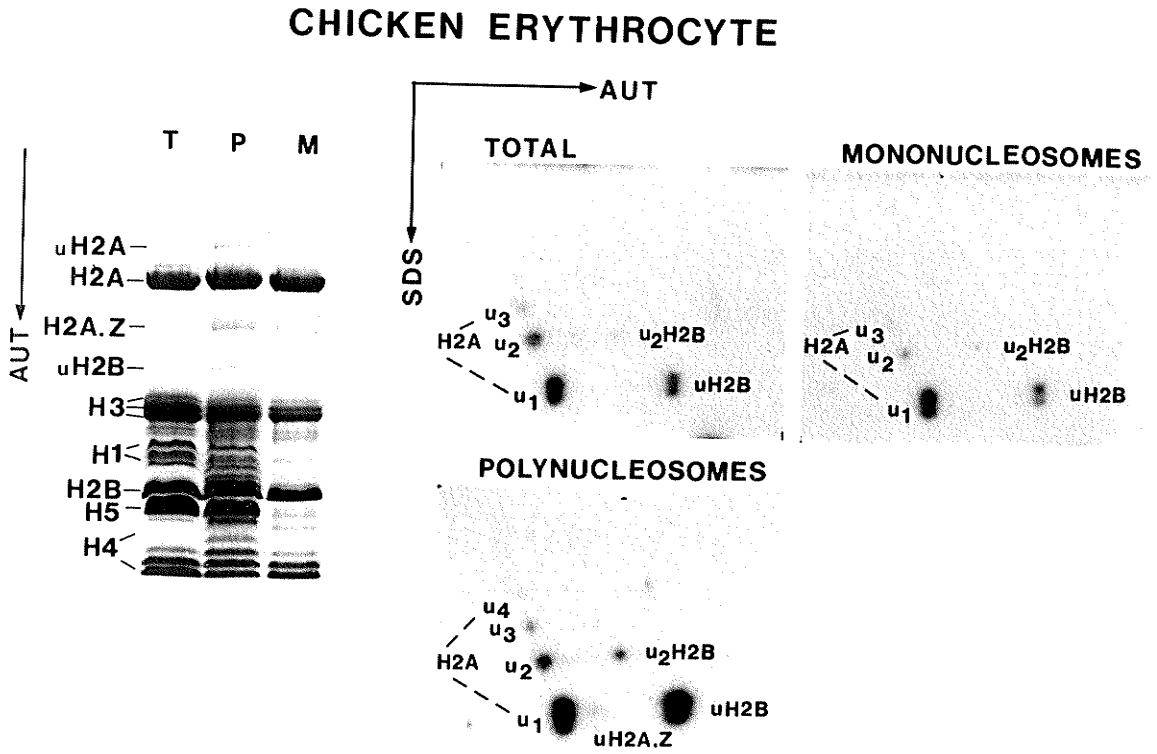

Figure 34. **Ubiquitinated histone species are enriched in the polynucleosome size fraction of salt soluble mature chicken erythrocyte chromatin.**
Chromosomal proteins from EDTA solubilized chromatin (T or total), salt soluble polynucleosomes (P or polynucleosomes) and salt soluble mononucleosomes (M or mononucleosomes) were analyzed by AUT 15% PAGE and as described in Figure 24.

ubiquitinated H2A, but most striking was the increase in levels of uH2B and u_2H2B (Figure 34).

Analysis of chromosomal proteins from the transcriptionally active macronuclei and transcriptionally inactive micronuclei of Tetrahymena demonstrated that polyubiquitinated H2A is present in both types of nuclei, but the level is greater in macronuclei (Figure 35). The level of ubiquitinated H2B species associated with macronuclei is much greater than that associated with micronuclei.

c. Polyubiquitinated histones and proteolysis

It has been suggested that the striking changes observed in chromatin at very high rates of transcription may be due to the proteolytic removal of nucleosomal proteins from the activated chromosomal region and that ubiquitin "tags" the target proteins for degradation (Varshavsky et al., 1983). It is uncertain if the arrangement of additional ubiquitin molecules on histone H2A differs from the arrangement of multiple ubiquitin molecules on proteins destined for degradation via the ATP-dependent non-lysosomal proteolytic pathway found in the cytoplasm. A number of authors have suggested that polyubiquitination of proteins for degradation occurs at multiple sites along the molecule (Hershko et al., 1980; Bachmair et al., 1986; Kanda et al., 1986). Characterization of in vitro synthesized polyubiquitinated lysozyme showed that ubiquitination occurred along the lysozyme molecule as well as in a ubiquitin to ubiquitin arrangement (Hershko and Heller, 1985). If there is a difference in the pattern of ubiquitination between cytoplasmic

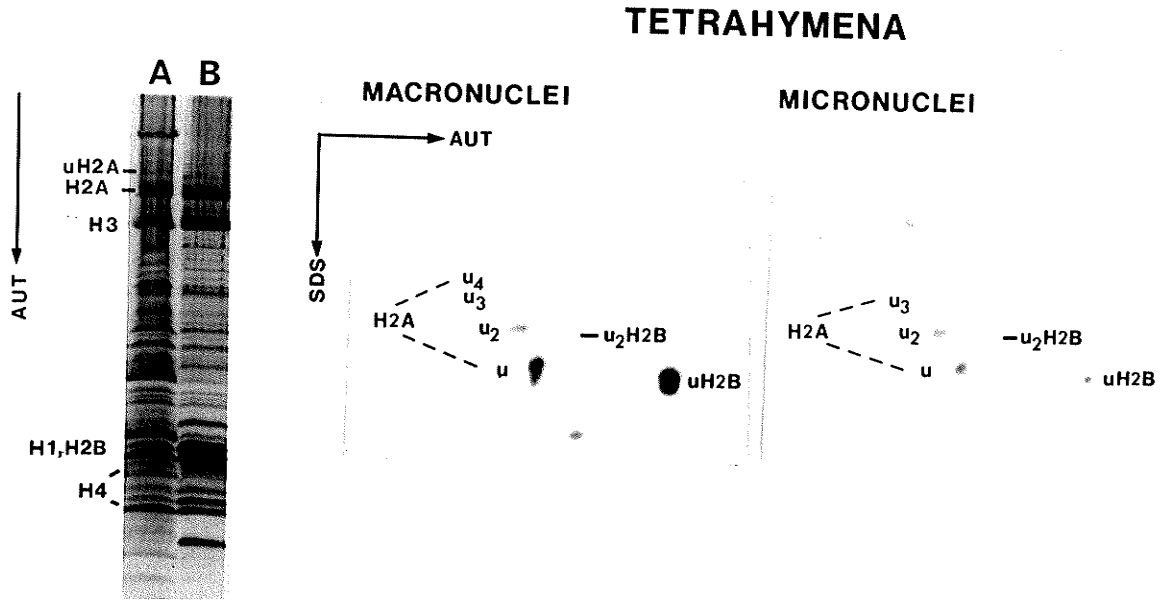

Figure 35. **Ubiquitinated histone species are enriched in the transcriptionally active macronuclei of Tetrahymena.**
Chromosomal proteins from Tetrahymena macronuclei and micronuclei were analyzed by AUT 15% PAGE (lane A, micronuclei, lane B, macronuclei) and as described in Figure 24.

proteins and chromatin, it may be functionally significant. The turnover of ubiquitin conjugates has been studied in intact cells and some ubiquitin conjugates are relatively stable (Hershko et al.,1982). Perhaps this stable population of ubiquitin conjugates differs in the pattern of ubiquitination and involves some other function than protein degradation. Recent evidence that transcription elongation proceeds through the nucleosome core without changing the position of the histone octamer (De Bernardin et al., 1986, Losa and Brown, 1987) suggests that ubiquitin may function by altering chromatin structure of active genes rather than "tagging" the histones for proteolytic removal.

When the molecular weights of lysozyme with up to five molecules of ubiquitin attached were estimated by SDS PAGE, the observed values in the case of lysozyme with two and five ubiquitins attached were less (about 4 kDa) than the calculated values (Hershko et al., 1980). The authors suggest this is due to proteolysis. When the log molecular weight of ubiquitinated H2A species from chicken erythrocytes (up to six ubiquitin attached) is plotted vs the mobility on SDS 15% PAGE gels, the points lie on a straight line (Figure 36). This suggests that the H2A molecule is not being degraded and argues against polyubiquitination of histones being a signal for their degradation.

d. Summary

A seven residue fragment of histone H2A containing two lysine residues (positions 118 and 119) can be conjugated to at least three ubiquitin molecules. It is likely that due to steric hindrance that

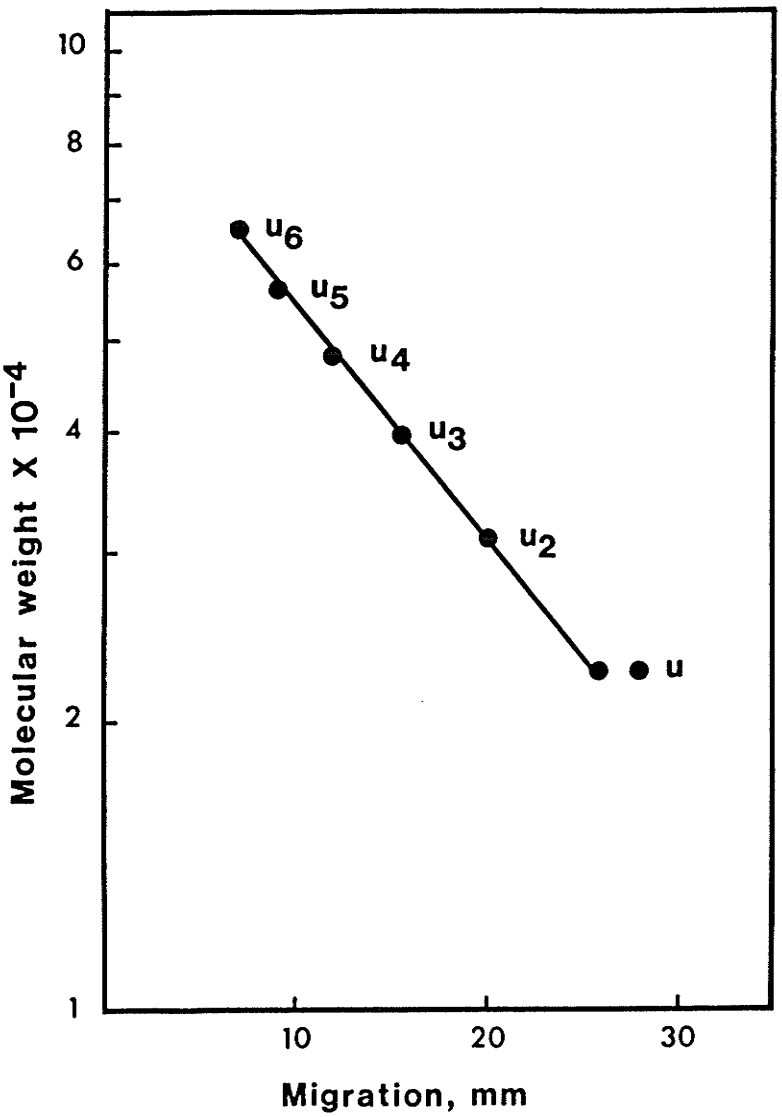

Figure 36. Estimated molecular weights of polyubiquitinated H2A vs migration distance in mm.
Ubiquitinated histones from the salt soluble, polynucleosome fraction of mature chicken erythrocyte chromatin were analyzed as described in Figure 24. The molecular weight of histone H2A was assumed to be 14 kDa. Each ubiquitin moiety would increase this molecular weight by 8.5 kDa. Monoubiquitinated H2A gives two species on SDS 15% PAGE.

only lysine 119 is used to attach ubiquitin to histone H2A and additional ubiquitins are attached through lysine residues of the first ubiquitin. It is unclear whether this pattern of polyubiquitination differs from that of cytoplasmic proteins destined for degradation via the ATP-dependent non-lysosomal pathway. An increase in the levels of uH2A, uH2A.Z and polyubiquitinated H2A is associated with transcriptionally active/competent fractions of chromatin. However, the most dramatic difference between chromatin fractions is the increased level of uH2B found in fractions enriched in transcriptionally active/competent genes.

IV. CONCLUDING REMARKS

In eukaryotic cells, the conversion of transcriptionally inactive chromatin to a transcriptionally active state requires at least two steps. The first step is the generation of transcriptionally "poised" or competent chromatin, that is, chromatin capable of being transcribed. The second step involves the actual induction and regulation of expression of potentially active genes. Both steps are believed to involve the interaction of trans-acting factors and cis-acting DNA sequences. In a review on the structure of active chromatin Pederson et al. (1986) have suggested that there are at least two families of trans-acting factors involved in nuclease hypersensitive regions. One family consists of proteins that create a nuclease hypersensitive region upon binding, while the second family consists of proteins that bind only at preexisting nuclease hypersensitive regions. The protein-DNA complexes that generate nuclease hypersensitive sites are sometimes organized at or near the bases of chromosomal loops and can direct an entire linked domain to form a transcriptionally poised state (Gross and Garrard, 1987). Once chromatin is in the transcriptionally poised state it does not require the continued interaction between trans-acting factors and cis-acting DNA sequences for maintenance or propagation. Specific histone variants, a depletion in histone H1 and increased levels of HMGs 14 and 17 may be responsible for maintaining the poised state of chromatin. Certain post-translational modifications such as acetylation and ubiquitination are also correlated with transcriptionally active chromatin. It is not known if the open structure of active chromatin increases the

accessibility of the histones to the enzymes responsible for these modifications or if these modifications may play a role in maintaining the open structure of active chromatin. Changes in the domain structure of chromatin generated by the binding of trans-acting factors, may increase the accessibility of histones to the enzymes responsible for acetylation and ubiquitination. These modifications may then be responsible for maintaining the poised chromatin state.

I have shown that there is an increase in the levels of uH2A, uH2A.Z, polyubiquitinated H2A and especially uH2B associated with transcriptionally active chromatin. In addition, the arrangement of ubiquitin in polyubiquitinated H2A may differ from that found in cytoplasmic proteins destined for degradation. This arrangement may be functionally significant and polyubiquitination of histones may serve to alter chromatin structure. Chromatin structure can be altered at the level of the nucleosome, as well as at the level of higher order chromatin structure.

In nucleosome core particles, the N-terminal and C-terminal tails of the core histones are not strongly protected from reductive methylation which suggests they are not involved in DNA binding (Lambert and Thomas 1986). Nuclear magnetic resonance studies on histone and DNA dynamics in nucleosome cores also suggest that the basic residues in the terminal histone sequences are not strongly involved in nucleosome structure, but may instead help to stabilize higher order chromatin structure (Hilliard et al., 1986). Modification of histones H2A and H2B by ubiquitination within the C-terminal regions of these molecules may, however, influence the binding of DNA to the

histone octamer. Oliva et al. (1987) have observed by electron microscopy that nucleosomes containing hyperacetylated histones are more elongated in shape than control nucleosomes. Therefore, it is possible for histone modifications to alter histone-DNA interactions and nucleosome structure.

In nucleosome cores the C-terminal sequence of histone H2A is accessible to proteolytic enzymes while the C-terminal sequence of histone H2B is not (Hatch et al., 1983; Rosenberg et al., 1986) . The same accessibility is expected with respect to the enzymes catalyzing the addition and removal of ubiquitin. The increased accessibility of the C-terminal tail of histone H2A compared to the relative inaccessibility of the C-terminal portion of histone H2B may explain why H2A can be ubiquitinated to a greater extent than H2B. The elevated levels of uH2B found associated with transcriptionally active chromatin may be the result of an alteration or disruption of nucleosome organization during transcription such that the C-terminal of histone H2B becomes accessible to the modifying enzymes.

The accessible carboxyl terminus of histone H2A is involved in formation of the dimer-tetramer contact interface in the histone octamer (Hatch et al., 1983). At the level of a single nucleosome, the presence of two uH2A molecules does not appear to affect the overall structure (Kleinschmidt and Martinson, 1981). However, the effect of polyubiquitinated H2A and uH2B on the formation of the dimer-tetramer interface is unknown. The disrupted nucleosomal ladder seen when transcriptionally active genes are digested with micrococcal nuclease may be the result of histone hyperacetylation and/or histone

ubiquitination. Fractionation of transcriptionally active chromatin by accessibility of the SH-group of histone H3 provides evidence for an altered nucleosome structure in active chromatin. Nucleosomes with accessible histone H3 SH-groups contain increased levels of hyperacetylated core histones, but it is not known if they also contain elevated levels of uH2B and/or polyubiquitinated H2A.

Histone H1, which is believed to be responsible for both the formation and stabilization of the 30 nm fiber, is depleted in chromatin fractions enriched for transcriptionally active/competent genes. Since detailed folding patterns of H2A and H2B in the nucleosome are not yet determined, the exact positions occupied by the ubiquitin moieties and their influence on the formation of the 30 nm fiber are unknown.

Some of the characteristics associated with active chromatin are also associated with chromatin undergoing replacement of histones with protamines during spermatogenesis. In the late stages of testis development H2A.Z and uH2A.Z disappear. There are two possible explanations for this. First, H2A.Z is associated with transcriptionally active chromatin and the level of transcription in late stage testis is extremely low or second, histones in nucleosomes containing H2A.Z are selectively converted to nuclear protamine. There were only minor changes in the levels of ubiquitinated histones between early and late stage testis. Assuming increased levels of ubiquitinated histones are associated with transcriptionally active chromatin, then their function in spermatogenesis when transcription is low or absent may be to act synergistically with hyperacetylated

histones to aid in the process of protamine replacement.

I have shown that the transcriptionally active protamine genes of developing trout testis are preferentially associated with the insoluble material remaining after fractionation. The presence of the ubiquitinated histones in the pellet fraction following fractionation of trout testis chromatin by the Sanders' (1978) procedure may be the result of two processes, transcription and protamine replacement, occurring at the same time. Both processes are thought to require a disruption of higher order chromatin structure.

In addition to polyubiquitin genes, ubiquitin coding sequences have also been found fused to unrelated "tail" sequences that code for putative metal-binding, nucleic acid-binding domains (Lund et al., 1985; Özkaynak et al., 1987). Özkaynak et al. (1987) have hypothesized that each tail functions as part of the ubiquitin containing fusion protein by binding to specific DNA sites in vivo (trans-acting factors interacting with cis-acting DNA sequences), thereby greatly increasing the concentration of ubiquitin in the vicinity of such sites. The intrinsic proteolytic activity of ubiquitin described by Fried et al. (1987) may be responsible for the release of ubiquitin from the DNA bound fusion protein. This in turn may lead to the ubiquitination of specific sites in the chromatin and an alteration in chromatin structure. In yeast the DNA repair enzyme, RAD6, codes for an enzyme that is specific for conjugating ubiquitin to histones (Jentsch et al., 1987). It is possible that either the same enzyme or a similar one could be responsible for the conjugation of the ubiquitin released from the fusion protein to histones.

Varshavsky et al. (1983) have found that the degree of nucleosome ubiquitination varies within the transcription unit with the 5'-proximal nucleosomes being more heavily ubiquitinated than nucleosomes near the end of the gene. This would agree with the model suggested above where the ubiquitin fusion protein binds to DNA in the promoter region, ubiquitin is removed and then conjugated to the histones in the area, especially histone H2B. Polyubiquitination as well as acetylation may then take place in the same region due to the increased accessibility of the histones to the enzymes responsible for these modifications. The increase in modified histones in this region may be responsible for altering the structure of the 30 nm fiber by disrupting the cooperative binding of histone H1.

V. FUTURE DIRECTIONS

The aim of future experiments should be to define more clearly the role of ubiquitin in chromatin. Presently it is unclear whether histone ubiquitination is the cause or a result of the "open" structure of transcriptionally active chromatin. The results of earlier two-dimensional hybridization mapping experiments demonstrated an association of ubiquitinated histones with transcriptionally active chromatin. These results have recently been questioned by Huang et al. (1986) who have used isopeptidase to show that the altered mobility of nucleosomes containing the transcriptionally active kappa immunoglobulin gene was not due to ubiquitinated histones. I believe it is still possible that transcriptionally active genes are associated with ubiquitinated histones. Histone acetylation can alter the shape of nucleosomes (Oliva et al., 1987) as well as their electrophoretic mobility (Bode et al., 1983; Imai et al., 1986). Ubiquitination and acetylation may act synergistically to alter nucleosomal structure and this altered structure may persist following removal of ubiquitin by isopeptidase. In addition, it has not been shown that treatment with isopeptidase can remove ubiquitin from uH2B and polyubiquitinated H2A. Experiments should be done to determine whether ubiquitin is removed from polyubiquitinated H2A and uH2B by digestion with isopeptidase.

With the development of methods to purify genes as chromatin and direct compositional comparisons, it may be possible to define more clearly the role of ubiquitin in chromatin. It may be possible to determine whether ubiquitinated histones are involved in the generation of transcriptionally poised chromatin or in the switch from

transcriptionally poised to active chromatin, using minichromosomes that contain bovine papilloma virus and a hormone responsive element that functions as a transcriptional enhancer element. These minichromosomes exist as extrachromosomal elements in cultured murine cells and can be purified about 2000 fold while maintaining the native structures of chromatin (Ostrowski, 1987). Since the presence of hormone generates features associated with transcriptionally active chromatin, a comparison of ubiquitinated histones from these minichromosomes in the presence and absence of hormone should provide information on the role of ubiquitin in transcriptionally poised vs transcriptionally active chromatin.

Further experiments to localize uH2B and polyubiquitinated H2A within specific genes may explain the role ubiquitination plays in altering the structure of transcriptionally active chromatin. In the model discussed above histones in the 5' region of an active gene are expected to be more heavily ubiquitinated than those 3'. The exact pattern of ubiquitination may be important functionally. Isolation of nucleosomes containing polyubiquitinated H2A and uH2B and examination of their associated DNA sequences may provide some answers.

VI. LITERATURE CITED

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