ISBN 0-315-54961-0

Canada

THE STRUCTURE OF TRANSCRIPTIONALLY COMPETENT CHROMATIN: THE ROLES OF H1 HISTONES AND ACETYLATION

A Thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

James Andrew Ridsdale

November, 1989.

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ACKNOWLEDGEMENTS

I wish to thank, first and foremost, my advisor for this project, Dr. Jim Davie. His help, in many different forms, and always freely given, was of inestimable value in the execution of this work.

I'd also like to thank those who were my main labmates: Diane, Genevieve, Barb, Darcy, Lissa and Jon. Each of generally cheerful demeanour they made my lab-time much less stressful than it might have been. (I exclude Mike here as he just made it much more interesting).

Drs. Stevens, Matusik, Dakshinamurti and Lewis acted as my advisory committee, and I'd like to thank them for their efforts on my behalf.

I'd like to acknowledge some material contributions to this work: those of Dr. Genevieve Delcuve for making the DNA measurements with diphenylamine, and of Lissa Desbarats for doing most of the work represented in Figures 19 and 20.

Finally I'd like to acknowledge the financial support of The Faculty of Graduate Studies and the Manitoba Health Research Council.

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LIST OF ABBREVIATIONS

AUT	acetic acid/urea/Triton X-100
A260	absorbance at 260 nm
bp	base pairs
CM	carboxymethyl
ddH2O	deionized distilled water
DNase I	deoxyribonuclease I
EDTA	(ethylenedinitrilo) tetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)] tetraacetic acid
EM	electron microscopy
h	hours
HMG	high mobility group
kbp	kilobase pairs
kD	kilodaltons
NP-40	nonidet P-40
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PIPES	piperazine-N, N'-bis (2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
RNase	ribonuclease
s	seconds
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N' -tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

ABSTRACT

Only a small fraction of the genome of multicellular eukaryotic cells is expressed at any time. All of the genome is packaged into chromatin. The distinguishing characteristics of chromatin which is competent for transcription have been subject to extensive investigation. Expressible genes appear to be assembled with structurally altered nucleosomes and in a more open conformation than the bulk of the genome. The H1 (or linker) histones probably have a key role to play in the compaction and stabilization of the transcriptionally repressed state. The work presented here demonstrates that the 0.15 M NaCl-soluble polynucleosomes from mature or immature chicken erythrocyte nuclei are highly enriched in transcriptionally active (e.g., histone H5 and β-globin) and competent (e.g., ϵ-globin) genes. These salt-soluble polynucleosomes are enriched in acetylated species of histones H2B, H2A.Z and H4, poly- and monoubiquitinated species of H2A and H2B, and histone variants H3.3 and H2A.Z. Moreover, these active/competent-gene-enriched chromatin fragments are complexed with linker histones H1 and H5. Reconstitution experiments revealed that active/competent-gene-enriched chromatin fragments are much more resistant than repressed gene chromatin fragments to exogenously-added-linker-histone-induced precipitation in 0.15 M NaCl. These

observations suggest that some feature of the active/competent gene chromatin fragments prevents the linker histones from folding the fibre into a stable higher-order structure. The possible role of acetylation as such a factor was explored by using hyperacetylated and partially deacetylated chromatin. This chromatin was used in fractionation and reconstitution experiments like those described above. The results support the hypothesis that core histone acetylation is a major factor in determining the altered solubility properties of competent-gene-chromatin, and that this occurs by changing the mode of association of the linker histones with the chromatin fibril.

GENERAL INTRODUCTION

As this thesis is the major requirement for the ascension of the candidate to the degree of Doctor of Philosophy, a philosophical introduction does not seem out of place. Not long ago what we now call "science" was "natural philosophy". The major expansion in scientific fact finding over the last number of years has forced the individual scientist to ever increasing degrees of specialization. In my experience some of the more abstract philosophical questions receive very little reflection amongst practising experimental scientists. Therefore, before embarking on the more specific details of the current work I will indulge briefly in some such reflection.

I will first ask the reader's forbearance for the use of the personal pronoun in this section. Understanding that it is considered poor form in scientific writing, I will use it nonetheless as it will make the present bit of writing easier to do.

I feel that some awareness of the philosophical underpinnings and history of scientific thought is important for the practising scientist. This is because, whether the individual scientist is aware of it or not, the questions asked and the answers found are significantly influenced by a world view which itself is the result of the historical evolution of human thought. I hope that what

I mean by this will be made somewhat clearer in what follows.

The various treatises and expositions on the history and philosophy of science are voluminous. It is safe to say that abbreviated generalizations about any particular thinker or era in the history of thought will fail to do them justice. I will merely give a short personal viewpoint of what I feel are some of the more important points.

History

The particular history of Western thought is usually begun with the Hellenistic thinkers of the last few centuries B.C. Of the large variety of ideas which survive from this era those of Aristotle had the greatest influence over European concepts of the nature of things from about the first century B.C. until the 17th century. From the time to the establishment of the Byzantine empire, reflection on Nature was further influenced by Christian theology.

It should be borne in mind that Aristotle developed a remarkably complete and coherent set of explanations for most of the readily observable natural phenomena. For these many centuries most observation and explanation was made to be consistent with the Aristotelian world view. In

this (teleological) world view all natural events were considered to have as their final cause a reflection of the will of the deity.

The decline of Aristotelianism and the rise of the modern world view is referred to as the Scientific Revolution. Its roots can most readily be traced to Galileo (b. 1564), Bacon (b. 1561) and Descartes (b. 1591). Galileo is famous for his astronomical observations which supported the heliocentric cosmology which had been suggested earlier by Copernicus. This work of Galileo is only part of the larger reconceptualization of causation from which purpose is excised. In the new world view the concept of purposefulness is replaced by mechanism. Each event or state of being in the world has an underlying mechanism rather than purpose. The essential reasons for the decline of Aristoltelianism during the scientific revolution is explained (from the Encyclopedia Britannica):

"...Aristotle was condemned because of his unsatisfactory account of local movement, and its consequences in mechanics, dynamics, cosmology and astronomy. His downfall in the 17th century was the result, above all, of his failure to create ... a language that allowed him to describe forms of things and events in precise or approximate mathematical formulas and his failure to lay sufficient stress, in his philosophy of experience, on the need for experiments."

The power of mechanics and mathematics as ways of explaining natural phenomena reaches its earliest important culmination in the work of Newton (who was born the year Galileo died). Newton's success in the development of a mathematical description on a broad range of natural phenomena led Laplace to make his famous comment that, given sufficient knowledge of the state of the universe, he could predict its entire future course.

Philosophy

I will turn now to a brief epistemological reflection. Epistemology deals with questions about the nature of knowledge: What constitutes knowledge?; Are there different types of knowledge?; How does knowledge progress?; and so on.

David Hume (b. 1711) made a central and important contribution to epistemology by the application of determined scepticism. In dealing with questions such as "How do I know that my existence is not merely the dream of an evil genie?" (the starting point of Cartesian philosophy), Hume would reply that one cannot, in fact, know about such things in the same way that you may know that, given a certain set of axioms, a certain other set of statements is true (as in mathematics). He suggested that pure reason cannot be a guide to judgements about matters of fact without appeal to the senses and observation.

Hume argues that observation must be the basis for knowledge. Pure reason is too imperfect to be, alone, a guide toward better understanding.

These ideas have been extended in this century to become an important part of the philosophy of science. A large number of different ideas form the basis of what is generally referred to as positivism. A positivist response to a question like that above, about existence being the

dream of a genie, would be that such a question is meaningless. Any assertion must be considered meaningless without some reasonable way of demonstrating its truth by its relation to empirical observables. I may be the dream of an evil genie, or I may have been created a few seconds ago with all my memories as they are; but without some way of indicating that this is or is not the case, then the problem is meaningless (or at least unscientific). This is a metaphysical problem (meta, meaning beyond or beside). Scientific problems, on the other hand, are physical problems.

This pragmatic theory of meaning and its various offshoots might be fairly considered to be the current status of the modern scientific world view. This is one in which, in general, empirical observations are considered to be of greater value than theories.

The major criticism of this point of view is that it does not do away with the theory dependence of observation, especially when dealing with complex phenomena. The types of measurements scientists make is dependent on their conception of the problems to be dealt with. An experimental scientist chooses to make a measurement based on speculation, and his or her conceptual understanding of a large body of other data. A "true fact" in isolation from the increasingly complex conceptualizations about the natural world, again, really has no meaning.

The large scale concepts or patterns of thinking about natural phenomena (commonly called paradigms) may not be "wrong", so to speak, but they may be incomplete. A well known example of this occurred with the development of relativistic physics. Until the turn of this century a Euclidian geometry of space was assumed because it is intuitively obvious. Experimental science based on the Euclidian-geometry assumption could be correctly executed, but could not contribute much to an increased understanding because that understanding only came with an abandonment of tacit assumptions and the development of a more useful mathematical language. So much of the experimental data generated within a paradigm does not contribute to increased understanding (although it may contribute to an increased well being for society at large).

It is through these sorts of criticisms and understanding of its history that science has come to be increasingly seen as a social process where major conceptual patterns are shifted by a sort of mass action process amongst the scientific community.

Mechanistic explanation and mathematics

Bearing Hume in mind, Kant asked: "How are a priori synthetic judgements possible?" (How can we make judgements about matters of fact without prior knowledge of them?) At

least some of the judgements Kant discussed in this context were of the sort: two different objects cannot occupy the same space, two events cannot be both sequential and simultaneous etc. These judgements that are axiomatic (or a priori) to at least some aspects human understanding are, I think, what would be commonly called physical intuition. These are pre - rational sensations about the physical world that develop in the brain of the infant interacting with its environment.

In a less abstract sense, it could be said that the young human **understands** many basic physical concepts (eg. objects are made of matter and have mass, mass under the influence of gravity has potential energy etc.) well before he or she is able to speak.

These concepts such as number, mass and energy are basic to the physical sciences. Physical intuition underlies a mathematical description of nature and forms the basis of the most epistemologically solid aspect of modern science. The reason for this is that almost every human (and many other creatures for that matter) **knows** what mass, time etc. are without ever having been taught.

These mathematical descriptions and explanations of natural phenomena are formal. That is, mathematics is the way of describing the form of the relationships which are physically intuitive. If I were to write f = ma, for example, I give a succinct formal description by which I

mean: If someone holds a mass above the earth, for example, a force must be applied to maintain its position. If the object is released, it will accelerate towards the earth. The force necessary to suspend the object is dependent on the extent of mass (a measurable quantity) and the rate of acceleration (the second derivative of space with respect to time) it experiences when released.

Using such formal mathematical descriptions scientists have generated statements about the world that are counter-intuitive, or, at least, challenge our intuitive grasp of the world. A famous example follows from Einstein's special theory of relativity that, contrary to the Kantian a priori judgement, two events may be either simultaneous or sequential depending on the frame of reference from which they are observed. The counter-intuitive nature of some of Einstein's conclusions is the result of the application of mathematical analysis to the results of instrumental observation (Einstein, 1953). In the end, however, it is mathematics which is the most important element in increasing scientific understanding. Starting with observations, mathematics can lead (to important new understanding, for example) where physical intuition alone is not able to follow.

Physical and biological explanation in biochemistry and molecular biology.

In biology explanation is basically functional: observable phenomena are related directly or indirectly to the conferring of selective advantage. The theory of evolution through natural selection is the cornerstone of all biology. Suppose we ask a question: why are certain amino acids in certain configurations in the haemoglobin molecule? The answer is: because this gives the haemoglobin molecule its particular affinity for oxygen, because oxygen must be carried to peripheral tissues, and so on. Finally we reach the ultimate biological explanation: because it helps to maximize the reproductive potential of the individual.

In a sense contemporary biochemistry and related areas are in a peculiar state in terms of using explanation that often lies between physical and biological (formal and functional) types of explanation. The paradigm of organism or cell as machine (mechanical) is well established and viable. At the same time almost all observations in this field are related to function. That is to say, sense is made of biochemical data by relating it (often in a very indirect route - as illustrated in the above example about haemoglobin) to the benefits derived in terms of increased fitness. "What is the physiological significance? " or "What does this mean in terms of cell function?" are the sorts of questions most often asked in order to gain a

perspective on the **meaning** of data coming from biochemical studies.

In molecular cell biology function is assigned to molecules; and the macroscopic behaviour of a cell is thought to be the result of a complex network of **functional** interactions amongst these molecules. DNA is often considered to be the "key" molecular type. This is because it is the "key" factor determining the heritability of traits, which is itself basic to the mechanism of natural selection.

The **physical** state of individual molecules is often considered to be sufficiently independent of each other that functional interactions are predominantly diffusion limited. But, what is the physical state of interaction amongst molecules within the cell? and what are the likely consequences of that sort of interaction? These questions are part of another question which could be stated more generally: How do we relate the microscopic (molecular level) properties of a system with its large scale macroscopic properties (eg. the behaviour of cells)?

One of the most influential contributions toward this goal came from Ludwig Boltzman's derivation of some of the basic ideas of thermodynamics. Boltzman used a mechanical description of interacting molecules and derived a powerful description of the macroscopic properties of systems in general: the second law of thermodynamics. When modelling

interactions amongst molecules as collisions between hard spheres, a probabilistic element was assumed and introduced into the mathematical function describing the spatial and temporal evolution of particle distribution. The assumption was that if molecules are jostling each other around like so many billiard balls; and there are a very large number of them, then the occupation of any appropriately chosen spatial element by a particle will be random. It is from this assumption that the concepts of the H quantity and entropy arise (A much more complete discussion of this, and related points is in Prigogine, 1980).

The hard sphere model of molecular interactions has proven to be very effective in describing a broad range of physical phenomena. Many other systems deviate from ideal hard sphere behaviour. In fact, the physically intuitive model of the predominance of elastic collisions amongst molecules leads to the second law (as discussed above), that the entropy of an isolated system increases with time. There are, however, very many macroscopic systems, including living cells, which seem to deviate from this law.

I think that this point is very important: **the second law is a necessary consequence of a system which consists (microscopically) of predominately hard sphere type interactions.** This leads to a well known dilemma in

science: if the second law is all pervasive how can we explain so much of the apparent regularity in the universe? How can blind forces lead to such intricate order?

These questions are beginning to find answers. Very many formerly diverse areas of study are finding varying degrees of interrelatedness under what is generally considered a sub-discipline of the physical sciences known as "Complex systems". The fact is, many natural phenomena are not well described by hard sphere interactions. In the more modern mathematical descriptions used by physicists today "broken symmetries" and "instabilities" play an important role where, at some level of description, some of the statistical "rules", such as those by derived Boltzman in his work, do not apply.

In abstract complex systems a few simple elements of the microscopic properties of the system make possible the emergence of macroscopic organizational properties. In this way order emerges from chaos: the complexity and creativity of the universe are consequences of blind forces only.

I think that for cell biologists there are important insights provided by the work of physicists that will help give a more sophisticated intuitive grasp of the kind of object a cell is; especially in the ideas that there are certain organizational properties that are universal to systems with a large number of coupled degrees of freedom.

I am thinking in particular of Kenneth Wilson's use of the renormalization group (Wilson, 1983) and the recent discussion by Rammal et al. (1986) about the spin glasses. In both these cases a very simple model system whose basic features can be easily understood, is found to have complex, hierarchical structural integration which is a natural property (follows as a mathematically necessary consequence) of the model itself. I think it would be fair to call this organizational structure an emergent property of the system. That is to say, a property that is not predictable from a more complete understanding of the nature of the subunits of the system. The greatest value of this work, for me, is that it gives some physical meaning to the way in which microscopic properties are related to emergent macroscopic ones.

As I understand it, the mathematical problem posed by the renormalization group transformations is to find values for a set of parameters and recursion relations amongst them that are stable under reiteration. The result is to find a functional (in the purely mathematical sense of the word) relationship between the physical properties of the system on one scale and that of another that does not vary with scale. The system thus characterized is fractal, where density fluctuates on all scales, but the relationship of the observable density on different scales is constrained by the fractal dimension. Understanding how

and why such a mathematically derived description relates to what occurs in real systems is a challenge to physical intuition.

For the most part the implications of such ideas are not fully incorporated as a motivation for research in the current practice of cell biology. One implication is, of course, that certain functionally important processes occur via molecular interactions which are **necessarily not observable at some intermediate level of structural integration.** We know that the cell is not merely a bag of enzymes, but a more precise idea of exactly what it is will probably require more detailed observation of its behaviour as an intact entity.

What is most important, in underpinning biochemical science, is functional explanation. Clearly, a cell must grow, divide, etc.; and each functional process, usually, has a physical basis in specific macromolecules. Most biochemical phenomena are interrelated by explanations which deal directly or indirectly with what are understood to be necessary life processes. A problem with functional explanations is that they are human rationalizations and necessarily reflect the conceptual limitations of those who generate them. It may be necessary for cells to grow and divide; but is it necessary, for example, that each chemical reaction that occurs in cells have a distinct protein catalyst?

Perhaps I can illustrate what I mean by looking at the idea of artifact. Any preparations of cells or cellular material are by definition artifacts, that is, something which is made or constructed. In cell biology the word is more generally used to mean those preparations that do not reflect the true state of the object of study. Such a definition of truth, however, is highly dependent on the current opinion and hypothesis.

Although a good deal of valuable understanding has, and will continue to come out of biochemical approaches, I do not feel that it is premature to consider integrating some of what is already known in order to generate a more coherent picture of what a cell is like. In order to do so, however, we will have to appeal to the physical intuition provided by consideration of the qualities of abstract complex systems.

CHROMATIN FUNCTION

In the general introduction it has been argued that the assignment of function to some structure or property of a biological system must appeal, ultimately, to the selective pressure which gave rise to that structure or property. The highly conserved nature of histone proteins suggests that the nucleosome may be the most conserved of all stable macromolecular complexes. This means that the function of the nucleosome is probably essential to cellular survival and that only small variations in structure can be tolerated in order for that function to be carried out.

It has been suggested that the primary function of the nucleosome is to allow for the orderly compaction of the genome. This is necessary because of the enormous length of the polymeric DNA molecules which make up the genome. These DNA molecules may be in the tens of centimetres in length, and though only about 1 nm in diameter, they must be packaged into a nucleus which is a few microns in diameter. While allowing for this orderly compaction, the nucleosome must also allow for ready access to the DNA for replication and transcription to occur. In this way the nucleosome would be the product of two simultaneous and, in a sense, opposite selection pressures: one to keep the DNA packaged and one to allow it to be readily opened up again.

The nucleosome may perform these functions by playing an architectural role: the orderly folding of DNA follows as a consequence of the trajectories that are possible for it when complexed with nucleosomes.

The foregoing argument assumes that there has also been some selection pressure which gave rise to increasing genome sizes. Although there may not be an obvious reason for this to occur the fact remains that the transition from the prokaryotic to eukaryotic cell form was accompanied by a significant increase in average genome size so that eukaryotic genomes are about 3 orders of magnitude larger than the average prokaryotic genomes in terms of DNA content.

CHROMATIN STRUCTURE

The areas of chromatin structure and those structural differences which characterize transcriptionally active or competent chromatin have been thoroughly reviewed (e.g. Pederson et al., 1986; Reeves, R., 1984). The purpose here, then, is not to provide a literature review, per se, but rather, to give a critical overview and assessment of some of the main trends in the field.

The looped domain and replication-expression models are discussed at some length. This is because these models explain the role of chromatin structure in the regulation of gene expression and give a mechanism by which different chromatin structures arise.

Chromatin structure: histones and DNA

The DNA of eukaryotic cells is complexed with an almost equal mass of protein. The greatest bulk of these proteins are the histones. The histones are all small basic proteins.

The histones, together with the DNA form the nucleosome. The nucleosome is the basic repeating subunit of chromatin structure. It consists of two each of the histones H2A, H2B, H3 and H4. This octameric complex forms the core of the nucleosome, and 146 bp of the DNA is bound

radially to the outside of this core. The continuous DNA strand links the nucleosomes together giving this basic form of chromatin a "beads on a string" structure which can be seen in electron micrographs of native chromatin prepared under low ionic strength conditions. The linker DNA can vary in length between and even within tissues. Linker lengths are, on average, around 30 bp, but different lengths up to around 80 bp can occur (as in sea urchin sperm) (Wu et al. 1986).

The H1 class of histones (which includes H5) are thought to interact with the linker DNA exiting the nucleosome. For this reason these histones are also called linker histones.

Histone structure

Sequence analysis of the core histones from various species, ranging form yeast to higher plants and mammals, indicate that they are amongst the most highly conserved of all proteins. The degree of conservation is such that H4 > H3 > H2B > H2A. All the core histones except H4 have variant forms. These variants are products of different genes. It should be borne in mind that complete consistency in the nomenclature of the variants between species and between different laboratories working on them has not been achieved (Wu et al., 1986). A schematic

drawing showing the sequences of each histone class is shown in Figure 1. A general structural feature of the core histones is a high concentration of basic residues near the amino terminus.

H4 and H3 are arginine rich histones. H4 is 102 amino acids in length and H3 is 135 amino acids in length. The variants of H3 are called H3.1, H3.2 and H3.3. The mass content of each of these H3 variants, as with other histone variants, differs according to cell and tissue types, as well as in cells during functional changes. For example, there is a relative increase of the H3.2 and H3.1 variants during the activation of human lymphocytes (Wu et al., 1983). Furthermore, the relative levels of expression of the variants change during the stages of the cell cycle (Wu and Bonner, 1981).

The H2 histones contain relatively more lysine compared to H3 and H4. H2B contains 125 amino acids and H2A 128 or 129, depending on the species, but variant forms may contain slightly more or fewer amino acids. The most common H2B is called H2B.1 and the main variant is H2B.2.

H2A is the most evolutionarily divergent core histone and also shows the greatest number of variants. The most common forms of H2A are called H2A.1 and H2A.2. A fairly extreme variant form, which makes up between 5 and 20 percent of the vertebrate H2A, is H2A.Z. This variant, when described in the chicken, has also been called H2A.F.

The H1 (or linker) histones are found in all eukaryotes with the exception of yeast. Most vertebrates studied have three or four main forms of H1, which are found in different levels in different tissues. These molecules are of about 220 amino acids in length. Their structure consists of extended tail regions at both the carboxyl and amino termini, and a globular central region. The most extreme variant of the H1 class is called H5 and is limited to nucleated erythrocytes. H5 will be discussed at greater length below.

In most cases the synthesis of the histones is tightly coupled to the DNA replication phase of the cell cycle (S phase). The synthesis of some histone variants has been found not to be coupled to the cell cycle; but, instead, these variants are synthesised at a basal rate throughout the cell cycle. In Chinese hamster ovary cells, for example, H3.3 and H2A.Z are two such basally synthesised histone variants (Wu et al., 1982).

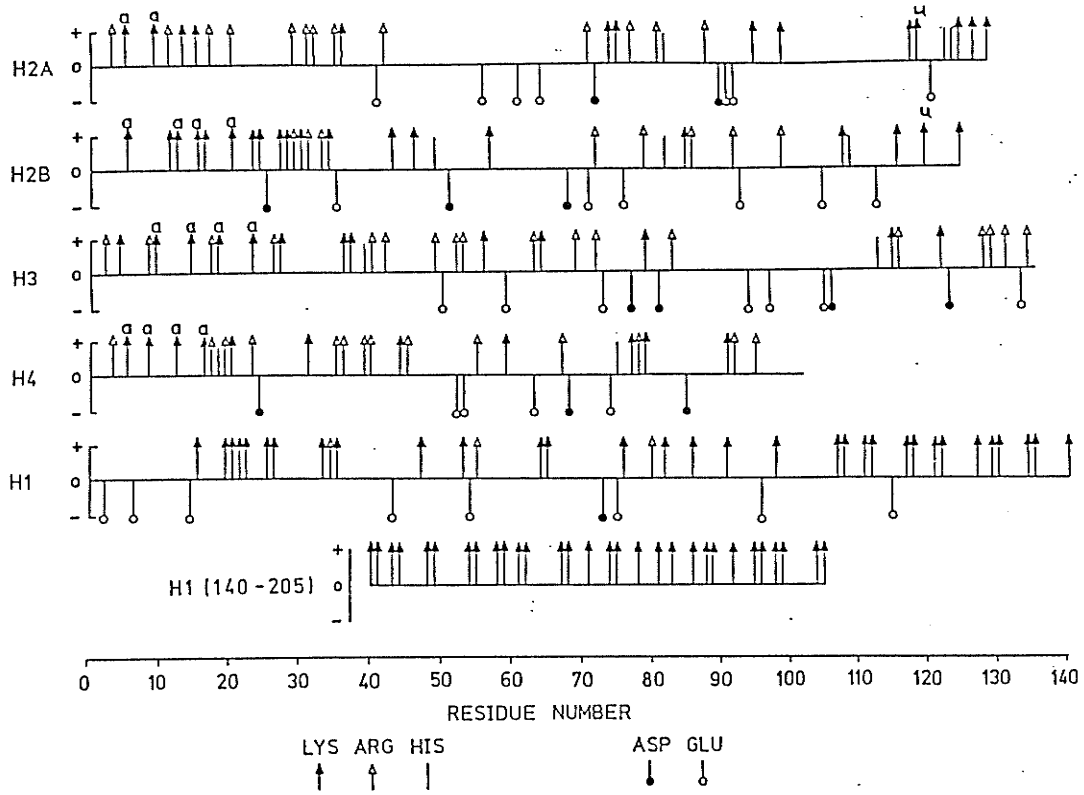

Figure 1

Schematic drawing of the sequences of the bovine histones. The positions of positive charge bearing residues are shown above the line and negative charge bearing residues below. a: indicates the sites of side chain acetylation; u: indicates the sites of ubiquitin attachment.

Core particle structure

The nucleosome makes up the basic repeating subunit of chromatin. Nucleosomes may be prepared from chromatin by digestion with micrococcal nuclease, which has a much greater preference for linker DNA over that bound by the histones. Amongst the prominant DNA products of a micrococcal nuclease digestion of chromatin is a fragment about 166 bp in length. This particle is called the chromatosome, and is thought to include the DNA necessary for the binding of H1 histones to the outside of the nucleosome core. The nucleosome core particle itself may be operationally defined as the main product of limiting micrococcal nuclease digestion of chromatin from which the H1 histones have been removed. Preparations of these particles from chicken erythrocytes have produced crystals such that a structure has been determined by X - ray crystallography at 7 Å resolution (Richmond et al. 1984). The DNA forms a left-handed solenoid (single start helix) around the outside of the octamer. The DNA path is clearly defined in this structure. Interestingly, this path is not continuously smooth, but is kinked in four places. At this resolution, the electron densities that arise from the individual histones of the octameric core cannot be distinguished. The structure is disc-shaped and is about 5.7 nm in height and 11.0 nm in diameter.

The histone octamer itself appears to be a tripartite structure consisting of an $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers. This model is supported, for the most part, by various studies carried out by the Moudrianakis group (eg., Eickbush and Moudrianakis, 1978). In 1985 this group published a structure for the octamer, without DNA, established from X - ray crystallographic data at 3.3 Å resolution (Burlingame et al., 1985). This structure was somewhat bigger than that of Richmond et al. (1984). Most of the increased volume in the octamer structure is due to a solvent channel which lies between the central $(H3\text{-}H4)_2$ tetramer and the flanking H2A-H2B dimers. Neither of the solved structures is of sufficient resolution to define the path of polypeptide backbones or assign regions corresponding to known sequence elements for any but very limited regions of the structure.

Figure 2 is a schematic diagram of the basic shape of the two structures. Note that the structure of Burlingame et al. (1985) is considerably elongated compared to the Richmond et al. (1984) structure. Although the Burlingame et al. (1985) structure does not include DNA, it is reasonable to suggest that the DNA would be situated on the ridges of the core as it is in the other structure. Such a placement of the DNA would probably lead to diverging trajectories of the DNA helical axes exiting the nucleosome compared to converging trajectories in the Richmond et al.

(1984) structure.

The problem of the variant structures is probably adequately resolved. Part of the explanation may be that the core is compacted by being squeezed by the DNA. Uberbacher et al. (1986) used neutron scattering methods and Park and Fasman (1988) used circular dichroism to investigate core particle structure as a function of changing solvent conditions. Both concluded that the nucleosome core is a flexible structure; and that the different conformations observed in the two X - ray structures are the result of the different conditions under which the crystals were prepared.

It is interesting to note that the amino terminal regions of the core histones can be removed without significantly disrupting the structure of the nucleosome. This was determined by digesting chicken erythrocyte cores with trypsin and characterizing the products. Although the carboxyl terminus of H2A was also cleaved in low ionic strength conditions, all amino termini were cleaved under various ionic conditions conditions. The digested particles remain intact (Wu et al., 1986).

Higher order structures of the chromatin fibril

Transitions in the structure of the extended beads-on-a-string form of chromatin to a more compacted fibril as a function of ionic strength have been observed by a number of methods. As yet, however, there is no consensus as to what the form(s) of the higher-order structure is nor the way in which the transitions take place (Pederson et al., 1986).

Williams et al. (1986) discuss the various models at some length and present evidence for a crossed-linker model. This model is presented in Figure 3 in order to demonstrate how the transitions from the extended beads-on-a-string form of chromatin to the 30 nm fibril might occur. Note that the trajectories of the helical axes of the DNA exiting the nucleosome change from being divergent to convergent during the transition.

The transition to higher-order structure does not occur in the same way in chromatin fibrils that have had their linker histones removed (Thoma et al., 1979). This phenomenon may be the structural correlate of the observations linking H1 histones with gene repression. This point is discussed at length below.

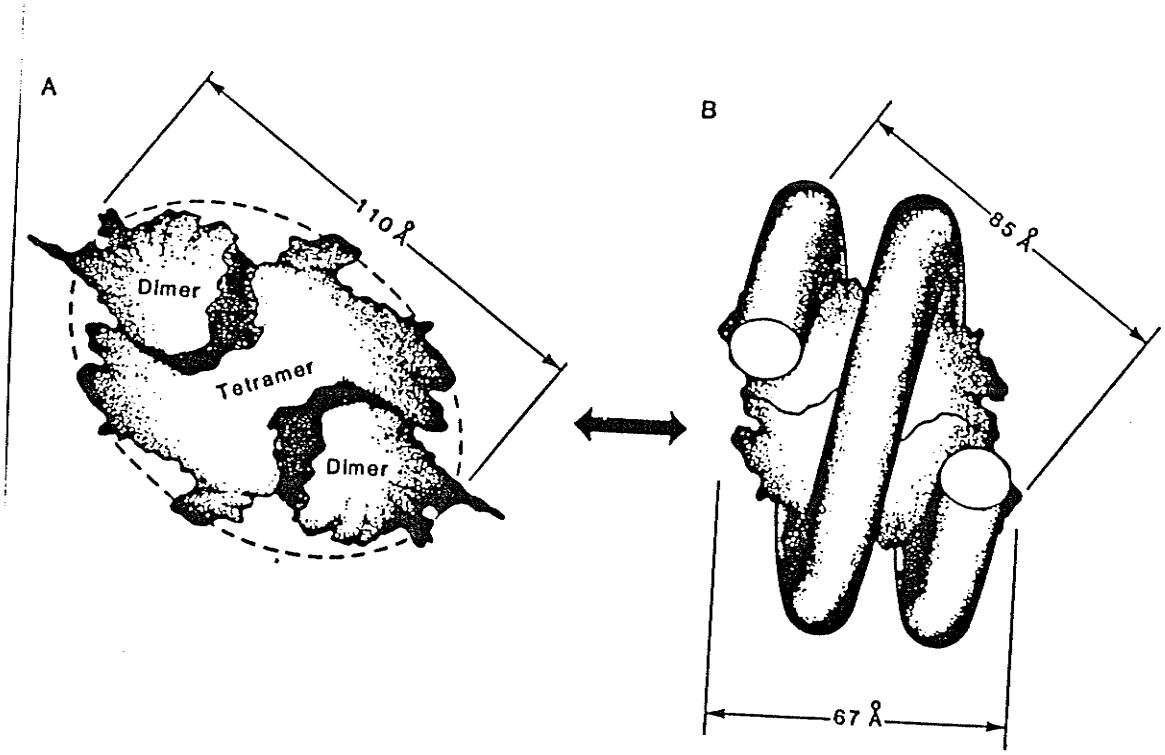

Figure 2

The nucleosome core may have conformational flexibility. Schematic drawings comparing the structures of (A), the histone octamer as determined by Burlingame et al. (1985); and (B), of the nucleosome core as determined by Richmond et al. (1984). Both are shown in the same orientation. Note the solvent channels between the H2A-H2B dimers and the H3-H4 tetramers of structure (A), (From Uberacher et al., 1986).

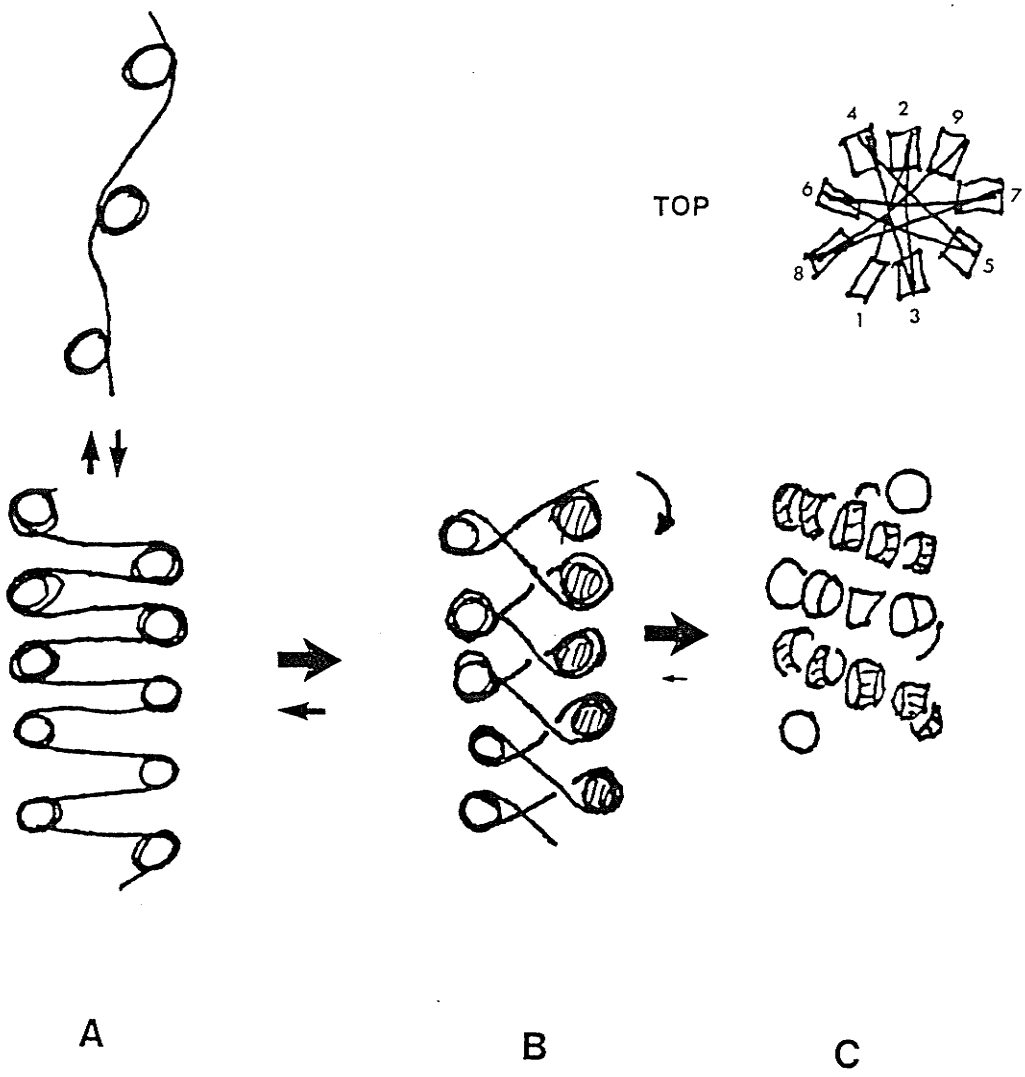

Figure 3

The transition from beads-on-a-string to a higher-order form of chromatin fibrils. A schematic drawing of the possible structural transitions that occur during the transition of chromatin fibrils to the 30-nm type structure. A: Extended and "zig-zag" forms of "beads-on-a-string" chromatin that are seen in low ionic strength preparations by EM. B: Transition to a crossed-linker form of higher-order structure. C: The fully compacted higher-order fibril. Note the changes of the trajectories of DNA exiting the nucleosomes during the transition, (Redrawn from Williams et al., 1986).

Modifications of histones and their possible functional significance.

All of the histones undergo post-synthetic modifications *in vivo*. Various chemical or enzyme treatments can also modify the histones *in vitro* (Wu *et al.*, 1986). The naturally occuring modifications are very likely to have functional significance. These modifications have all been linked to alterations of chromatin structure, probably occurring through the disruption of higher-order folding of the chromatin fibril. Different histones have been found to undergo methylation, acetylation, phosphorylation, ubiquitination, glycosylation and poly(ADP-ribosyl)ation (Wu *et al.*, 1986).

The controversy over the basic structure of chromatin at the level of the nucleosome and the 30 nm fibril is significant in light of the arguments given above that the primary function of chromatin is a architectural one. Much of what is discussed in this section deals with structural alterations in chromatin which is related to its capacity for transcription. There is a good deal of evidence that there are different types of chromatin structures and there are, also, biochemical correlates of those alterations. However, there is, as yet, no good detailed structural model which is able to relate biochemical changes with probable structural ones. When a set of atomic coordinates

of sufficient resolution is available for the nucleosome core, then computational methods such as molecular dynamics can be applied to determine how modifications might alter the structure. If, also, a better model of the transition to higher-order structure is available, then these changes in core structure can be related to possible changes in the stability of higher-order structure. In this way, a much more complete description of the probable role of histone modifications in the alteration of chromatin structure may be given.

Amongst the various modifications of the histones ubiquitination, and acetylation have been most implicated in transcriptional control (Reeves, 1984).

Ubiquitination

Ubiquitination occurs on histones H2A and H2B. Ubiquitin is a highly conserved 76 amino acid polypeptide that is attached to lysine residues near the carboxy termini (lysine 119 of H2A (Wu et al., 1986), lysine 120 of H2B (Thorne et al., 1987)) of the histones via an isopeptide linkage. Ubiquitin has a well established role in an ATP dependent protein degradation pathway (Hersko, 1983). This does not appear to be the case with H2A because the ubiquitin moiety turns over much more rapidly than the histone itself (Seale, 1981).

Some evidence for a role of histone ubiquitination in

the control of transcription is that of Varshavsky and co-workers. They used a particle gel electrophoresis method where variously modified nucleosome species can be resolved by their differing mobilities. Hybridization of the associated DNA suggested that ubiquitinated nucleosomes were preferentially associated with transcriptionally active regions (Barsoum and Varshavsky, 1985). There is some controversy about these results, however, as the efficacy of the particle gel technique in resolving the ubiquitinated nucleosome cores has been called into question (Huang et al., 1986). Huang et al., (1986) suggested that core histone modifications other than ubiquitination could account for the results of Varshavsky's group.

More recently, Nickel et al. (1989) used ubiquitin specific antibodies to show an association of the ubiquitinated form of H2B and transcriptionally competent chromatin from several different sources. Much smaller enrichment was observed for ubiquitinated H2A.

Acetylation

Acetylation is the most thoroughly investigated histone modification, both in terms of the structural changes in the nucleosome that occur and its possible functional significance. The possible role of histone acetylation in the maintenance of trancriptionally

competent chromatin is discussed below.

Acetylation occurs in each of the core histones at ϵ-amino groups of lysine and at the amino terminal serines on H2A and H4. The amino terminal acetylation is of the cotranslational type which is fairly common and is also irreversible. The sites of lysine side chain acetylation are as follows (amino acid number from amino terminus): H2A - 5, 12; H2B - 5, 10, 13, 18; H3 - 9, 14, 18, 23; and H4 - 5, 8, 12, 18 (see Figure 1). Side chain acetylation is reversible, and several acetylases and deacetylases have been characterized (Wu et al., 1986). Sodium butyrate strongly inhibits most histone deacetylases and, when applied to living cells, increases the level of highly acetylated histones (Wu et al.,1986).

Chicoine et al. (1986) have presented evidence which suggests that the different sites of side chain acetylation may be distinguished in terms of possible function in Tetrahymena. They showed that two side chain acetylation sites of histone H4 are the only ones used during replication, while an additional site is exclusive to transcriptional function.

The nuclear matrix

The nuclear matrix is often thought of as a stable form of skeleton structure which is responsible for the high order architecture of the nucleus. As will be argued below, the matrix may also be (or at least include) those components of the nucleus that are the most dynamic. In any case, there is evidence associating the matrix in all major nuclear functions (replication, transcription, mRNA processing, and transport; Verheijen et al., 1988; W.G. Nelson et al., 1986).

The nuclear matrix is a structure which is defined in purely operational terms: the type and number of its constituents depends on how it is prepared. There is a good deal of evidence to suggest that complexes involved in nascent RNA turnover and processing make up the greatest mass of most types of matrix preparations (presented in reviews by Verheijen et al., 1988; Bouteille et al., 1983). In some types of preparations, at least, the matrix is highly enriched in heterologous nuclear ribonucleoproteins (hnRNPs) (Fey et al., 1986, Gallinaro et al., 1983) which are thought to be the processing complexes associated with nascent RNA. There is good evidence that transcription occurs on the nuclear matrix (Jackson and Cook, 1986; Razin and Varovaya, 1986); and strong evidence that RNA processing occurs there as well.

The work by Zeitlin et al. (1987), for example, demonstrated that nascent RNAs could be processed and released from the nuclear matrix by soluble splicing extracts prepared from HeLa cells.

This hnRNP containing component of the matrix would consist of relatively short lived complexes. It should be noted that the majority of RNA synthesized in nucleus is also rapidly degraded there (Schroder et al., 1987). Bodnar (1988) suggests that matrix attachment sites are of two types: stable attachment which is common to all cell types, and dynamic sites which are due to transcription related events. The dynamic association sites are, therefore, cell type specific. The hnRNP components of the matrix, then, would be representative of the dynamic type of attachment sites.

It should be pointed out in concluding this brief perspective on the structure of the components of the nucleus that a good integrated perspective on the relationship between nuclear structure and its functions is lacking (compared, for example to the other organelles). The fact that the nucleus as a whole undergoes rotational motion within cells (De Boni and Mintz, 1986), for example, does not have a clear functional explanation.

THE RELATION BETWEEN CHROMATIN STRUCTURE AND FUNCTION

Chromatin structure and the regulation of transcription: the expression-replication model

The following discussion will focus on the role of chromatin structure in the regulation of gene expression. This role of chromatin is probably played through its compaction of the genome. The tendency (more precisely, the lower energy configuration of the system) of histone interaction with DNA is to compact the DNA, and results in a lower probability of interaction with transcriptional machinery. The increased regional probability of transcription, then, would require an input of energy to prevent the formation of the compacted structure.

The effectiveness of the eukaryotic mechanism of differential gene expression was pointed out by Weintraub (1984): The level of expression of growth hormone mRNA in nonexpressing cells is less than 10^{-9} that in expressing cells. In prokaryotic cells the ratio of mRNA levels in the repressed and derepressed state is about 10^{-3}.

The interrelation of the functions of chromatin as a factor in the regulation of transcription and in genome compaction is explained by what has been called the replication-expression hypothesis, which embraces the looped domain hypothesis. These are probably the most

powerfully explanative ideas in current chromatin research.

Most of the ramifications and predictions, and some supporting data for the looped domain/replication-expression model have been presented in an extended form by Bodnar (1988).

A schematic representation of the domain model and its possible role in gene expression is shown in Figure 4. Looped domain hypothesis suggests that, although the genomic DNA strands are continuous within each chromosome, it is divided into regions or domains which are, on average, of about 50 kbp each. The domain is a loop because it is held at the ends in the nuclear matrix. Each domain is considered to be more or less isolated from its neighbours. That is to say, transcriptional and/or replicative functions may be occurring on one domain while a neighbouring domain may be in a repressed state. The replication expression hypothesis suggests that the potential for gene expression within each domain is "inherited" from the parent template domain at the time of replication, so that domains that are transcriptionally competent at the time of replication have a much greater probability of being assembled into competent chromatin than a domain that is repressed.

In eukaryotes the specific interaction of DNA binding proteins with cis-acting DNA sequences is probably a key step towards establishing the transcriptionally competent

chromatin domain. It is clear from experiments with transgenic mice that cis-acting DNA sequences are sufficient for the tissue-specific expression of some genes (Goring et al., 1987; Quaife et al., 1987; Grosveld et al., 1987). Present evidence indicates that the interaction of trans-acting factors with cis-acting DNA sequences has a key role in establishing but not maintaining the transcriptionally competent chromatin structure (Gross and Garrard, 1987; Maniatis et al., 1987). It is likely that few different (perhaps around 30) types of regulatory DNA-binding proteins are found in a particular eukaryotic cell, and that it is their combination which is important for gene regulation (discussed in Bodnar, 1988).

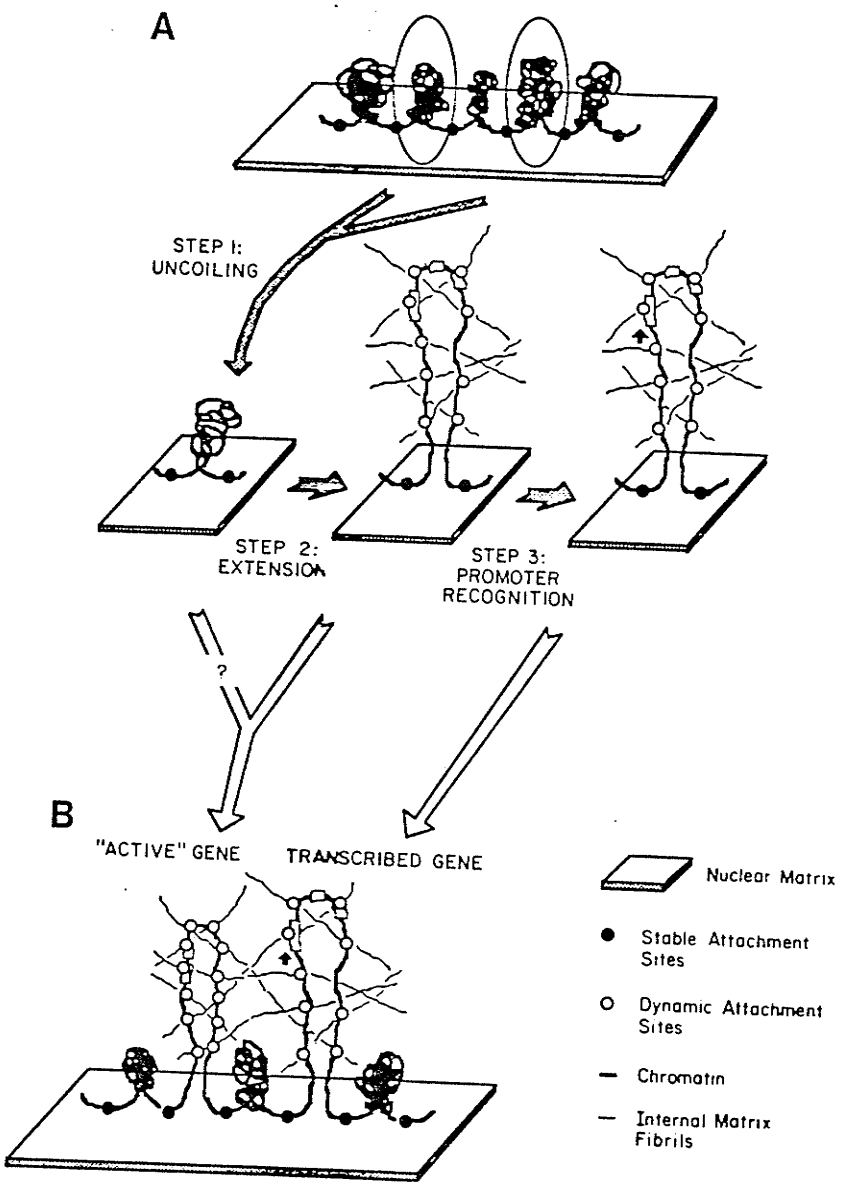

Figure 4

The domain model and gene expression. A: The domains are shown as condensed loops which uncoil. Domain uncoiling is required for transcription, which is indicated by small arrow and open boxed region. B: Three types of looped domains can be distinguished: repressed highly condensed domains, and uncoiled domains that may or may not be actively transcribed (From Bodnar, 1988).

Evidence for the looped domain and replication-expression models.

Several lines of evidence can be interpreted as supporting the looped domain model. The most direct comes from electron micrographs of deproteinized metaphase chromosomes that show the DNA looping out from a central axis (Paulson and Laemmli, 1977) . Studies by Coffey and coworkers on interphase nuclei, where most of the histones had been removed, suggest that the DNA is contained mainly as loops which are also topologically constrained (Volgelstein et al., 1980).

Mirkovitch et al. (1986) mapped DNA regions showing association with the nuclear matrix (matrix associated regions or MARs) across a 320 kbp region of the Drosophila genome. The possibility of such a mapping indicates that the genome is bound to the matrix at fairly specific regions. Furthermore, although several genes were included within the region, no RNA coding sequence is interrupted by a matrix attachment site. This is consistent with the interpretation of transcriptional independence of domains. A similar approach was taken by Cockerill and Garrard (1987) who showed specific binding of DNA sequences associated with enhancer elements to matrix preparation.

A number of in vitro studies have addressed the replication-expression model more or less directly. All findings are consistent with this general mechanism:

histones inhibit the interaction of transcriptional machinery with DNA (with linker histones adding a further degree of inhibition beyond that of nucleosome cores alone); but the influence of the histones can be significantly reduced by the presence of *trans*-acting factors on functional elements of the DNA before, but not after, the binding of histones. This is consistent with the idea that, in general, nucleosome formation represses gene expression, and that gene regions are kept competent for gene expression by preventing normal nucleosome formation on at least some parts of the DNA.

Some specific results are as follows: An *in vitro* reconstitution experiment indicates that the TFIIIA transcription factor is able to displace DNA from nucleosome cores. Nucleosomes complexed with linker histone may prevent this (Rhodes, 1985).

Hannon *et al.* (1984) measured the transcriptional initiation by exogenous RNA polymerase in a reconstituted chromatin system as a function of the composition of linker histones. In chromatin without linker histone, RNA chain initiation is restricted to linker DNA. With the addition purified linker histones, initiation was further inhibited. The inhibitory effect of nucleosomes probably occurs primarily at the initiation stage, as transcriptional elongation can occur through DNA regions bound to nucleosomes (Losa and Brown,1987; Lorch *et al.*, 1987).

Schissel and Brown (1984) found that complexes of assembled chromatin with both the transcription factor TFIIIA and histone H1 were stable and could not be displaced by one another. They concluded that, in active chromatin, stable transcription complexes protect 5S RNA genes from histone H1-mediated repression and H1 binding prevents the formation of stable active transcription complexes. Workman et al. (1988) found that transcription potentiated by the immediate early (IE) protein of pseudorabies virus was inhibited by nucleosome assembly if the assembly occurred before the IE protein was present.

The role of linker histones in gene repression.

General

Transcriptionally competent gene chromatin is in a state which is sensitive to attack by DNase I relative to the bulk of chromatin (Weintraub and Groudine, 1976). The region of DNase I sensitivity extends well beyond the coding region of the gene, and probably delineates a looped domain (Yaniv and Cereghini, 1986). An example of this is the glyceraldehyde-3-phosphate dehydrogenase gene which is contained in a 12 kbp DNase I sensitive domain in chicken (Alvey et al., 1984). The most complete evidence that altered chromatin structure is a property of a looped domain comes from work with the chicken lysozyme gene in hen oviduct. Phi-Van and Stratling (1988) showed that the genomic regions, which delineated the boundries of the approximately 19 kbp DNase I sensitivity domain, were also matrix attachment regions.

Sensitivity to DNase I suggests an open or exposed chromatin structure which would be more accessible to the transcription machinery than the bulk of chromatin. The repressed class of chromatin structures may be effectively invisible to the elements involved in transcriptional regulation.

The factors involved in maintaining the transcriptionally competent chromatin structure are poorly

understood. Factors thought to be involved include post-synthetic histone modifications (e.g. histone acetylation and ubiquitination; Gross and Garrard, 1987; Ferenz and Nelson, 1985; Nelson et al., 1986; Alonso et al., 1987) and the loss of histone H1. Since these processes are reversible, the maintenance of the transcriptionally competent chromatin structure should be considered as a dynamic process (Alonso et al., 1987). One of the key steps in this process is thought to be the disruption of histone H1-H1 contacts essential for the binding of the H1 histone to chromatin (Weintraub, 1985). Several lines of evidence suggest a role for H1 histones in gene repression (Weintraub, 1984; Brown, 1984). Chromosomal components, which interfere with the binding of H1 histones to the nucleosome, will prevent the chromatin fibre from forming higher order, repressed chromatin structures.

An experiment, which demonstrates the role of linker histones in repression of transcription by inducing the formation of higher-order structures in a fairly direct way, is that of Hannon et al. (1984). They reconstituted different types of linker histone with linker-stripped chromatin. They assayed the chromatin as a template for RNA synthesis and examined it for higher-order structure by electron microscopy. They found that the extent of repression of RNA chain initiation correlated with the capacity of the different linker histones to induce the

formation of higher-order structure.

Chicken erythrocytes: histone H5

In chicken erythrocytes the preponderance of the linker histone is of the H5 type which appears to give a high degree of stability to higher-order structures and may contribute to the very low transcriptional activity of these cells (Thomas and Rees, 1983; Thomas et al., 1985; Bates et al., 1981). Histone H5 is accumulated up to a H5/H1 ratio of slightly greater than 2 (Bates and Thomas, 1981) during the terminal stages of erythrocyte differentiation. One of the intriguing questions still to be answered is whether histone H5 replaces H1 in specific locations along the chromatin fibre. This question is addressed in the first part of the experimental work presented here.

Several workers have shown that a chromatin fraction, apparently less compact than the bulk, is enriched in globin sequences which are expressed at high levels during erythrocyte maturation (Weintraub, 1984; Kimura et al., 1983). Weintraub (1984) electrophoretically resolved two populations of supranucleosome particles, one which contained globin genes and another which contained vitellogenin sequences, a gene that is not expressed in erythroid cells. The integrity of the vitellogenin supranucleosome particles did not depend on the continuity

of the DNA strand. Apparently, this structure was held together by the interactions of contiguous H1 histones. The globin gene containing supranucleosomes, which contained H1 and H5, differed from the vitellogenin supranucleosomes in that they did not have the DNA size independent stability nor did they take up exogenous histone H1, as did the vitellogenin supranucleosome species. Kimura et al. (1983) used a different approach to study the distribution of the H1 histones in chicken erythrocyte chromatin by determining the sedimentation rates of various gene chromatin fragments in sucrose gradients. They found the erythrocyte-specific retardation of β-globin gene chromatin to be consistent with a partial depletion of H1 histones.

The role of acetylation in destabilizing chromatin compaction.

Transcriptionally active/competent chromatin is thought to be in an unfolded conformation (as reflected, for example, by an increased sensitivity to DNase I). Most active-gene-enriched chromatin fractions contain increased levels of modified and variant histone species. Nucleosomes containing such histones may vary somewhat in structure from the bulk and thereby affect nucleosome dependent transitions to higher-order structures. Differences in nucleosomal structure that result in differences in higher-order structure may be of primary importance in determining the role of chromatin structure in the regulation of transcription.

One of the most universally observed features of active-gene-enriched chromatin is the presence of hyperacetylated nucleosomal histones (Reeves, 1984; Vidali et al., 1988; Loidl, 1988). Hyperacetylation of the amino-terminal region of the nucleosomal histones (H2A, H2B, H3, and H4) may be linked to the chromatin solubility in the presence of Mg^{+2} and destabilization of chromatin higher-order structures (Perry and Chalkley, 1981, 1982; Allan et al., 1982; Annunziato et al., 1988). Numerous reports have suggested that histone acetylation functions in the maintenance or control of the transcriptional and/or

replicative capacity of chromatin regions (see Loidl, 1988 and Vidali et al., 1988 for discussions).

Although not all data make a clear distinction, acetylation also seems to affect the structure of the nucleosome (a good discussion of this point is in Oliva et al., 1987). Imai et al., (1986) show by neutron scattering that histone hyperacetylation does not effect the size of nucleosome core particles. On the other hand, Oliva et al. (1987) show an elongated nucleosome shape associated with hyperacetylation. It might be that the shear forces to which the particles are subjected during preparation for EM are sufficient to allow for changes in shape which are less pronounced in the unstressed particles present in solution which are used for neutron scattering.

Other data show some differences in the physical properties of hyperacetylated nucleosomes compared to normals. Bode et al. (1980) and Ausio and van Holde (1986), for example, both show similar data demonstrating changes in circular dichroism (CD) spectra and thermal denaturation profiles of nucleosomal DNA, although Ausio and van Holde (1986) characterize the changes in the CD spectrum as small. These points are discussed further in Part IV of the current work.

The effect of histone acetylation on the condensation of the fibril into higher-order structures has also been investigated by McGhee et al. (1981) and Annunziato et al.,

(1988). The conclusions of both these studies is that acetylation does not completely prevent condensation of chromatin, but that the degree of condensation is not so great as it is with unacetylated chromatin.

In the erythrocytes from anaemic chickens (which are in the late states of differentiation and still expressing the globin genes), approximately 3.7% of modifiable histone lysine sites are undergoing acetylation and deacetylation (Zhang and Nelson, 1986, 1988a,b). These sites are rapidly acetylated (half-life of 12 min) and deacetylated (Zhang and Nelson, 1988a,b). The histones undergoing dynamic acetylation appear to be those hyperacetylated species that are associated with active/competent DNA (Nelson et al., 1986; Alonso et al., 1987; Zhang and Nelson, 1988a; Hebbes et al., 1988). Nelson and colleagues have shown that altering the level of acetylated histones has a profound effect on the solubility of active/competent gene chromatin fragments in buffers containing 3 mM $MgCl_2$, with increased levels of acetylated histones resulting in enhanced solubility.

Hebbes et al. (1988) demonstrate a direct link between core histone acetylation and transriptionally active chromatin. They did this by using an antiserum specific for hyperacetylated histone H4. Using the antibody to fractionate chicken embryo erythrocyte chromatin they demonstrated a 15- to 30-fold enrichment of globin gene

sequences in chromatin bound to the antibody, but no enrichment of the inactive ovalbulmin gene sequences.

MATERIALS AND METHODS

Tissues

Mature chicken erythrocytes.

Blood from adult White Leghorn chickens was collected at a local slaughter house. The major vessels of the neck were severed by an automatic mechanical process, and the blood was collected into a tray containing ice cold blood collection buffer (75 mM NaCl, 25 mM EDTA and 30 mM sodium butyrate). The blood cells were pelleted, and then washed twice by centrifugation (2000 x g, 10 min) in the same buffer and most of the buffy coat was removed at each wash by aspiration. Aliquots of the cells (of about five ml packed volume) were packed into 14 ml tubes by centrifugation for 3 min in a clinical centrifuge, frozen and stored at -70°C.

Immature chicken erythrocytes.

Anaemia was induced in mature White Leghorn hens by the administration of phenyl hydrazine (acetyl form) at the concentration of 2.5% w/v in 40% ethanol. Subcutaneous injections were given according to the following six day schedule: day 1, 0.7 ml; day 2, 0.7 ml; day 3, 0.6 ml; day

4, 0.4 ml; day 5, 0.7 ml; day 6, 0.8 ml. On the seventh day the birds were sacrificed by decapitation and the blood was collected, washed and stored as described above. In the case of cells to be used for incubation in media in the absence of sodium butyrate, this compound was also omitted from the blood collection buffer.

The erythroid cells in the peripheral blood of phenylhydrazine treated hens are 95% post-mitotic polychromatic erythrocytes and about 3% mature erythrocytes. In untreated hens the peripheral blood consists of over 99% mature red cells (Williams, 1972; Gasaryan, 1982).

Cell incubations.

In some experiments immature red blood cells, prepared as described above, were incubated in media in the presence or absence of sodium butyrate. In these instances, the cells washed once in blood collection buffer, were washed once more with cold Swim's S-77 medium (Gibco, or Sigma) pH 7.2 with or without the addition of sodium butyrate to 10 mM. The cells were then incubated in the same media for one hour at 37°C before being packed into 14 ml tubes, frozen and stored at - 70°C.

Experimental Procedures

Isolation of nuclei

The frozen cells were lysed by thawing in a 37°C bath after the tubes were filled with RSB [reticulocyte standard buffer: (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM PMSF)] with 30 mM sodium butyrate. Periodic mixing of the tube during thawing assures a homogeneous lysate. The nuclei were prepared from the lysed cells by washing 4 times. Each wash was in a volume of RSB about 10 times the volume of the packed cells and containing 0.25% NP-40 (Sigma) in the first two washes. Washes consisted of resuspension of the pellet in a small portion of RSB with a cut off Pasteur pipette, and homogenization of the nuclei in the remaining volume of wash buffer with a Potter-Elvenjem homogenizer and centrifugation (2000 x g for 10 min). Butyrate was not included in the nuclear preparation from the immature cells which were incubated in its absence.

Digestion of nuclei with micrococcal nuclease

Washed nuclei were resuspended in the digestion buffer (1 M hexylene glycol, 10 mM PIPES (piperazine-N,N' bis[2-ethane sulphonic acid]), pH 7.0, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1%

thiodiglycol, 1 mM PMSF, 30 mM sodium butyrate) to a DNA concentration of 50 A_{260} units/ml and incubated with 25 A_{260} units/ml micrococcal nuclease (Pharmacia) at 37°C for 30 min. Shorter digestion times were sometimes used as indicated in the text of the results section. Digestion was stopped with the addition of EGTA to 10 mM and nuclei were collected by centrifugation. Butyrate was excluded from the digestion buffer used for those nuclei prepared from the immature cells incubated in the absence of this compound.

Modified Sanders' fractionation of digested nuclei

After micrococcal nuclease digestion the nuclei were collected by centrifugation and resuspended to a volume equivalent to that during digestion in 50 mM Tris HCl, pH 7.0, 2 mM $MgCl_2$, 75 mM KCl, 10 mM EGTA, 1% thiodiglycol, 30 mM sodium butyrate, 1 mM PMSF and 200 mM NaCl and left on ice for 30 min. When collected by centrifugation, the pellet fraction is P0.2. The supernatant (S0.2) was dialysed against 1 mM EDTA, pH 7.0, and then made to 100 mM KCl to precipitate most of the linker histone containing chromatin. The precipitated chromatin (S0.2 ppt.) was collected by centrifugation (12000 g, 20 min) and resuspended in 1 mM EDTA. The supernatant (S0.2 sup.) was dialysed against 1 mM EDTA.

Selective removal of H1 from nuclei with citric acid/phosphate buffer

Isolated nuclei were washed twice in 0.25 M sucrose, 25 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ in citric acid/phosphate buffer pH 2.4, at 4°C for 30 min. The citric acid phosphate buffer was made as described by Gomori (1955). For 10 ml final volume, for example, 4.8 ml of 0.1 M citric acid and 0.2 ml of 0.2 M dibasic sodium phosphate were mixed. This was then mixed with 5 ml of 2X wash buffer (0.5 M sucrose , 2 mM $CaCl_2$ and 2 mM $MgCl_{2)}$. The acidic buffer treated nuclei were equilibrated in digestion buffer for 1 h before digestion with micrococcal nuclease.

Salt precipitation and gel filtration of soluble chromatin

Digested nuclei were resuspended into a volume of 10 mM EDTA, pH 7.2, 1 mM PMSF, equivalent to that used in the digestion, and left on ice for 2 hours to release chromatin fragments into solution. Insoluble nuclear debris was removed by centrifugation (12,000 g, 20 min). The supernatant (SEDTA) contained 80.3 ± 3.8% (n=4) of the total nuclear 260 nm absorbing material from mature chicken erythrocyte. The pellet is called PEDTA. SEDTA was diluted to a 260 nm absorbance of 30 units/ml by the

addition of 10 mM EDTA, and NaCl was added with vigorous stirring to 0.15 M from a 4 M stock. The resulting precipitate was collected immediately by centrifugation (12,000 g, 20 min.).

The supernatant (10.8 ± 0.4% of SEDTA, n=4, from mature cells) was concentrated by lying a 3.5 kD cutoff dialysis bag filled with this solution into a tray of solid polyethylene glycol. The concentrated supernatant was then applied to a 2.5 x 110 cm Bio-Gel A5m column in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.15 M NaCl and run at a flow rate of approximately 50 ml/hr. 3.75 ml fractions were collected.

Digestion of nuclei with DNase I

Nuclei were digested with DNase I as described by Villeponteau et al. (1984). Nuclei were suspended in RSB (10 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 3 mM $MgCl_2$) to a concentration of 13 A_{260} units/ml. DNase I (Sigma) was added to 1 µg/ml and the nuclei were digested for 10 min at 37°C. Digestion was terminated by the addition of EDTA to 10 mM and placing the digest on ice. DNA was prepared for slot blot analysis as described below.

Preparation and reconstitution of stripped chromatin fragments and linker histones.

Dry CM Sephadex (30 mg/ml) was added to SEDTA and then NaCl to 0.35 M from a 4 M stock (with stirring) and the suspension was stirred at 4°C for 1 hour. The chromatin fragments stripped of the linker histones were collected by filtration using Whatman Number 1 filter disks. The CM Sephadex was first washed with 0.6 M NaCl and then 1.0 M NaCl. This was done by resuspending the CM Sephadex in the appropriate ice cold salt solution and stirring for 10 min at 4°C and collecting the washes by filtration. The second wash contained the H1 and H5 histones, in proportions equivalent to that found in nuclei, which were essentially free from other proteins. The linker histone preparation was dialysed against water, lyophilized and stored as a concentrated solution at -20°C. The stripped chromatin was dialysed against 1 mM EDTA, 1 mM PMSF stored at 4°C and used within a few days.

Stripped chromatin was reconstituted with varying quantities of the mixed H1 and H5 preparation essentially according to the method of Nelson et al. (1979). Both chromatin and histones were dialysed against 10 mM Tris-HCl, pH 8.0 and 0.5 M NaCl. Varying dilutions of the linker histones in the same buffer were added to a constant amount of chromatin and dialysis was continued for

2 hours, then for one hour against 0.25 M NaCl, 1 mM EDTA and then against 1 mM EDTA overnight, both buffered with 10 mM Tris-HCl, pH 8.0.

Reconstituted material was fractionated by the addition of NaCl to 0.15 M as described above.

During initial studies the quantities of linker histones and chromatin used in the reconstitutions were determined by densiometric scanning of stained SDS PAGE gels of known amounts of the stock solutions. The relative densities of the two preparations were compared to that of a native chromatin standard (SEDTA). The volumes of the stocks to be used in reconstitution were then determined so that the nominal linker:core ratio of 1 was equivalent to that of the SEDTA chromatin. In later work these volumes were determined by assaying the quantities of chromatin and proteins in the stocks directly by the TCA (see analytical procedures) precipitation assay for the proteins and absorbance at 260 nm for the chromatin. A ratio of 5.3 A_{260} of stripped chromatin per 1 A_{400} of linker histone stock (using 10 µl sample) gives a linker:core ratio equivalent to that found in native chromatin.

Treatment of core particles with ethidium bromide

Core particles from EDTA released chromatin (SEDTA) and from salt-soluble polynucleosomes were prepared by a

method similar to that of Libertini and Small (1980). H1 and H5 histones were removed with CM Sephadex as described above and the stripped chromatin was dialyzed against 10 mM Tris - HCl pH 8.0 and 1 mM $CaCl_2$. The chromatin was made to 10 A_{260} units/ml and digested with 10 units/ml of micrococcal nuclease for approximately 10 min (digestion times were optimised for each preparation). Further purification of the core particles by gel filtration on Bio-Gel A5m in 10 mM Tris - HCl pH 8.0 and 1 mM EDTA did not affect the interaction with ethidium bromide. Treatment of the core particles with ethidium bromide and analysis of particle integrity by electrophoresis was carried out as described by McMurray and van Holde (1986).

Treatment of core particles with N-ethyl[^{3}H]maleimide

Core particles from EDTA released chromatin (SEDTA) and from salt-soluble polynucleosomes were prepared as described in the last section. These particles were treated with N-ethyl[^{3}H]maleimide as described by Wong and Candido (1978). This compound reacts with free thiols such as cysteine 110 of histone H3 to form a stable covalent adduct and should be, hence, a probe for core particle structure. N-ethyl[^{3}H]maleimide (New England Nuclear) was diluted to 80 mCi/mmol with unlabelled N-ethylmaleimide in heptane and stored at 4°C until use.

For labelling of 0.2 ml of chromatin at 2 A_{260}/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and NaCl to varying concentrations, 2 µl of N-ethyl[^{3}H]maleimide stock was added and mixed. The control for labelling was 0.2 ml samples of chromatin were mixed with 0.4 ml of 6 M urea, 4 M NaCl in 10 MM Tris-HCl, pH 7.4, 1 mM EDTA and labelled with 6 µl of N-ethyl[^{3}H]maleimide. These concentrations of urea and NaCl dissociate the particle and give maximum labelling of the nucleosome (Wong and Candido, 1978). Reaction with N-ethyl[^{3}H]maleimide was carried out at room temperature for 1 h.

To stop the reaction 150 µl of the mixture was added to 1 ml of ice cold 10% TCA and mixed. The TCA precipitable material was captured by filtration through 2.4 cm Whatman GF/C glass microfiber filters prewet with 10% TCA. The filters were washed with a further 10 mls of ice cold 10% TCA before being air dried and counted. The results were expressed as percent labelling where the level of labelling of NaCl-urea treated particles was 100%.

Analytical procedures

Measurement of DNA concentration by absorbance at 260 nm

Absorbance at 260 nm was used to estimate the DNA concentration of samples. One A_{260} unit is equal to 50 µg of pure DNA.

Measurement in nuclei

To determine the concentration of nuclei before digestion with micrococcal nuclease four 10 µl samples of the nuclear suspension were taken. These were dissolved in 990 µl of 5 M urea, 2 M NaCl. Care was taken to ensure the resulting viscous solution was transparent and monodisperse in the spectrometer cuvette. The spectrophotometer was adjusted to zero against 5 M urea, 2 M NaCl.

Measurement in chromatin

1/10 or 1/100 dilutions of chromatin solutions with 2 M NaCl were made to obtain measurements of DNA concentrations. At this concentration of NaCl the histones are dissociated from the DNA and there is little quenching of its absorbance. The spectrophotometer was adjusted to zero against 2 M NaCl. As chromatin consists of nearly

equivalent mass of DNA and protein, the concentration of protein in a chromatin sample may also be determined in this way.

Measurements of purified DNA

1/10 or 1/100 dilutions of purified DNA were made with ddH_2O. The spectrophotometer was adjusted to zero against ddH_2O.

Measurement of DNA by a diphenylamine assay

In some fractions (e.g. PEDTA) the presence of large amounts of protein precludes the accurate measurement of DNA by measurement of the 260 nm absorbance. To determine the actual DNA concentration of these fraction an assay utilizing diphenylamine was used (Giles and Myers, 1965). To the DNA containing sample in 10% perchloric acid was added an equal volume of 4% diphenylamine in glacial acetic acid, followed by 1/40 volume of 1.6% acetaldehyde. This was incubated at 30°C overnight. The optical density difference at 595-700 nm was determined. An difference of 0.15 is equivalent to a DNA concentration of 10 µg/ml.

TCA precipitation assay for protein

To measure the concentration of protein samples an assay involving precipitation with TCA was used. Depending on the sample concentration, either 10 or 100 µl of sample was made to 0.8 ml with H_2O. To the diluted sample, 0.4 ml of 50% TCA was added and mixed vigorously. The fine precipitate was allowed to develop for about 10 min and light scattering of the sample was measured by reading its absorbance at 400 nm. The spectrophotometer was adjusted to zero against 16.6% TCA. The protein concentration of the sample was calculated according to the formula (for 100 µl of sample):

$$A_{400} \text{ reading} \times 1290 = x \text{ µg/ml protein}$$

Preparation of DNA samples, electrophoresis and blotting

For Southern blotting, equal quantities (determined by A_{260}) of chromatin samples were dialysed against water and lyophilized. The lyophilised powder was redissolved in 2 x DNA sample buffer (0.08 % bromophenol, 5% Ficoll) containing 2% SDS. 10 µg per lane samples were subjected to electrophoresis on 1% agarose gels in standard electrophoresis buffer (30 mM Tris-acetate pH 7.2, 1 mM EDTA) containing 0.1% SDS. After electrophoresis gels were stained for about 45 min in 1 µg/ml ethidium bromide for photography. DNA denaturation and blotting was carried out by standard procedures exactly as described in Maniatis et al. (1982).

For slot blotting the DNA samples were purified utilizing RNase and pronase stocks prepared as follows:

Pronase (Boehringer-Mannheim, 165 921) was dissolved at 10 mg/ml in 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA and heated to 70°C for 1 min to destroy contaminating nucleases. After aliquoting the protease was stored at -20°C.

Bovine pancreatic RNase A and RNase T_1 (both from Sigma) were made to 5 mg/ml and 5000 u/ml, respectively. T_1 was dialysed for 16 h against ddH_2O before use. Aliquots were heated to 80°C for 10 min to destroy other nucleases before being stored at -20°C.

DNA samples (either as nuclei or chromatin fractions) were dialysed (where appropriate) and made to 25 mM EDTA, 160 mM NaCl, and 2.2% Na sarcosyl. Pronase (1/20 volume) was added and the samples were incubated at 37°C overnight. RNase (1/50 volume) was added and incubation continued for another 2 h. More pronase (1/50 volume) was added and the incubation continued for another 2 - 16 h.

After the enzyme digestions, the aqueous solutions were extracted once with an equal volume of phenol (redistilled, saturated with 100 mM Tris-HCl, pH 8.0, and including 0.1% 8-hydroxyquinoline as an anti-oxidant) and twice with equal volumes of IAC (24:1 chloroform and isoamyl alcohol as an anti-foaming agent).

After organic extraction 1/10 volume of 2 M sodium acetate was added to the aqueous phase and then two volumes of ice cold ethanol (95%) and the samples were kept at -20°C for at least two hours. The precipitated DNA was collected by centrifugation for 30 min at 12000 g. The pellets were dried under vacuum for about 5 min and dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. High molecular weight samples were heated to 60°C for about 10 min to improve the rate of rehydration.

For slot blotting the purified DNA was made to 0.3 M NaOH, incubated at 37°C for 30 minutes, neutralized by the addition of an equal volume of 2 M ammonium acetate, pH 7.0, and applied to nitrocellulose using a Schleicher and

Schuell slot blotting manifold.

Hybridization of blots with nick-translated probes

Hybridization with nick-translated probes was carried out essentially as described by Thomas (1979). The baked blots were wetted in 5 X SSC (1 X SSC = 150 mM NaCl, 15 mM Na citrate) and sealed into plastic bags. To this was added the prehybridization solution (50 % (v/v) formamide, 5 x SSC, 50 mM $NaPO_4$, pH 6.5, 1 x Denhardt's solution (0.02% of each of Ficoll, polyvinylpyrrolidone and bovine serum albumin-fraction V) with 200 µg/ml denatured salmon sperm DNA) which had been prewarmed to 42°C. After prehybridization for at least 4 h the prehybridization solution was drained from the bag and the hybridization solution was added. This solution was the same as the prehybridization solution, but included, in addition, the denatured ^{32}P - labelled nick-translated probe and 10% dextran sulphate. After hybridization for 16 h at 42°C the filters were removed from the bags and washed as follows: 2 x SSC, 0.5 % SDS, first for 5 min, and then 15 min, at room temperature; 0.1 x SSC, 0.5% SDS at 68°C, first for 2 h and then for 30 min. The hybridized filters were air dried, covered with Saran wrap and autoradiographed at -70°C using intensifying screens and Kodak XAR film.

The cloned DNA probes used for hybridizations

The cloned DNA probes used were:

pCBG 14.4, unique intronic sequence of the adult ß-globin gene, pCBG 18.7, a unique sequence of the embryonic ϵ-globin gene and pCBG 13 which contains the 5′ end of the adult gene and some flanking sequences were acquired from H. Martinson (Villeponteau et al., 1982).

pVTG 412, 5′ region of the chicken vitellogenin gene (Burch and Weintraub, 1983).

pH2A.F, received from J.R.E. Wells, is a cDNA clone of the gene of the histone H2A variant H2A.Z (Harvey et al., 1983).

pchV2.5B/H from A. Ruiz-Carrillo is a 2.5 kbp fragment of the histone H5 gene (Ruiz-Carillo et al., 1983).

pOV12 is the ovalbumin gene received from M.-J. Tsai (Woo et al., 1978).

pCkTk (Groudine and Casmir, 1984) and pMyc3.2SST (Schubach and Groudine, 1984), genomic clones for the thymidine kinase and c-myc genes, respectively, were acquired from M. Groudine.

pVIT, a genomic clone of the vimentin gene (Zehner and Paterson, 1983), was a gift from B.M. Paterson.

Polyacrylamide gel electrophoresis of proteins.

a) SDS PAGE

A discontinuous two part system was used. The 6 % acrylamide stacking and 15 % separating parts of the gels were made from mixing stock solutions in the following proportions:

	Stock solution	Stacking gel	Separating gel
1.	30% acrylamide, 0.8% bis acrylamide.	0.4 ml	3.5 ml
2.	1.5 M Tris-HCl pH 8.8	---	1.75 ml
3.	0.5 M Tris-HCl pH 6.8	0.5 ml	---
4.	10% (w/v) SDS	20 µl	70 µl
5.	10% (w/v) ammonium persulfate	20 µl	40 µl
6.	TEMED	2 µl	3.5 µl
7.	H_2O	1 ml	1.65 ml

The gels were 8 cm high (about 6 cm of separating gel) x 9 cm wide x either 0.5, 0.8 or 1 mm thick. The gels were run in an Idea Scientific (Corvallis, WA.) Minislab apparatus at a continuous 170 V, giving an approximate field strength of about 20 V/cm, for 1.5 h. The tank buffer consisted of 200 mM Tris, 1.52 M glycine and 0.4% SDS.

b) 5.4% acetic acid-6.6 M urea-0.375% Triton X-100 (AUT) PAGE

This discontinous two part system gives good resolution of basic proteins. The 6% stacking and 15% separating gel portions were made using stock solutions in the following proportions.

	Stock Solution	Stacking gel	Separating gel
1.	30 % acrylamide, 0.8 bis acrylamide	0.5 ml	4 ml
2.	4 % TEMED, 43.1 % acetic acid	---	1 ml
3.	Urea	0.8 g	3.2 g
4.	TEMED	20 µl	---
5.	0.004% riboflavin	0.2 ml	0.8 ml
6.	3 M K acetate pH 4.0	0.25 ml	---
7.	0.3 M Triton X-100	40 µl	160 µl
8.	Thiodigycol	20 µl	80 µl

The gel dimensions and apparatus used are the same as those of SDS PAGE, except that all AUT gels were 0.8 mm thick. The gels were run at 200 V, for an approximate field strength of 25 v/cm, for 3.25 h. The tank buffer was 0.9 N acetic acid.

c) Two dimensional (AUT - SDS) PAGE.

Better resolution of protein samples could be obtained by using the AUT and SDS PAGE systems sequentially. To do this the lane of interest from a stained AUT gel was excised with a knife and soaked for about 30 min in 125 mM Tris-HCl, pH 6.8, 4% SDS and 5% 2-mercaptoethanol. The gel slice thus equilibrated was applied to the top of the stacking layer of a 1 mm thick SDS gel. Electrophoresis was carried out at 150 V until the Coomassie Blue ran off the bottom of the gel (about 1.5 h).

Staining of gels

All gels were fixed and stained with 0.04% Serva Blue (Coomassie Blue) in 45 % methanol, 9% acetic acid. They were destained first in 25% methanol, 12.5% acetic acid for 0.5 - 2 h depending on the gel thickness and then transferred into 5% methanol and 7.5% acetic acid.

Preparation of chromatin proteins for electrophoresis.

In most instances, protein analyzed by AUT-PAGE, was prepared by a protamine release method adapted from Richards and Shaw (1982). In these cases a known quantity

of dialysed chromatin (determined by absorbance at 260 nm) was lyophilized. A 1% solution of protamine sulphate (w/v: Sigma grade X from salmon sperm) in 100 mM Tris-acetate, pH 8.8, 20% (v/v) glycerol, 8 M urea, 5 % (v/v) 2-mercaptoethanol, 2 % thiodiglycol and 1 % cysteamine-HCl was added to the lyophilized material. After sitting at room temperature for 10 minutes the sample tubes were spun in a benchtop microcentrifuge at top speed for about 5 minutes. The supernatant was used directly for AUT PAGE.

For SDS PAGE lyophilized chromatin was prepared as above. The powder was dissolved in 2X sample buffer (125 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol. 0.01% bromophenol blue), heated in a boiling water bath for 20 s and cooled to room temperature before being applied to the gel.

In some instances acid-soluble proteins were extracted from nuclei or chromatin samples. To do this samples were made to 0.4 N H_2SO_4 by slow addition from a 4 N stock . After sitting on ice for 30 min the insoluble material was collected by centrifugation (12,000 g, 10 min). The extracted material was first converted to the acetate form by dialysis against 0.1 N acetic acid and then dialysed against ddH_20 before being lyophilized.

Electrophoresis of chromatin particles

Good resolution of short chromatin fragments (about tetramer size or shorter) could be accomplished by electrophoresis on 4% polyacrylamide gels which were given greater mechanical strength by including 0.5% agarose. The gels were approximately 9 cm wide x 18 cm long x 0.8 mm in thickness, and in a 1/5 strength DNA electrophoresis buffer (1X DNA electrophoresis buffer = 30 mM Tris-acetate pH 7.2, 1 mM EDTA).

For 14 mls of gel, 70 mg of agarose was measured into a glass test tube and then 2.8 ml of electrophoresis buffer, 1.75 ml acrylamide:bis stock (30%, as above) and 9 ml of water. The tube was placed in a boiling water bath until the agarose was melted. The tube was then allowed to cool before the addition of 50 µl of 10% ammonium persulphate and 5 µl of TEMED. The temperature at which the gel was poured was critical as the rate of polymerization of the acrylamide is greatly increased at high temperatures but the agarose will set at low temperatures. The optimal temperature is approximately 45°C, however, the rate of acrylamide polymerization at this temperature is still much faster than at room temperature.

Chromatin samples used for this type of electrophoresis had to be free of salt and of sufficient

concentration that at least 10 µg could be loaded per lane. Samples were made to 1X DNA sample buffer (with xylene cyanol = 0.042% bromophenol blue, 0.042% bromophenol blue and 2.5% Ficoll) before loading.

The gel was run with buffer circulation at 150 V until the xylene cylanol was about 4 cm from the bottom of the gel. The gel could be stained with either 1 µg/ml of ethidium bromide or in the Coomassie Blue stain described above.

Quantitation of Data by Densitometry

Autoradiograms from hybridized slot blots were scanned with a densitometer. Peak heights were determined to quantitate the extent of hybridization. The relation between signal and the amount loaded was essentially linear.

The DNase I sensitivity of the various gene chromatins were determined as described by Villeponteau et al., (1984). All hybridization data for DNase I treated nuclei were first normalized to the hybridization of total genomic DNA (from micrococcal nuclease treated nuclei) and then calculated as a percentage of ovalbumin hybridization to identical slots.

Protein content was measured by densitometric scanning of Coomassie Blue stained polyacrylamide gels. The amounts of modified and variant H2A and H2B species that were in different chromatin fractions were compared as percents of total H2A or H2B. The enrichment of acetylated H4 were compared as a weight sums of total acetylation:

Acetyl H4 level = $\Sigma n\delta_n$ / $\Sigma\delta_n$, where n is the number of acetyl groups assigned to a protein band and δ_n is the densitiy of the band.

PART I

A major part of the current work deals with fractionation of chromatin and the characterization of those fractions. It is argued that the biochemical characteristics of certain fractions may reflect those of chromatin that is competent for gene expression while other fractions reflect the state of repressed chromatin. These arguments are based on the enrichments of different gene sequences that are known to be in functionally different states (competent and repressed) within the chicken erythrocyte.

The ways in which the different genes may be described as active and/or competent or repressed depends on various criteria that may be used in defining these functional states. The likely state of the gene can be reasonably assumed from knowledge of the tissue distribution of the protein products: β-globin and histone H5 proteins are specific for the erythrocyte; c-myc, thymidine kinase, glyceraldehyde-3-phosphate dehydrogenase and vimentin are found in numerous tissues, including the erythrocyte; and vitellogenin and ovalbumin are expressed specifically in the liver and oviduct, respectively, but not in the erythrocyte. These three functionally distinct classes of genes are described as being tissue-specific, housekeeping,

and repressed.

Two different cell preparations are used in these studies, immature erythrocytes from anaemic chickens and mature erythrocytes from normal animals (see "Tissues" section in Materials and Methods, pg. 51). With this in mind other ways of assaying the state of different genes can be described. These assays are: the presence of cytoplasmic mRNA, transcription of RNA in isolated nuclei (nuclear runoff), and DNase I sensitivity. As some genes may be negative in the first, and also (sometimes) the second, of these assays, and positive in the third, it is convenient to distinguish genes as active and/or competent in expression using these assays. Mature erythrocytes contain essentially no mRNA. Various genes in these cells are, however, sensitive to digestion with DNase I (Weintraub and Groudine, 1976, also see Figure 20). The chromatin state that is sensitive to DNase I but does not give rise to mRNA is termed transcriptionally competent. Immature erythrocytes contain high levels of mRNA, especially that of the globin genes. The globin genes of these cells, then, are transcriptionally active.

The usefulness of the active-competent distinction in chicken erythrocytes lessened somewhat when Affolter et al. (1987) showed that, in mature erythrocytes, the histone H2A, H5 and β-globin genes produced RNA in a nuclear runoff assay. This assay is thought to give an indication of the

presence of transcriptional complexes within a gene region (Marzluff and Huang, 1984). Perhaps "poised" is a better adjective for the description of the state of such chromatin.

The significance of such a finding is unclear, and several things should be borne in mind. For example, because most nascent RNA is turned over, transcriptional initiation may not be the most important stage at which the regulation of the formation of mature, cytoplasmic mRNA occurs. Also, the relationship between various proteins associated with DNA elements which are important in the regulation of transcription, and the presence of transcription complexes assayable by nuclear runoff remains obscure. Certainly, the accurate initiation and elongation of RNA transcription is a multi-component, multi-step process. It is likely that some of these components are always associated with non-repressed chromatin and may give rise to transcripts in nuclear runoff-type assays.

Although the competent-active distinction may be operationally obscure, that between competent and repressed chromatin is not. Genes specific for the terminally differentiated states of different cell lineages do not show detectable mRNA, nuclear runoff activity or DNase I sensitivity.

In this section competent gene sequences are from the β-globin domain, which contains genes for β chains of

haemoglobin. Vitellogenin, which is an egg protein produced by the liver, is taken as an example of a gene which is repressed in the erythrocyte.

A previous study (Rocha et al. , 1984) demonstrated that the β-globin gene domain was selectively enriched in the chromatin fractions eluted with low salt (50-200 mM NaCl) from micrococcal nuclease treated nuclei while transcriptionally inactive genes, vitellogenin and ovalbumin, were concentrated in the less salt-soluble fractions.

In this section the distribution and type of linker histone complexed with various gene chromatins (β-globin gene domain and vitellogenin gene) are investigated in mature chicken red blood cells. To this end, we redesigned the chromatin fractionation procedure used by Rocha et al. (1984) and studied the extraction/solubility properties of the various gene chromatins isolated from nuclei that had H1 selectively removed.

One of the questions addressed here is whether histone H5 replaces H1 in specific locations along the chromatin fibre. Mazen et al. (1982) studied the distribution of H5 along the polynucleosome chains of chicken erythrocyte chromatin with anti-H5 antibodies and immunoelectron microscopy. These investigators demonstrated that there existed histone H5- and H1- enriched domains. However, this study did not determine whether H5 molecules were

preferentially localized with specific DNA sequences.

Cole and co-workers have demonstrated that the distribution of H1 is nonuniform in chromatin (Huang and Cole, 1984; Jin and Cole, 1986). Huang and Cole (1984) separated two regions of chromatin by their solubility properties at physiological ionic strength: an aggregation-resistant region (chromatin soluble at physiological ionic strength) depleted in H1 and an aggregation-prone region (chromatin insoluble at physiological ionic strength containing more H1 than the former region). In a recent study, Jin and Cole (1986) reported that H1 exchange was limited to their respective regions: H1 exchange would not occur between aggregation resistant and aggregation-prone regions.

RESULTS

β-globin oligonucleosomes are enriched in low-salt-eluted chromatin fractions.

Previous results (Rocha et al., 1984) demonstrated that the β-globin domain was enriched in the low salt eluted chromatin fractions (50, 100 and 200 mM NaCl eluted fractions). This fractionation procedure, similar to that originally developed by Sanders (Sanders, 1978) has been modified such that the micrococcal nuclease digested nuclei were extracted directly with a 200 mM NaCl containing buffer. This treatment liberated about 10% of the 260-nm-absorbing material (Table I) which consisted predominately of mononucleosomes (Figure 5, DNA, lane c). This chromatin fraction (S0.2) was enriched in β-globin sequences (approximately 4 to 5 fold over total) containing about 45% of the total globin sequences. The vitellogenin sequences were present at approximately the same level as the bulk DNA in this fraction S0.2.

The hybridization results suggest that the monomers were extracted with a low degree of sequence discrimination as they contained both globin and vitellogenin sequences (Figure 5). The most striking observation was that, although the amount of dinucleosomes and longer chromatin fragments in the salt extract was minor, they hybridized strongly to the globin probe and minimally to vitellogenin.

An embryonic globin gene (ϵ-globin) gave the same hybridization pattern as adult β-globin (Figure 5). This observation suggested that the β-globin domain in mature chicken erythrocyte nuclei was selectively eluted as oligomer-sized chromatin fragments.

β-globin domain is associated with aggregation-resistant and aggregation-prone regions.

The 200 mM NaCl extracted fraction (S0.2) contained the four core histones (H2A, H2B, H3 and H4) and an enrichment of the HMG proteins, and a reduced amount of the H1 histones with an increased relative content of H1 to H5 (Figure 5, protein). It should be noted that HMG proteins 1 and 2, and to a lesser extent 14 and 17 are extracted from undigested nuclei at this ionic strength (Rocha et al., 1984). The mononucleosome is the predominant species in this fraction. This would explain the depletion of the H1 histones in this fraction since this particle is prone to losing its H1 histones (Jin and Cole, 1986). To ascertain the extent to which the H1 histones were associated with extracted globin oligomers, this fraction was separated into aggregation-resistant fraction (depleted in H1 histones) and aggregation-prone fraction (Huang and Cole, 1984; Jin and Cole, 1986). This was achieved by making the dialysed salt eluted fraction to 100 mM KCl (Simpson, 1978). The aggregation-prone, precipitated

fraction (S0.2 ppt.) contained both H1 and H5 with the relative content of H1 being greater than in total chromatin, and the aggregation-resistant soluble fraction (S0.2 sup.) was depleted in the H1 histones and enriched in the HMG proteins (Figure 5). The β-globin oligonucleosomes were greatly enriched (about 8-9 fold) in the precipitate. The ϵ-globin oligomers distributed in the same manner as the β-globin fragments (Figure 5).

To determine whether the 200 mM NaCl extracting buffer was causing a redistribution of the H1 histones contributing to the generation of the KCl-soluble globin chromatin, the chromatin fragments were released from micrococcal nuclease digested nuclei in a low ionic strength buffer. The chromatin fragments were split into aggregation-resistant KCl-soluble fraction and the aggregation-prone KCl-insoluble fraction. As expected, the H1 histones were depleted in the aggregation-resistant fraction (Figure 6). These results indicated that the extraction of the nuclei with the 200 mM NaCl solution was not responsible for the presence of the globin oligonucleosomes in the aggregation-resistant chromatin fraction.

Histone H1 can be selectively extracted from chicken erythrocyte nuclei.

In order to ascertain the role of H1 in the fractionation of the globin and vitellogenin sequences, we wanted a procedure which would selectively remove H1 from the chicken erythrocyte nuclei. Lawson and Cole (1979) developed a procedure which selectively released histone H1 from HeLa cell nuclei without disruption of the sequential arrangement of nucleosomes on DNA. We determined that for chicken erythrocyte nuclei resuspended in a citric acid/sodium phosphate buffer, pH 2.4, the selective removal of H1 appeared to be optimal and no detectable H5 was extracted from the nuclei. In addition to H1, HMG 14 and 17 and some high molecular weight proteins were extracted (not shown).

The integrity of the chromatin of the acid-treated nuclei was checked by electrophoretic analysis of the DNA fragment pattern after micrococcal nuclease digestion (Figure 7). Although the digested citrate/phosphate-treated nuclei yielded an electrophoretic banding pattern that was somewhat less distinct than that of control nuclei, it still clearly gave the characteristic nucleosomal DNA repeat. The initial rate of nuclease digestion of the acid-treated nuclei was greater than that of the untreated nuclei by about 2.3-fold. To further test

for nucleosome integrity after acid treatment, the treated or untreated nuclei were extensively digested with micrococcal nuclease. When the size distribution of DNA fragments was examined on agarose gels (Figure 7 B) and 4% polyacrylamide-non-denaturing gels (not shown), the monomeric-size DNA fragment predominated in both cases. Fragments migrating more rapidly than the monomeric fragment were found in the same abundance for both samples. The chromatin fragments from both samples were inspected by electrophoresis on agarose/polyacrylamide gels which resolve the nucleo-protein complexes (Figure 7 C). The H5-nucleosomes (MN2) and nucleosomes (MN1) without linker histones isolated from acid-treated nuclei migrated as discrete particles similar to their counterparts from untreated nuclei. Thus, by these different criteria, irreversible disruption of the nucleosome by this acid treatment was not occurring.

Removal of H1 alters the distribution of the globin sequences.

Following treatment with the acid buffer, the nuclei were digested with micrococcal nuclease and subsequently extracted with 200 mM NaCl. The removal of H1 did not markedly affect the amount of material that could be extracted from the nuclei (Table I) or the distribution of the β-globin and vitellogenin sequences among the various

sized chromatin fragments in the supernatant S0.2 fraction (comparing Figure 5 to Figure 8, lanes c). Note that globin, but not vitellogenin sequences were located in the extracted oligonucleosomes. The relative nuclease sensitivities of the globin and vitellogenin chromatin did not change markedly after H1 was removed (Figure 8, compare lanes a). As described above, the 200 mM NaCl extract was separated into two fractions, and the distribution of the globin and vitellogenin sequences was determined. When H1 was removed, the enrichment of the globin sequences in the aggregation-prone chromatin fraction was less pronounced compared to what was observed in the previous experiments (compare Figure 5 to Figure 8 β-globin, lanes e). This was also observed for the vitellogenin sequences in the aggregation-prone fraction, although the reduction of hybridization to the various sized DNA fragments was less acute than that observed for globin. It should be noted that the distribution of the 260-nm-absorbing material in the aggregation-prone (S0.2 ppt.) and aggregation-resistant (S0.2 sup.) fractions did not change significantly (Table I).

Table I: Distribution of Chromatin in Different Fractions

fraction	distribution of 260-nm-absorbing material (% of total)	
	+H1	−H1[a]
pellet P0.2	80.6 ± 0.5	76.2 ± 2.6
supernatant S0.2	9.3 ± 0.9	12.0 ± 2.7
KCl soluble S0.2 sup.	8.1 ± 0.1	10.2 ± 0.2
KCl insoluble S0.2 ppt.	1.2 ± 0.1	1.8 ± 0.2

[a] Nuclei treated with citric acid/phosphate buffer, pH 2.4.

Table I. The distribution of chromatin in different fractions. The absorbance of the various chromatin containing fractions at 260 nm was measured. The values given are expressed as the total absorbance of micrococcaal nuclease digested nuclei, and are expressed as means ± standard deviations for 4 different experiments.

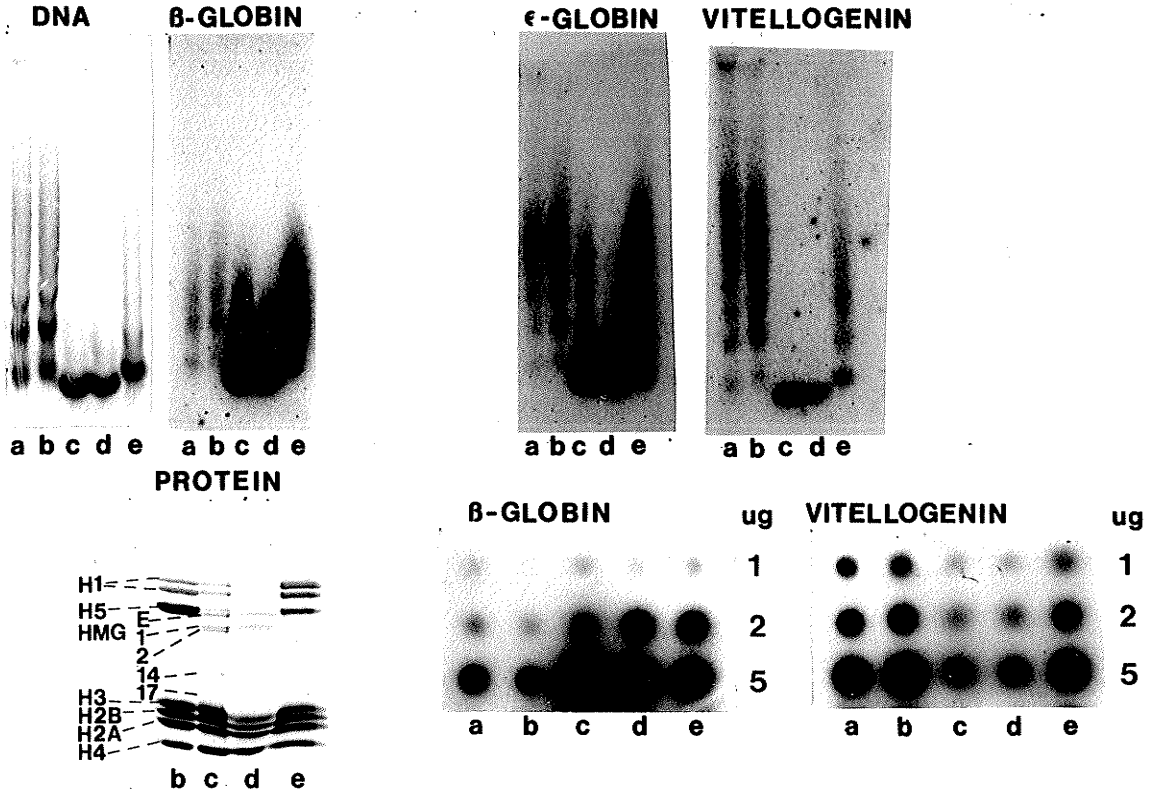

Figure 5

ß-globin domain chromatin is preferentially extracted from micrococcal nuclease digested nuclei as oligonucleosomes. Mature chicken erythrocyte nuclei were resuspended in digestion buffer to a DNA concentration of 2.5 mg/ml and incubated with micrococcal nuclease. DNA was purified from each fraction and used for Southern blot and dot blot analysis. The DNA (10 µg/lane) was electrophoresed in 1% agarose minigels and stained with ethidium bromide. Proteins were electrophoretically resolved on a SDS gel and stained with Coomassie Blue. The lanes correspond to the following: a, total; b, fraction P0.2; c, fraction S0.2 ; d, fraction S0.2 sup.; e, fraction S0.2 ppt.

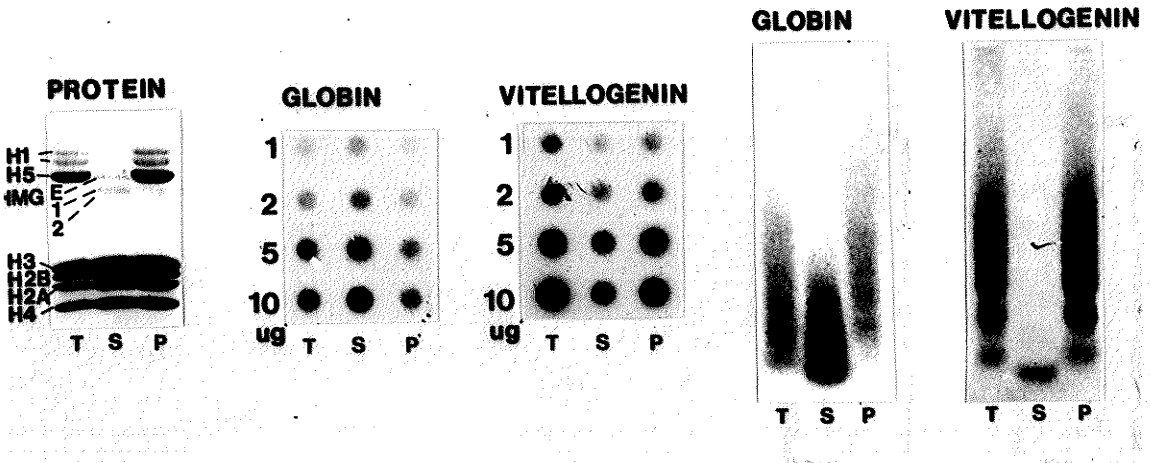

Figure 6

β-globin chromatin fragments are enriched in an aggregation-resistant, KCl-soluble fraction. Nuclei were digested as described in Figure 1 and resuspended in 10 mM EDTA, pH 7.0. EDTA solubilized chromatin (T) was made to 100 mM KCl and centrifuged. The KCl-soluble, aggregation-resistant fraction (S) contained 30% of the 260 nm-absorbing material in fraction T while the KCL-insoluble, aggregation prone chromatin (P) contained the remaining 70%. The globin probe was pCBG 14.4.

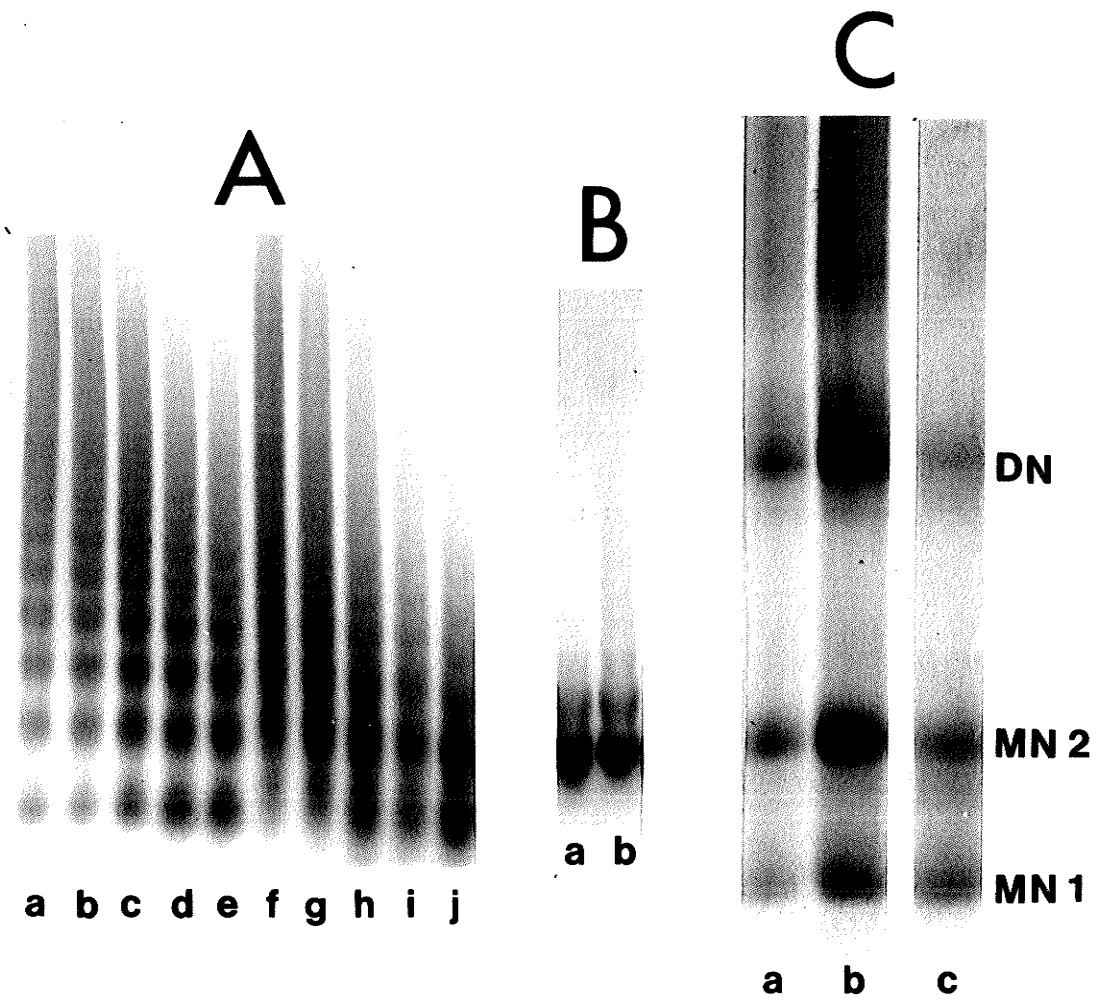

Figure 7

Structure of nucleosomes is not disrupted by treatment of nuclei with citric acid/phosphate, pH 2.4, buffer. (A) Untreated nuclei (lanes a - e) or nuclei treated with citric acid/phosphate buffer, pH 2.4 (lanes f- j) were digested for various times with micrococcal nuclease. DNA was isolated and electrophoretically resolved on a 1% agarose gel which contained ethidium bromide (1 μg/ml). Times of digestion were as follows: a, 10 min; b, 15 min; c, 20 min; d, 30 min; e, 40 min; f, 5 min; g, 10 min; h, 15 min; i, 20 min; j, 30 min. (B) Untreated nuclei (Lane b) or nuclei treated with the acidic buffer (lane a) were digested with 100 units/ml of micrococcal nuclease for 25 and 20 minutes, respectively. The DNA fragments were electrophoretically resolved. (C) Chromatin fragments from acidic buffer treated nuclei (lanes a and b) or untreated nuclei (lane c) were electrophoretically resolved on a 4% polyacrylamide/0.5% agarose composite gel. The gel was stained with Coomassie Blue. MN1 are mononucleosomes without linker histones, and MN2 are mononucleosomes with linker histones. DN are dinucleosomes.

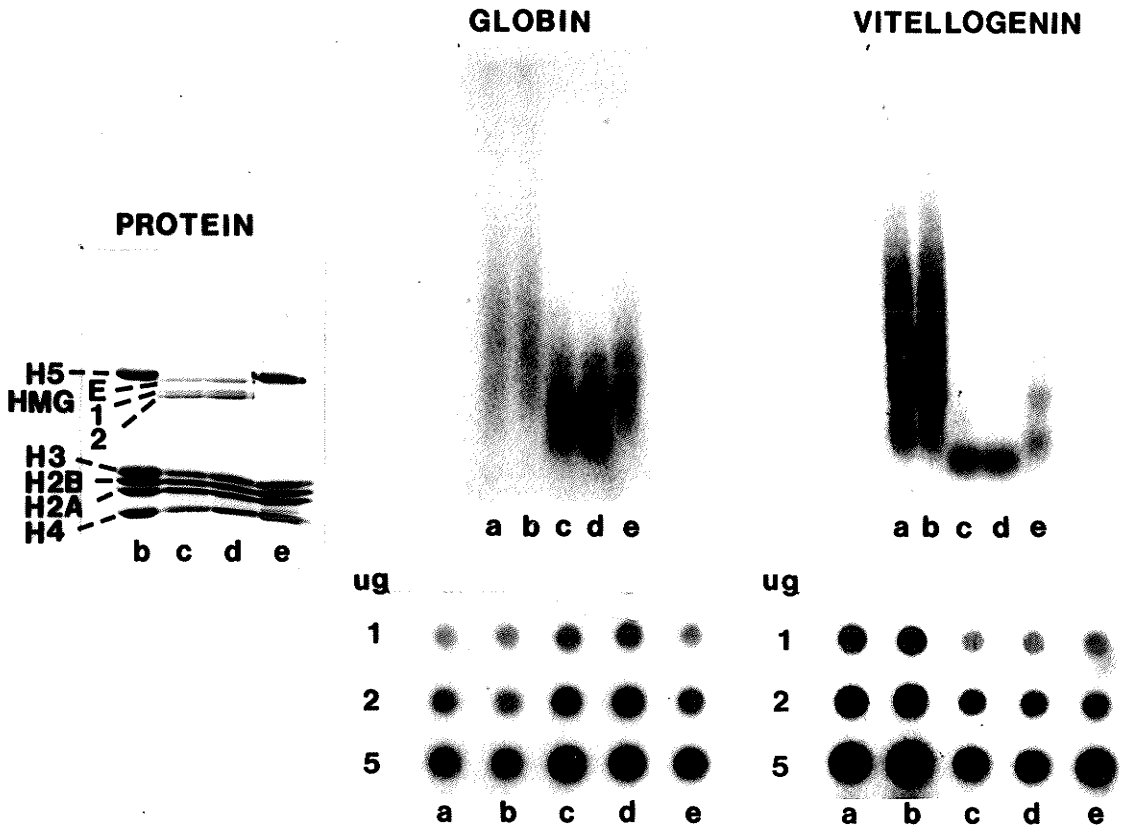

Figure 8

Fractionation of globin and vitellogenin sequences after removal of H1. Nuclei treated with the acidic buffer were digested with 50 units/ml of micrococcal nuclease for 20 min. The DNA was isolated and analyzed by Southern and dot blot analysis. The ß-globin probe used was pCBG 14.4. Protein was analyzed by SDS PAGE. The lanes correspond to the following: a. total EDTA solubilized chromatin; b, fraction P0.2; c, fraction S0.2; d, fraction S0.2 sup.; e, fraction S0.2 ppt.

DISCUSSION OF PART I

Rocha et al. (1984) previously demonstrated that the β-globin domain chromatin was preferentially extracted from mature chicken erythrocyte nuclei by low ionic strength buffers (buffers containing 50 and 100 mM NaCl). The principal nucleosome species in these fractions was the mononucleosome and it was assumed that the globin enrichment in the low-salt fractions was due to a selective extraction of competent gene mononucleosomes. Our present analysis demonstrates that, although the salt-extracted nucleosomes are primarily of monomer size, the globin nucleosomes are present as oligomers.

Our results demonstrate that the β-globin chromatin (a transcriptionally competent, DNase I sensitive gene) is present in both aggregation-prone and aggregation-resistant fractions. The aggregation-prone chromatin (fraction S0.2 ppt) was greatly enriched in globin sequences and in H1. After removal of H1 from the nuclei prior to fractionation, the extent of globin enrichment in this fraction diminished. Although the content of the vitellogenin sequences in the S0.2 ppt. fraction from the H1 stripped nuclei was less than that for untreated nuclei, the decline in vitellogenin sequences in this fraction was not as acute as that observed for globin sequences. This observation suggests that a portion of the globin domain is

present as H1-enriched chromatin regions. Our analysis indicates that the β-globin domain in mature chicken erythrocyte is a mosaic of aggregation-resistant and aggregation-prone regions. The aggregation-resistant chromatin may be either depleted in linker histones and/or contain a full complement of H1 histones which are bound differently to a chromatin which is resistant to aggregation. The aggregation-prone regions are associated with both H1 and H5. The pattern of distribution of the vitellogenin sequences is not markedly affected by the removal of H1. For example, the removal of H1 did not result in the solubilization of vitellogenin oligonucleosomes in the supernatant S0.2. Moreover, when EDTA-released chromatin from the H1-depleted nuclei was separated into aggregation-resistant and aggregation-prone fractions as described in Figure 6, globin, but not vitellogenin polynucleosomes were found in the aggregation-resistant chromatin fraction (not shown). These observations suggest the vitellogenin gene is principally associated with aggregation-prone chromatin regions which are H5 rich.

It has been suggested that the β-globin genes are selectively soluble at approximately physiological ionic strength as an indirect result of their greater nuclease sensitivity and smaller average size: the smaller fragments may lose the histones H1 and H5 to the larger

chromatin fragments (Komaiko and Felsenfeld, 1985). To test this hypothesis, chicken erythrocyte chromatin was released from micrococcal nuclease digested nuclei into low ionic strength buffers and separated into aggregation-resistant and aggregation-prone regions (100 mM KCl soluble and insoluble, respectively) which is similar to the procedure used by Jin and Cole (1986). It is important to note that the vitellogenin and globin chromatins are digested at similar rates by micrococcal nuclease (Bloom and Anderson, 1979). If the small fragments lost their H1 histones to the longer fragments, these smaller fragments would become aggregation resistant and one should observe both small globin and vitellogenin fragments in similar concentration in the KCl soluble fraction. Clearly, this did not occur (Figure 6). It is conceivable that globin chromatin is more prone to losing its H1 histones. Since we isolated salt-extracted oligonucleosomes containing H1 histones (S0.2 KCl ppt, Figure 5), this result suggests that the globin oligonucleosomes are capable of retaining their H1 histones and that there may indeed be a portion of the globin chromatin which is depleted in H1.

Ferenz and Nelson (1985) and Nelson et al. (1986) demonstrated that globin oligonucleosomes were selectively eluted from micrococcal nuclease digested immature chicken erythrocyte nuclei into solutions which were of approximately physiological ionic strength. Thus, our

results are in accord with theirs. It should be noted that the β-globin chromatin in immature erythrocyte is actively transcribed and in a micrococcal nuclease sensitive state (Bloom and Anderson, 1979). For cells used in our study, the globin chromatin may not be transcribed and has lost its sensitivity for micrococcal nuclease. (The globin chromatin is still in a DNase I sensitive state). Therefore, although the active and competent β-globin chromatin fragments differ in micrococcal nuclease sensitivities, both must share similar compositional features which allow them to be extracted as oligomers at physiological ionic strength.

In summary we find that the β-globin chromatin domain is a mosaic of aggregation-resistant and aggregation-prone regions. Such a chromatin composition may reflect a destabilization of higher-order structure which could readily explain the preferential solubility at physiological ionic strength, the altered sedimentation rates of erythrocyte β-globin chromatin (Kimura et al., 1983), and the destabilized supranucleosome structure of β-globin chromatin (Weintraub, 1984).

PART II

The greatest part of this section addresses the problem of the nature of transcriptionally competent chromatin through the isolation and characterization of a class of polynucleosomes, highly enriched in competent gene sequences, and soluble at physiological ionic strength.

Evidence is also presented which suggests that the active-gene-enriched class of chromatin has core particles which are structurally altered compared to bulk nucleosomes.

RESULTS

Chromatin fragment precipitation by NaCl as a function of concentration.

Figure 9 shows the relationship of the degree of solubility of chromatin fragments in 0.15 M NaCl as a function of their concentration. Clearly, a greater fraction of the chromatin is precipitated when the EDTA extracted material (SEDTA) is at a greater concentration.

Figure 10 shows Southern blot analyses of the salt-soluble material (S150) hybridized with probes for the histone H5 gene and the vitellogenin gene. Note that the competent histone H5 gene chromatin is soluble as polynucleosomes but that the repressed vitellogenin gene chromatin is present only as mononucleosomes.

Preparation of salt-soluble polynucleosomes.

The micrococcal nuclease digestion of the mature chicken erythrocyte nuclei was of the extent to give a release of 6.0 ± 0.5% of the 260 nm absorbing material, most of which is acid soluble, into the digestion buffer. The chromatin was solubilized in 10 mM EDTA, and this solution was made directly to 0.15 M NaCl.

In order to separate the competent-gene-enriched polynucleosomes from the bulk of the material the 0.15 M NaCl soluble fraction was subjected to gel filtration. The

Bio-Gel A5m column profile of the chromatin fragments soluble in 0.15 M NaCl and the four fractions pooled are shown in Figure 11. The major peak occurs at fraction number 93 at 3.83 A_{260} units/ml. The four pooled fractions (I, II, III, and IV) contained 6.9, 10.4, 19.8 and 62.9%, respectively, of the 260 nm absorbing material loaded on the column. The fragment sizes in the column fractions are as follows: fraction I, larger than octamers; fraction II, dimers to about dodecamers; fraction III, mainly mononucleosomes; and fraction IV, only core sized mononucleosomes (Figure 12, DNA).

Salt-soluble polynucleosomes are highly active-gene-sequence enriched.

The DNA from the pooled column fractions was resolved on agarose gels and corresponding hybridization results are shown in Figure 12. The autoradiographic intensity of CBG 13 (β-globin) hybridization for the column fraction I is well beyond the saturation level of the film when most of the other fractions are not yet visible. In order to better quantify the extent of hybridization in each of these fractions, gels were loaded with varying amounts of chromatin and the autoradiograms were measured by densitometry as described in the Materials and Methods. The column fraction "I" was found to be almost 50-fold enriched over total for β-globin sequences (see Table II).

CBG 18.7 (ϵ-globin) gives essentially the same pattern (Figure 12). Identical blots probed with vitellogenin show that the 0.15 M NaCl soluble material contains little of these sequences. Measurements of the extent of vitellogenin hybridization in the 0.15 M precipitate indicated that this fraction contained the same enrichment of these sequences as the EDTA-released material (within the limits of the precision of the measurement technique).

The protein content of the salt-soluble polynucleosomes.

The protein composition of the various fractions were analyzed on AUT gels (Figure 13) and on two dimensional (AUT into SDS) gels (Figure 14). The first column fraction was highly enriched in the modified histone species including the acetylated species of H4 (Figure 14, lane d and Figure 13), H2A.Z (also called M1 and H2A.F (Urban et al. 1979; Sung et al., 1977) (Figure 15) and H2B (Figure 14; note that in panel C, H2B trails toward the anode on the AUT gel). We have made these assignments based on the following information: firstly, Sung et al. (1977) demonstrated that, in mature erythrocytes, histone phosphorylation and methylation cease to occur; secondly, Nelson et al. (1986) has shown that all of the erythrocyte nucleosomal histones, including some of the histone variants, can be acetylated; thirdly, Pantazis and Bonner (1981) reported that mouse H2A.Z had three acetylated species but no phosphorylated forms; and, fourthly, the ubiquitinated histone species have been identified as such with an anti-ubiquitin antibody (Nickel and Davie, 1989). The enrichment of uH2B is especially striking since in this fraction its content is similar to that of uH2A (Figure 14, panel C and Figure 15). The content of uH2A in chromatin is typically three fold greater than that of uH2B (Figure 14, panel A and Finley and Varshavsky, 1985). The first

column fraction appears to be enriched in the histone variants H2A.Z and H3.3 (compare panel C to panel A in Figure 14 and Figure 15). We are concerned that the enrichment of H3.3 may be an artifact due to oxidation. In other analyses, however, where oxidation does not seem to be apparent, increased amounts of H3.3 were observed. Quantitation by densitometry of Coomassie Blue stained gels was carried out for those proteins which could be sufficiently resolved by electrophoresis. The results are given in Table III.

The content of the modified histone species diminished as the size of the chromatin fragments decreased (Figure 13, lanes d to g). For column fraction IV (Figure 13, lane g) the level of modified histone forms was similar to that in unfractionated chromatin (Figure 13, lane a). It can also be seen on the AUT gel (Figure 13) that the HMG proteins 14 and 17, which are enriched in the 0.15 M NaCl-soluble fraction are preferentially bound to smaller nucleosome oligomer-, monomer- and core particle sized species.

A number of proteins, which are present in the 0.15 M NaCl soluble fraction, are absent from the first column fraction (compare panels B and C in Figure 14). This indicates that they are not bound to these polynucleosomes at this ionic strength. These proteins include the HMG proteins e, 1 and 2; and include a protein (shown by the

arrow in Figure 14 panel B) which, to our knowledge, is heretofore undescribed and uncharacterized. This species has a mobility similar to H1a on SDS PAGE and is present in soluble chromatin prepared by different salt fractionation schemes (Rocha et al., 1984; Komaiko and Felsenfeld, 1985). It is likely that this species has been misidentified as H1a leading to the suggestion of the enrichment of this histone in certain active-gene-enriched fractions (Komaiko and Felsenfeld, 1985). The peptide map of this protein indicates that it is not similar to any of the linker histones (not shown).

In experiments where the EDTA solubilized chromatin (SEDTA) polynucleosomes were obtained by gel filtration on Bio-Gel A5m in low ionic strength and subsequently precipitated with 0.15 M NaCl (inverting the order of salt precipitation and gel filtration), a similar degree of globin-sequence enrichment is found in the soluble polynucleosomes. Under these conditions, however, these proteins (HMG e, 1, 2 and others) are found in the supernatant fraction (not shown). This indicates that these proteins are not bound to chromatin fragments at physiological ionic strength but are bound under low ionic strength conditions and that their presence in salt-soluble chromatin may be the result of indiscriminate extraction.

In order to compare the content of the linker histones

of the polynucleosomes in the column fraction I, the histones isolated from this fraction and from total chromatin were electrophoretically resolved on a SDS polyacrylamide gel (Figure 16). The salt-soluble polynucleosomes had a partial depletion in the linker histones, containing about 70% of the total complement of linker histones associated with unfractionated chromatin.

Core particles of salt-soluble polynucleosomes are sensitive to ethidium bromide-induced dissociation.

McMurray and van Holde (1986) reported that binding of ethidium bromide to chicken erythrocyte core particles results in a dissociation of the structure which is reversible, time dependent and independent of DNA concentration. Total dissociation was complete within 25 h. The electrophoretic analysis described in this section was carried out by Dr. J. Davie. Nucleosome core particles were prepared from total and salt-soluble polynucleosomes by removing the linker histones with CM - Sephadex and digesting the stripped fragments with micrococcal nuclease. The DNA fragment size of the core particles thus prepared is shown in Figure 17. The extent of dissociation of nucleosome core particles of total (T) or salt-soluble polynucleosomes (I) at various concentrations of ethidium bromide was determined by electrophoretic analysis (Figure

17). In the absence of ethidium bromide both types of core particle migrated as a single band. At an ethidium bromide:core particle base-pair molar ratio of 0.10 a dissociation of the T core particles occurs to a small extent as seen by the appearance of free DNA. McMurray and van Holde (1986) calculated a 6% level of dissociation under these conditions. In contrast, at least 50% of the I core particles have dissociated. Increasing the ratio of ethidium bromide:base pair DNA to 0.3 resulted in the complete dissociation of the I particles and approximately 10% of the T particles.

Initially the core-particle - ethidium bromide complexes were incubated for approximately 15 h before analysis. Reduction in the incubation time did not alter the extent of dissociation of the I particles. Moreover, at ratios of 0.3 complete dissociation was observed when electrophoretic analysis was carried out immediately after mixing, indicating that ethidium bromide induced dissociation of I core particles is essentially immediate.

Core particles of salt-soluble polynucleosomes are no more reactive with N-ethyl[^{3}H]maleimide than bulk core particles.

Various reports (Chan et al., 1988; Allegra et al., 1987) have indicated that nucleosomes associated with active/competent genes have an enrichment of solvent

accessible thiol groups. These thiol groups are presumed to be those of cysteine 110 of histone H3 and, the increase reactivity may represent an open nucleosome structure. To probe the thiol reactivity of histone H3 of the salt-soluble-polynucleosomes, core particles prepared from this fraction, and from fraction P150 (taken as a representation of inactive chromatin), were reacted with N-ethyl[^{3}H]maleimide, essentially as described by Wong and Candido (1978). (see Materials and Methods section pg 59). These experiments were carried out with the assistance of Mr. Patrick Fredette.

Results of a typical experiment are shown in Figure 18. This figure shows the increasing accessibility of the H3 thiol as the nucleosome dissociates in increasing salt concentration, and is very similar to the result obtained by Wong and Candido (1978). There is little difference in the reactivities of the two types of core particles with N-ethyl[^{3}H]maleimide. Indeed, the particles from fraction P150 are more reactive with this chemical probe. This result is contrary to the expectation that a more open nucleosome structure, associated with competent chromatin, would be more accessible to N-ethyl[^{3}H]maleimide.

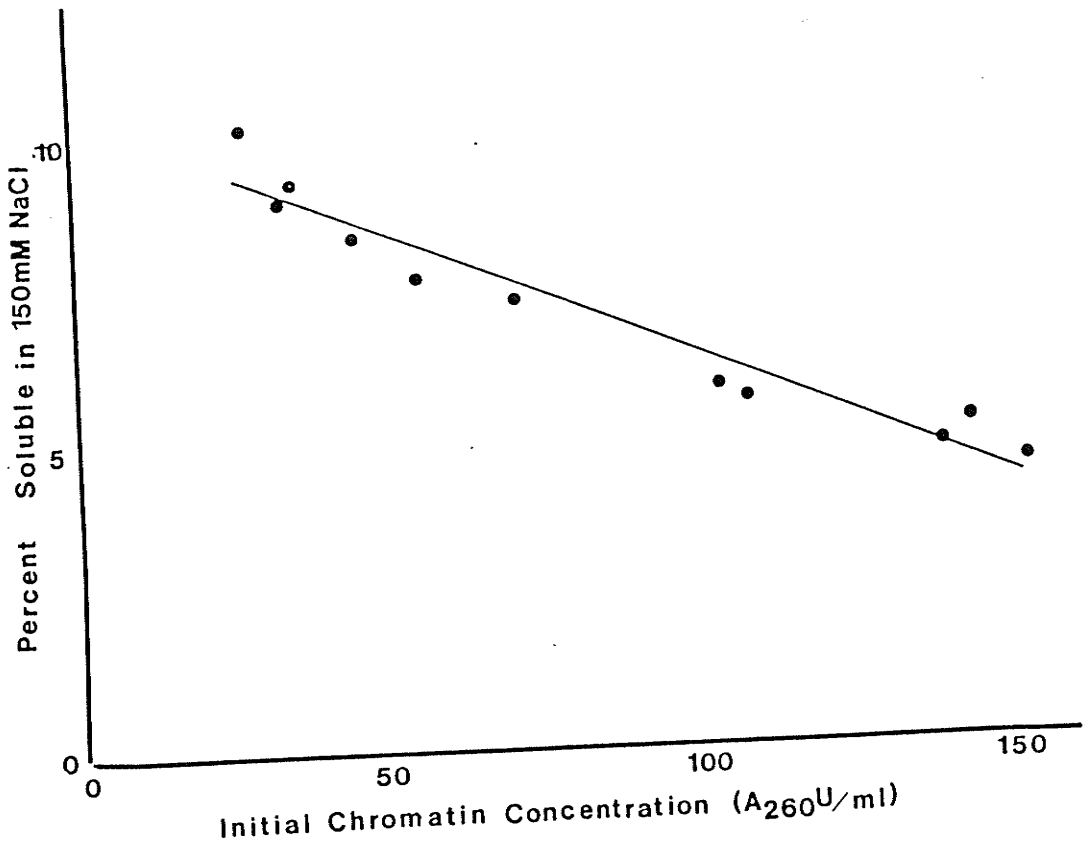

Figure 9

The extent of salt induced precipitation is influenced by the initial concentration of chromatin. Chromatin solublized from micrococcal nuclease digested nuclei by 10 mM EDTA at a concentration of 145 A_{260} units/ml was diluted with 10 mM EDTA to various concentrations and then made to 0.15 M NaCl. The resulting precipitate was collected by centrifugation and the chromatin concentration in the supernatant was determined and expressed as a percentage of the amount before precipitation.

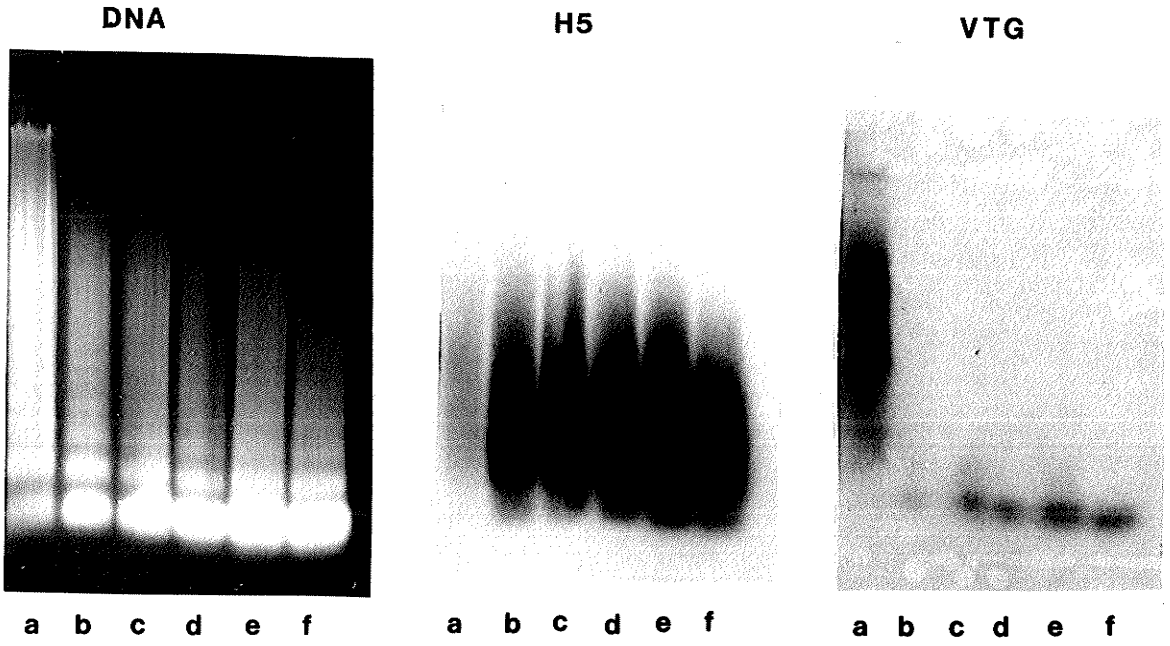

Figure 10

Histone H5 gene polynucleosomes are soluble at physiological ionic strength. Solubilized chromatin (SEDTA, lane a) was made to either 29, 48, 72, 109 or 145 A_{260} units/ml. Each of these samples was made to 0.15 M NaCl and the resultant precipitate was collected by centrifugation. The supernatants (lanes b to f) were dialyzed against water and used for electrophoresis on 1% agarose mini-gels, transferred to nitrocellulose filters and the DNA was hybridized to detect the indicated sequences (H5, histone H5; VTG, vitellogenin).

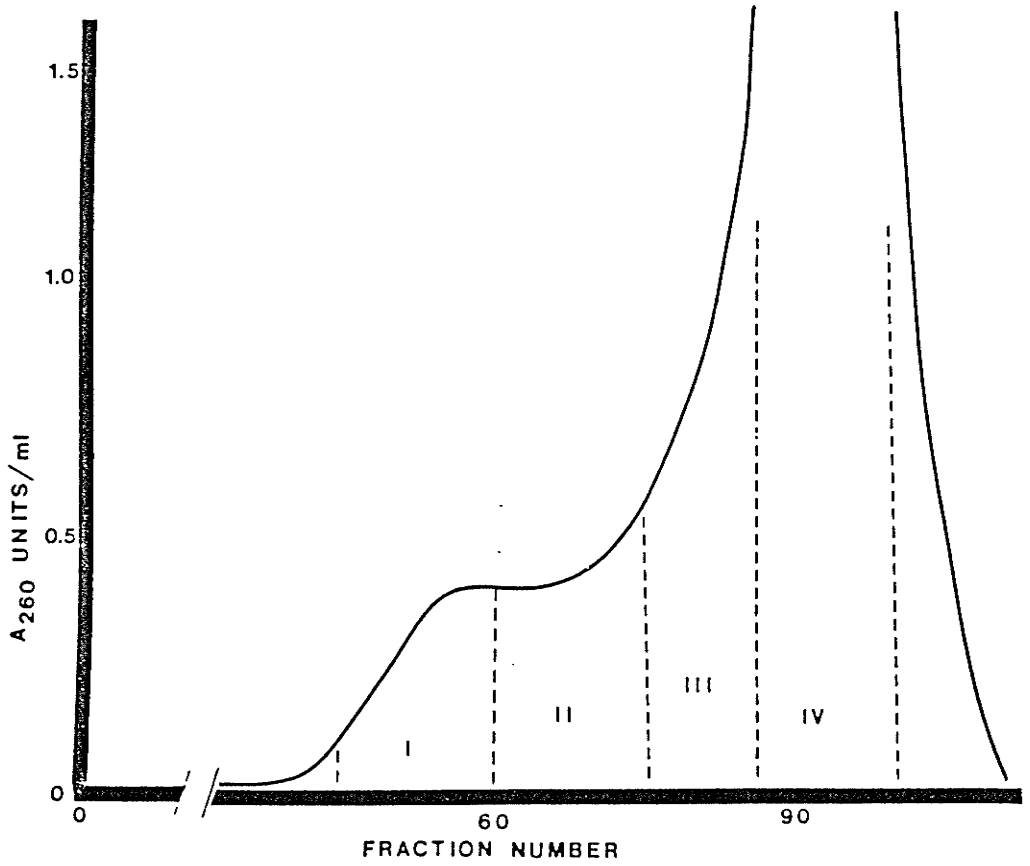

Figure 11

Gel exclusion chromatography of aggregation-resistant chromatin fragments. Aggregation resistant chromatin fragments were loaded on a Bio-Gel A5m column equilibrated with 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The four pooled fractions (I, II, III and IV) are shown.

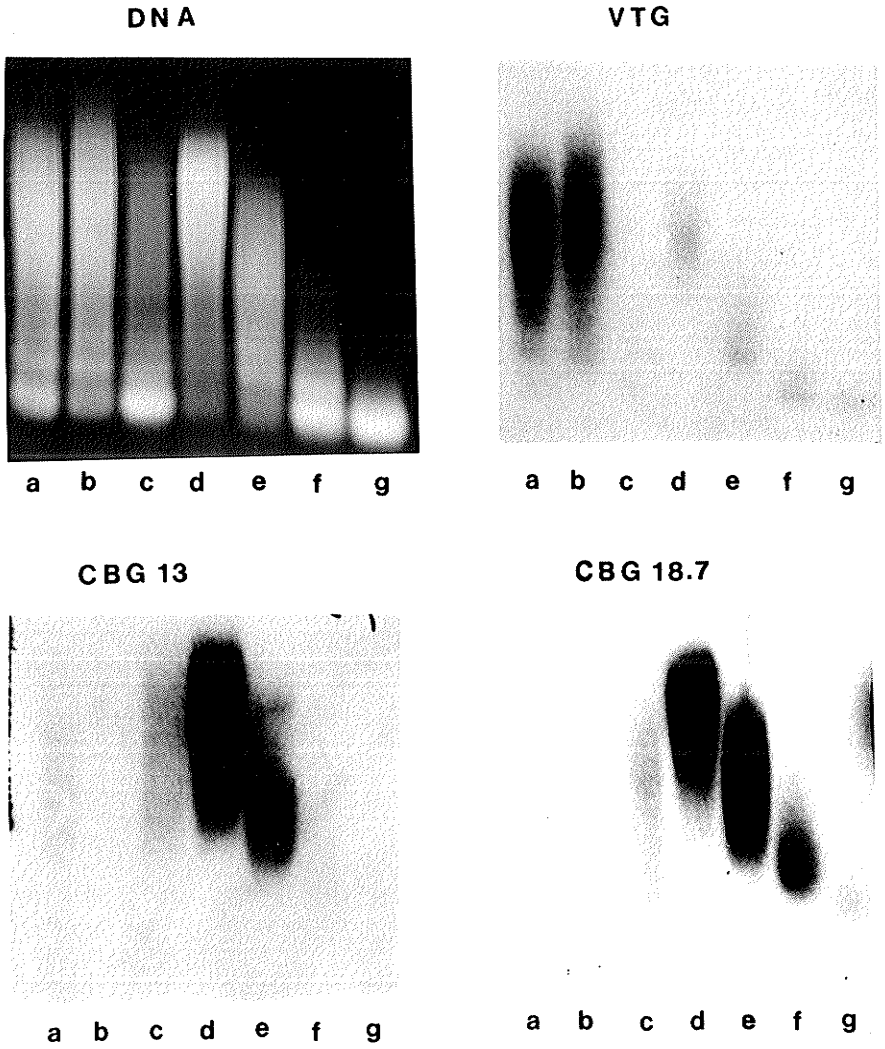

Figure 12

Polynucleosomes that are soluble at physiological ionic strength are highly enriched in β-globin sequences. DNA fragments isolated from unfractionated, EDTA released, chromatin (SEDTA, lane a), 0.15 M NaCl precipitate (aggregation-prone chromatin, lane b), 0.15 M NaCl supernatant (aggregation-resistant chromatin, lane c) and Bio-Gel A5m column fractions I, II, III and IV (lanes d, e, f and g, respectively) were electrophoretically resolved on a 1% agarose gel. After transfer to nitrocellulose filters the DNA was hybridized to the indicated sequences (CBG 13, β-globin; CBG 18.7, ϵ-globin; VTG, vitellogenin).

TABLE II

Fraction	Globin Sequences (% of SEDTA)	Enrichment Over Total
SEDTA	100	1.0 X
150 mM NaCl sup.	58	5.4 X
150 mM NaCl ppt.	42	0.5 X
Column fraction "I"	34	48.2 X

Table II β-Globin sequence distribution in fractionated chromatin. The extent of hybridization to probe pCBG 13 of the various chromatin fractions was determined as described in the Materials and Methods section. Enrichments are expressed relative to the SEDTA hybridization signal.

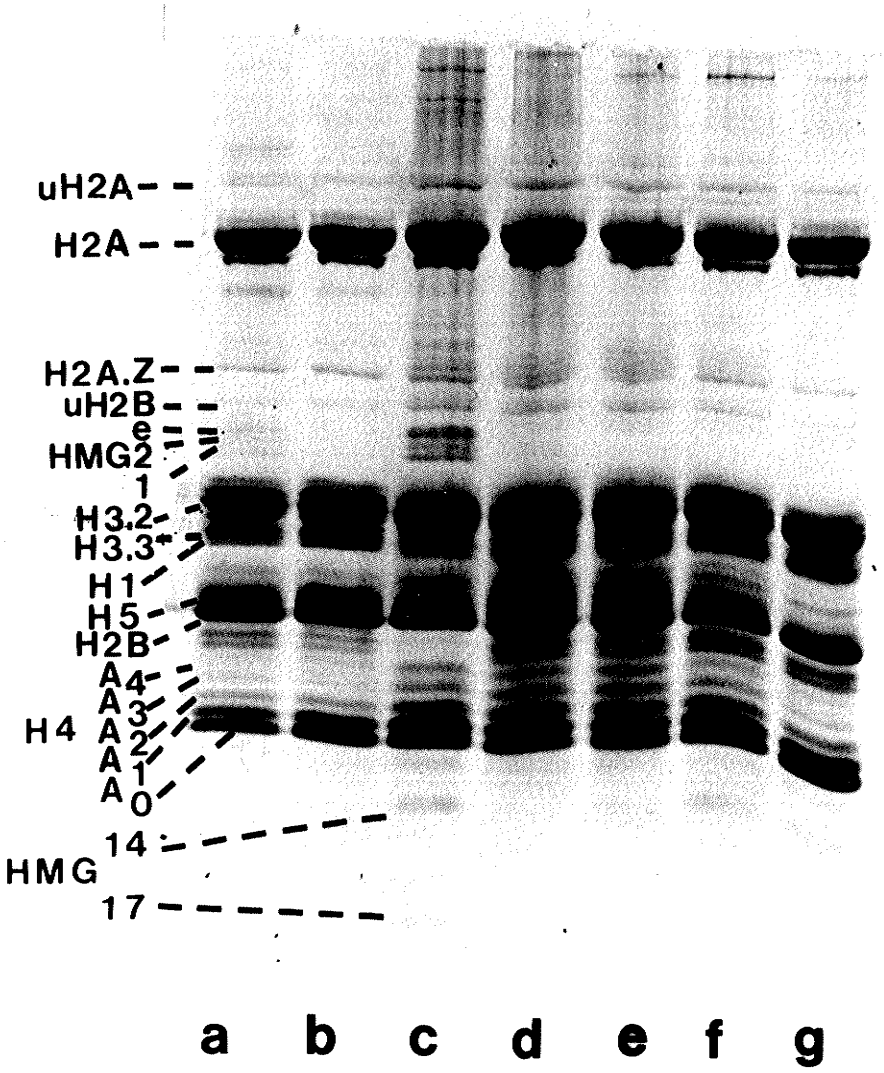

Figure 13

Analysis of the proteins present in the various fractions. Proteins isolated from unfractionated chromatin (SEDTA, lane a), 0.15 M NaCl precipitate (aggregation prone chromatin, lane b), 0.15 M NaCl supernatant (aggregation resistant chromatin, lane c) and Bio-Gel A5m column fractions I, II, III and IV (lanes d, e, f and g, respectively) were electrophoretically resolved on an acetic acid / 6.7 M urea / 0.375% Triton X-100 (AUT) 15% mini-slab polyacrylamide gel. The gel was stained with Coomassie Blue. A_0, A_1, A_2, A_3 and A_4 denote the un-, mono-, di-, tri-, and tetra-acetylated species of H4, respectively. The "u" prefix is used to indicate the ubiquitinated forms of H2A and H2B.

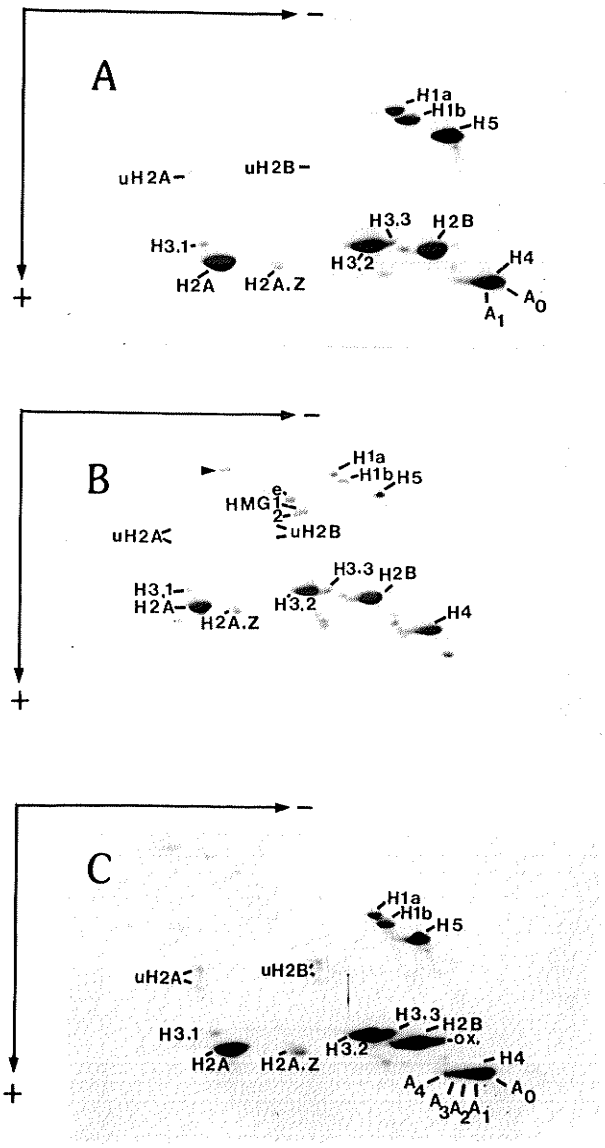

Figure 14

Two-dimensional gel electrophoresis of proteins present in various chromatin fractions. Proteins from unfractionated chromatin (A), aggregation-resistant chromatin (0.15 M NaCl supernatant, B), and polynucleosomes (column fraction I, C) were separated by two-dimensional gel electrophoresis (AUT into SDS). The gels were stained with Coomassie Blue. A_0, A_1, A_2, A_3, and A_4 denote the un-, mono-, di-, tri-, and tetra-acetylated species of H4, respectively. The "u" prefix is used to indicate the ubiquitinated forms of H2A and H2B.

TABLE III

Histone Type	Enrichment Over Total
uH2A	1.3 X
H2A.Z	2.8 X
uH2B	3.7 X
Acetyl H4	2.4 X
H1 & H5	0.7 X

Table III Comparison of the protein content of the salt-soluble polynucleosomes with total chromatin. Protein content was determined by densitometry of AUT and SDS PAGE gels stained with Coomassie Blue. The amount of the variant and modified H2A and H2B were compared as a percent of total H2A or H2B in each fraction. Acetylated H4 is compared as an weighted sum of total acetylation as described under Materials and Methods. Linker histones are compared as a ratio of the total of H1 and H5 and the total of core histone.

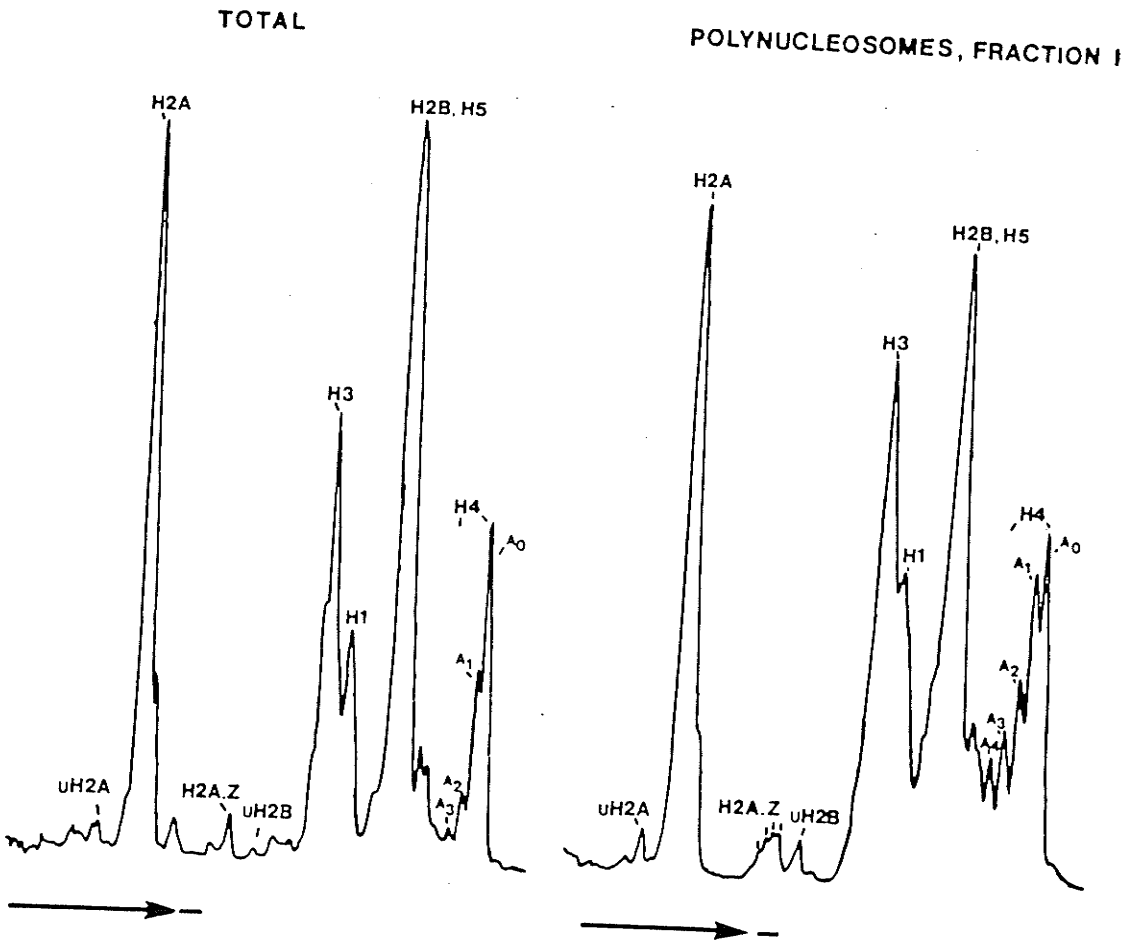

Figure 15

Densitometer scans of the AUT gel electrophoretic patterns. Proteins isolated from the unfractionated chromatin and the polynucleosome (column fraction I) were resolved on AUT gels and stained with Coomassie Blue. Densitometer scans of the electrophoretic patterns are shown. Modified species of H2A.Z are indicated by lines above the scan.

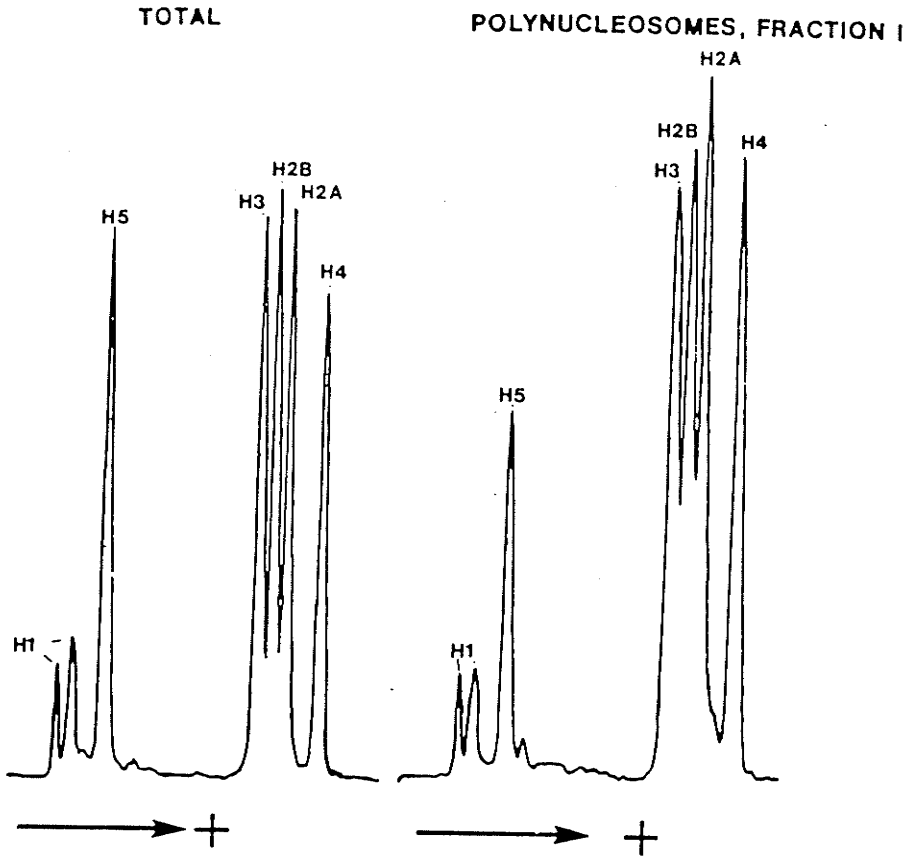

Figure 16

Densitometer scans of the SDS gel electrophoretic pattern. Proteins isolated from unfractionated chromatin (Total) and polynucleosomes (column fraction I) were resolved on SDS gels and stained with Coomassie Blue. Densitometer scans of the electrophoretic patterns are shown.

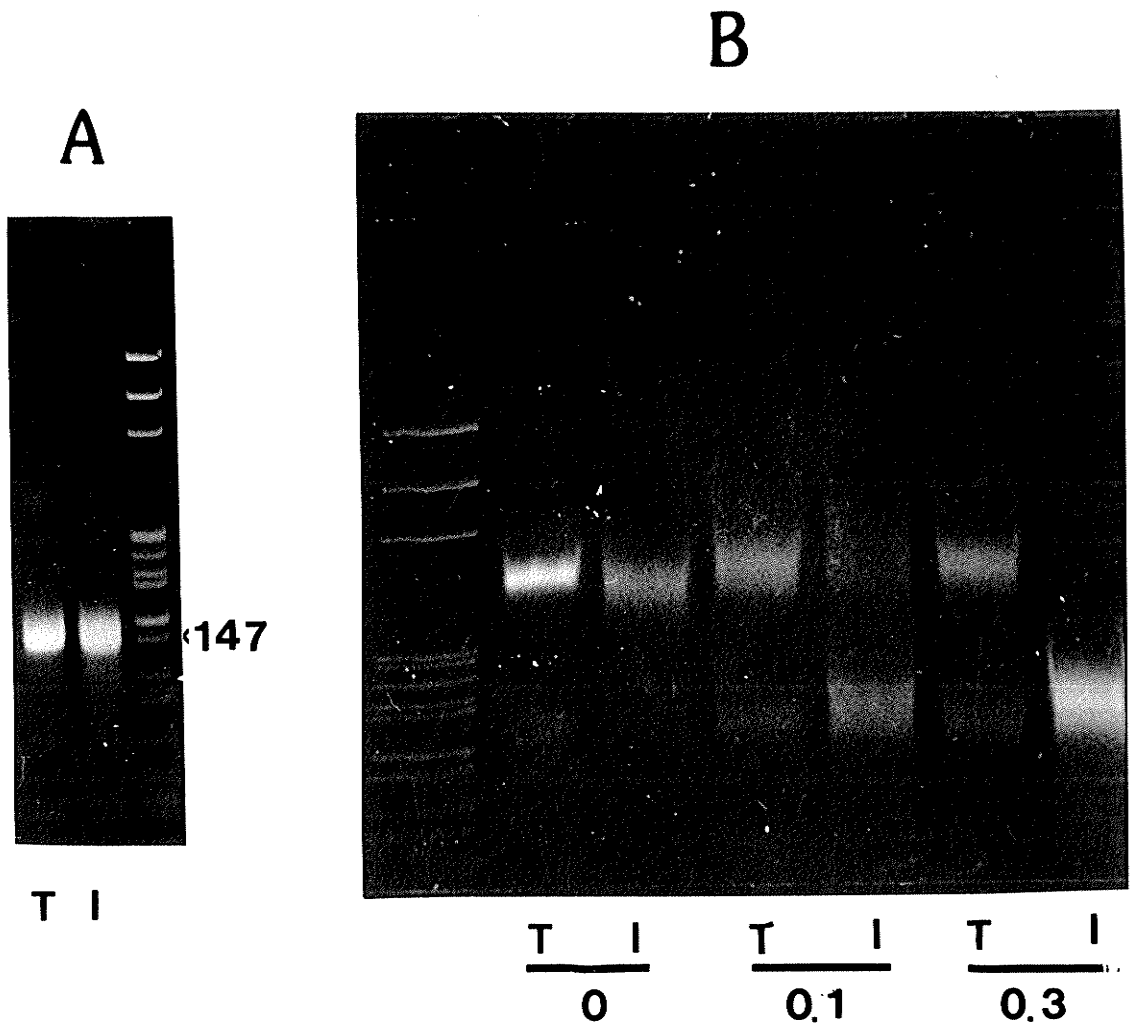

Figure 17

Core particle dissociation by ethidium bromide. **A:** 4.5% polyacrylamide gel electrophoresis of DNA from the core particle preparation of total (T) chromatin and salt-soluble polynucleosomes (I). **B:** Electrophoretic analysis of core particles preincubated with ethidium bromide. The number beneath each pair of lanes is the molar ratio of ethidium bromide:base pair DNA.

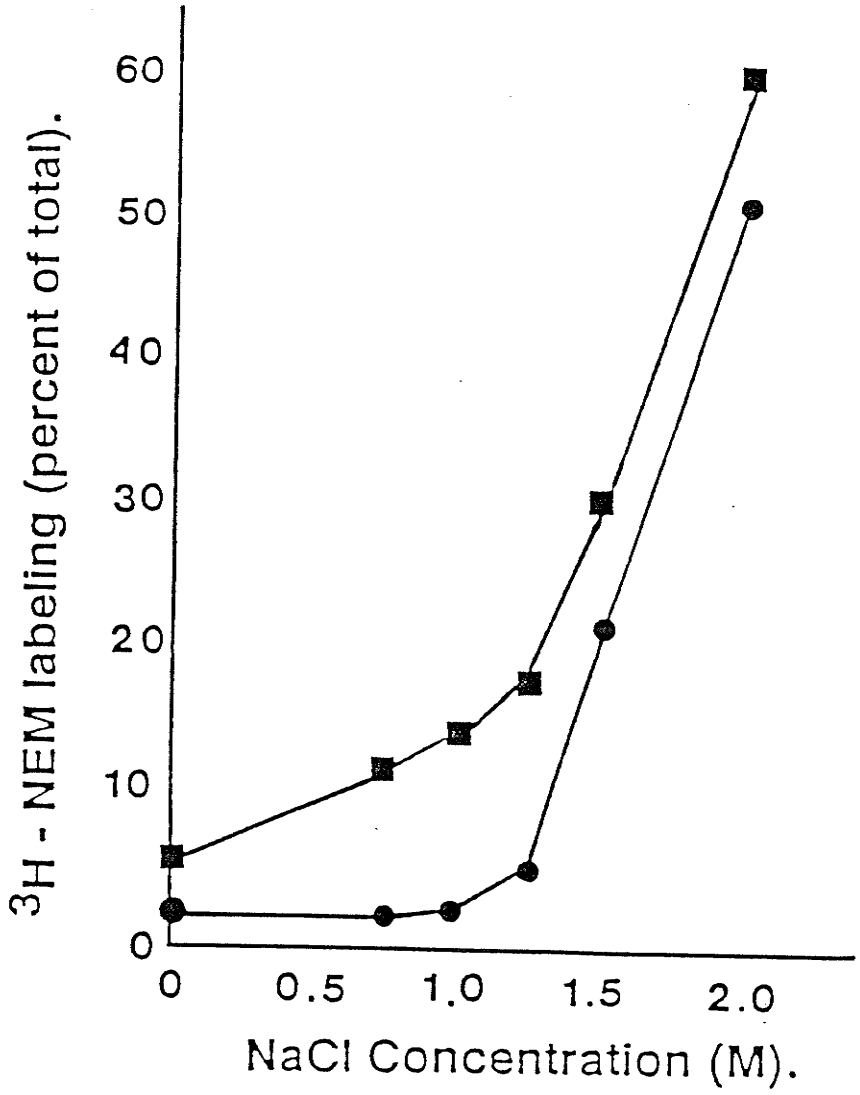

Figure 18

Core particle structures were probed by reaction with N-ethyl[^{3}H]maleimide. Core particles from column fraction "I" (●) and from fraction P150 (■) were reacted with N-ethyl[^{3}H]maleimide as described in the Material and Methods section (pg. 60).

DISCUSSION OF PART II

This series of experiments has been designed to optimize the solubilization of chromatin from micrococcal nuclease digested nuclei. The buffer used for micrococal nuclease digestion was designed so that no nucleosome sized material escapes from the nuclei. The solubilized nucleoprotein used represented about 95% of the total DNA of digested nuclei, as measured by the diphenylamine assay (Giles and Myers, 1965).

Most chromatin soluble in 10 mM EDTA is precipitated in 0.15 M NaCl. Of the 10 - 11% that remains soluble, most is monomer and core sized particles. Size fractionation of this soluble material indicates that the largest polynucleosomes are the most highly enriched in β- and ϵ-globin gene sequences. This suggests a size dependency of chromatin solubility at physiological ionic strength: shorter fragments are soluble mainly as a function of their size while longer fragments are soluble because of other distinct properties. This can be seen easily by examination of the globin hybridization pattern to any of the fractions soluble in 0.15 M NaCl (see Figures 12 and 10). The degree of hybridization increases substantially toward the top of the gel, where the bulk DNA distribution decreases (see Figure 12). Differences in the size dependent efficiency of hybridization alone will probably

not explain this.

A certain degree of caution must be exercised when interpreting the results of chromatin fractionation based on salt solubility because of the exchange of linker histones amongst chromatin fragments at even slightly elevated ionic strengths, and the preferred binding of these histones to longer oligonucleosomes (Caron and Thomas, 1981; Thomas et al., 1985; Thomas and Rees, 1983). Komaiko and Felsenfeld (1985), for example, found that the preferential solubility of globin-chromatin was lost as a function of the degree of redigestion of the salt-insoluble fraction when remixed with the soluble fraction. They concluded that the enrichment of globin-sequences in the salt-soluble chromatin was due to its micrococcal nuclease sensitivity and the loss of linker histones from the resultant shorter average sized fragments to longer ones. Jin and Cole, 1986, have directly addressed the problem of linker histone exchange and its possible influence on chromatin partitioning by salt-induced precipitation. They have shown that H1 histone in HeLa cell chromatin does not substantially equilibrate between soluble and insoluble polynucleosome fractions (approximately octamer size and greater) and conclude that these chromatin fractions are inherently different. The results presented here also support the model of inherent differences in chromatin types giving rise to differences in solubility. Our

finding that the longest salt-soluble polynucleosome fraction is the most enriched in the globin gene, for example, runs counter to the argument that globin gene-chromatin is soluble due to greater nuclease sensitivity and resultant shorter mean fragment size.

The globin-gene-enriched polynucleosomes were associated with hyperacetylated species of H4, H2A.Z and H2B, the ubiquitinated forms of H2A and H2B (particularly uH2B), and a partial depletion of H1 and H5. This chromatin fraction may also contain elevated levels of H2A.Z and H3.3. No evidence was found for a depletion of any of the nucleosomal histones including H2A and H2B.

It is interesting to note that H2A.Z and H3.3 have been shown to be sythesized throughout the cell cycle (Wu and Bonner, 1981) and would be in relative excess to other histones outside of S-phase. Their presence in active chromatin could be the result of exchange of histones that may occur during transcription and/or deposition on early replicating genes.

To what extent histone acetylation, linker histone depletion or other factors may contribute to the preferential solubility of the β-globin chromatin is, in part, the subject of Part IV of the current work. Ferenz and Nelson (1985) and Nelson et al. (1986) demonstrated that the preferential solubility of β-globin was dependent on both the acetylated state of the β-globin chromatin

and the presence of Mg^{+2}. Their results are in agreement with those of Perry and Chalkley (1981) who demonstrated that, in the presence of Mg^{+2}, histone acetylation increased the solubility of chromatin fragments. Since we are fractionating chromatin in the absence of Mg^{+2}, histone acetylation may not be responsible for the selective solubility of the globin enriched polynucleosomes. It is probably most economical in terms of explanation to ascribe the greatest contribution to determining the solubility of chromatin fragments in 0.15 M NaCl to the linker histones. Clearly, linker histones are required for NaCl-induced chromatin aggregation for, in their absence, chromatin fragments remain soluble (Jin and Cole, 1986; Davie et al., 1986; Huang and Cole, 1984). The partial depletion in histones H1 and H5 of the globin enriched polynucleosomes may explain why these chromatin fragments are soluble in 0.15 M NaCl. In vivo, histone acetylation, histone ubiquitination and reduced content of linker histones may all make important contributions to destabilizing higher-order structure and to promoting solubility of the β-globin domain. Since acetylation-deacetylation (Nelson et al., 1986; Jin and Cole, 1986; Brotherton et al., 1981), ubiquitination-deubiquitination (Finley and Varshavsky, 1985; Wu et al., 1981; Seale, 1981), and, perhaps, the association of linker histones with various regions of chromatin are in a dynamic equilibrium, the maintenance of

the altered structure of the β-globin domain should be considered as a dynamic process.

Structural alterations of nucleosome core particles in salt-soluble polynucleosomes.

Nucleosome structure of the salt-soluble polynucleosomes was analyzed by ethidium bromide-induced dissociation. The nucleosome core particles derived from the salt-soluble polynucleosomes were sensitive to ethidium bromide-induced dissociation. Complete dissociation of these core particles occurs at a much lower stoichiometric ratio of ethidium bromide : base pair of DNA compared to those required for bulk core particles (0.3 vs 2.0 (McMurray and van Holde, 1986)). It is possible that the increased sensitivity to ethidium bromide induced dissociation may be due, in part, to increased levels of acetylation. Recently Oliva et al. (1987) have demonstrated that histone hyperacetylation is associated with an elongated shape of the nucleosome. Also, several studies indicate that hyperacetylation loosens the histone - DNA contacts which may facilitate the ethidium bromide induced dissociation process (discussed in the Introduction, pg. 48).

In contrast to the results obtained using ethidum bromide dissociation of particles, labelling with NEM is

not able to distinguish major structural differences between the core particles of the active-gene-enriched salt-soluble polynucleosomes and bulk nucleosomes. This result suggests that the active-gene-enriched chromatin particles are no more open in the $(H3\text{-}H4)_2$ tetramer region than is bulk chromatin.

Other observations have shown that active chromatin may be more reactive to thiol reagents; and, although the reasons for this are not clear some possible explanations may be speculated upon. Chan et al. (1988), for example, demonstrated preferred reactivity to a thiol specific probe in active-gene-enriched chromatin. The thiol reactions of these studies were carried out in intact nuclei. It is possible, for example, that the continuity of the DNA strand in the intact nucleus may subject the nucleosome to different stresses than those that would occur in fragments in solution. In this way some aspects of nucleosome structure may only be distinguished by chemical probes while still within intact nuclei.

PART III

This section deals with two main subjects. The first is the relationship between the in situ state of chromatin and the active-gene-enriched salt-soluble polynucleosome fraction described in Part II. To this end the chromatin of several genes was assayed for sensitivity to DNase I and for solubility as polynucleosomes in 0.15 M NaCl. The degree of solubility of chromatin fragments as polynucleosomes in 0.15 M NaCl correlates well with the sensitivity to DNase I for several genes. Chromatin of repressed, housekeeping and erythroid-specific genes can be distinguished as distinct groups by the degree to which they display these properties.

The second main subject of this section is the relationship of linker histones with competent-gene-containing chromatin. To investigate this, NaCl precipitation of chromatin fragments stripped and then reconstituted with varying quantities of H1 and H5 (linker) histones was carried out. The results indicate that the polynucleosomes of erythroid-specific genes have altered interaction with these histones. In bulk chromatin and in the chromatin of the repressed ovalbumin and vitellogenin genes, the linker histones appear to interact cooperatively in the formation of salt precipitable structures.

Chromatin of erythroid-specific genes (histone H5 and β-globin) as well as that of the histone H2A.F gene is resistant to this type of interaction.

RESULTS

The level of polynucleosome solubility defines distinct classes of developmentally regulated genes.

Mature chicken erythrocyte chromatin fragments were separated into two fractions based on their solubility properties in 0.15 M NaCl. The salt-soluble chromatin fraction consists primarily of mononucleosomes while the majority of the polynucleosomes are salt-insoluble. In Part II it was demonstrated that the small percentage of polynucleosomes, which are salt-soluble, are highly enriched in β-globin gene sequences. Figure 19 shows the hybridization of a variety of additional cloned DNA sequences to fractions obtained by chromatin precipitation with 0.15 M NaCl. Examination of the extent of hybridization in the oligonucleosome region of the salt-soluble chromatin lanes reveals three distinct classes: (1) class 1 gene chromatin, which includes H5 and H2A.F gene sequences, is enriched several fold over total in the salt-soluble oligonucleosomes, (2) class 2 gene chromatin, which includes c-myc, vimentin and thymidine kinase, is not enriched, but shows a limited extent of hybridization to salt-soluble oligonucleosomes, and (3) class 3 gene chromatin, which includes the repressed ovalbumin gene sequences, is virtually absent in the salt-soluble oligonucleosomes.

Nuclei were digested with DNase I as described by Villeponteau et al. (1984). The digested DNA was placed onto nitrocellulose and hybridized to the indicated gene probe. The intensities of hybridization were quantified by densitometer scanning and expressed as a percentage of ovalbumin probe hybridization (Villeponteau et al., 1984). The correlation between DNase I sensitivity and salt solubility properties of the chromatin of the various genes is shown in Figure 20. Class 1 gene chromatin (e.g., H5, β-globin and H2A.F gene chromatin), which is potentially transcriptionally active (Harvey et al., 1983, Affolter et al., 1987), has the greatest sensitivity to DNase I as well as greatest salt-solubility suggesting a relationship between these two properties. Class 2 gene chromatin (e.g., that of the thymidine kinase and c-myc genes, housekeeping genes which are not expressed in these cells (Conklin and Groudine, 1986) has a low to moderate sensitivity to DNase I and is only partially soluble in 150 mM NaCl. Class 3 gene chromatin, which contains repressed gene sequences like those of ovalbumin, is salt-insoluble and relatively insensitive to DNase I digestion.

The nucleosome density of the salt-soluble polynucleosomes is unaltered.

Total chicken erythroid chromatin fragments and salt-soluble polynucleosomes obtained by gel exclusion

chromatography of salt-soluble chromatin, were examined by electron microscopy (Figure 21). EM studies were carried out by Dr. J.B. Rattner at the University of Calgary. The salt-soluble polynucleosomes have the same apparent nucleosome density as that of total chromatin fragments. Nucleosome free stretches of DNA were not observed in the salt-soluble polynucleosomes. The occasional irregularities in the linker length can be accounted for by stretching during specimen preparation. At this level of resolution the structure of the competent gene enriched chromatin did not differ from that of total chromatin.

Erythroid-specific gene polynucleosomes are resistant to exogenously-added-linker-histone-induced precipitation.

Analysis of the protein content of the salt-soluble polynucleosomes indicated that these chromatin fragments are depleted approximately 30% in the linker histones, H1 and H5, relative to levels found in bulk chromatin. In order to test whether the chromatin of erythroid-specific genes had an altered binding of linker histones, H1/H5-stripped total chromatin was reconstituted with varying quantities of purified linker histones (see Materials and Methods section) and then separated into 0.15 M NaCl-soluble and -insoluble fractions. Figure 22, DNA panel, shows the size distribution of the DNA fragments which remain salt-soluble. Without the addition of linker

histones, all of the chromatin fragments remain soluble. The amount of polynucleosomes present in the salt-soluble fraction declined with increasing linker histone added until the mononucleosome sized fragment predominated. The blot hybridized with the ovalbumin probe shows a decline in the extent of hybridization with increasing amount of linker histone added. The size distribution of the soluble ovalbumin chromatin fragments parallels that of total chromatin. In marked contrast, the degree of hybridization to the histone H5 gene probe shows an increasing enrichment in the soluble chromatin as the amount of linker histones is added, until an apparent maximum is reached, and then begins to decline slightly. The size distribution of the chromatin fragments that remain soluble is quite different than that of the bulk, with H5 gene sequences being localized in oligonucleosomes. β-globin and histone H2A.F gene probes yielded the same results as the histone H5 probe with the salt-soluble oligonucleosomes being enriched in these sequences. These observations indicate that class 1 gene chromatin, but not class 3 gene chromatin, has the inherent property to resist linker histone induced precipitation.

Figure 23 is a quantitative representation of hybridizations such as those shown in Figure 22. In this form the data clearly indicate that there is a qualitative difference in the way that linker histones associate with

competent chromatin compared to repressed chromatin to give rise to salt precipitable structures. The general sigmoid shape to the curve defined by total chromatin solubility as a function of linker histone density indicates that these histones interact cooperatively in the formation of salt precipitable structures. Although sufficient density of linker histones leads to increased precipitability of competent chromatin, this appears to occur in a non-cooperative manner.

In Part II it was shown that the largest chromatin fragments, which are soluble in 0.15 M NaCl, are highly enriched in β-globin gene sequence. These salt-soluble polynucleosomes are characterized by having a large number of modified and variant core histone species. The polynucleosome fraction from the reconstituted material, which is salt-soluble, has core histone modifications and variants which are similar in type and extent to those found in native chromatin. Most noticeable amongst these are significant increases in the levels of acetylation, ubiquitinated H2B and the variants H3.3 and H2A.Z (Figure 24).

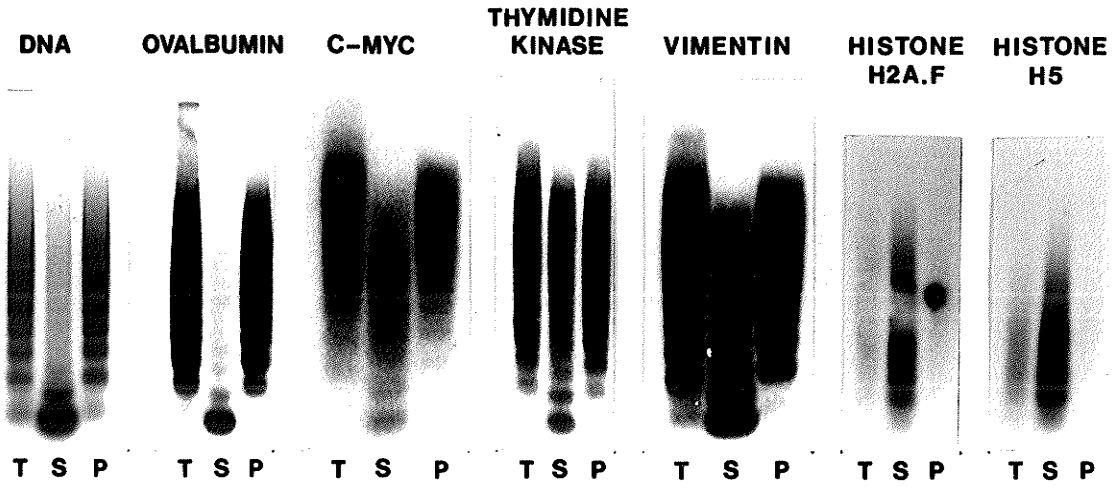

Figure 19

Polynucleosome solubility of chromatin of different genes. Purified DNA from total EDTA released chromatin fragments (T), and those soluble (S) or precipitable (P) in 0.15 M NaCl were electrophoretically resolved on a 1% agarose gel containing 1 μg/ml ethidium bromide, transferred to nitrocellulose and hybridized to various cloned DNA probes. Each lane contained 5 μg of DNA.

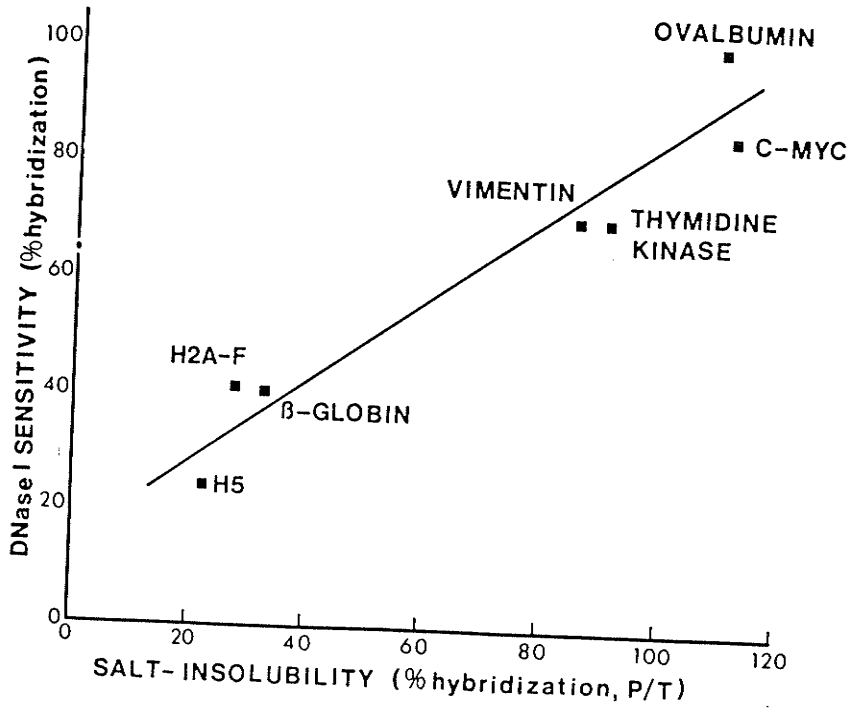

Figure 20

The relationship between DNase I sensitivity and polynucleosome insolubility. The DNase I sensitivity of various nuclear genes relative to the sensitivity of ovalbumin (100%) was determined as described in the Materials and Methods section. Salt insolubility is the ratio of the hybridization of DNA isolated from the 0.15 M NaCl insoluble chromatin (P) to that isolated from total EDTA released chromatin (T).

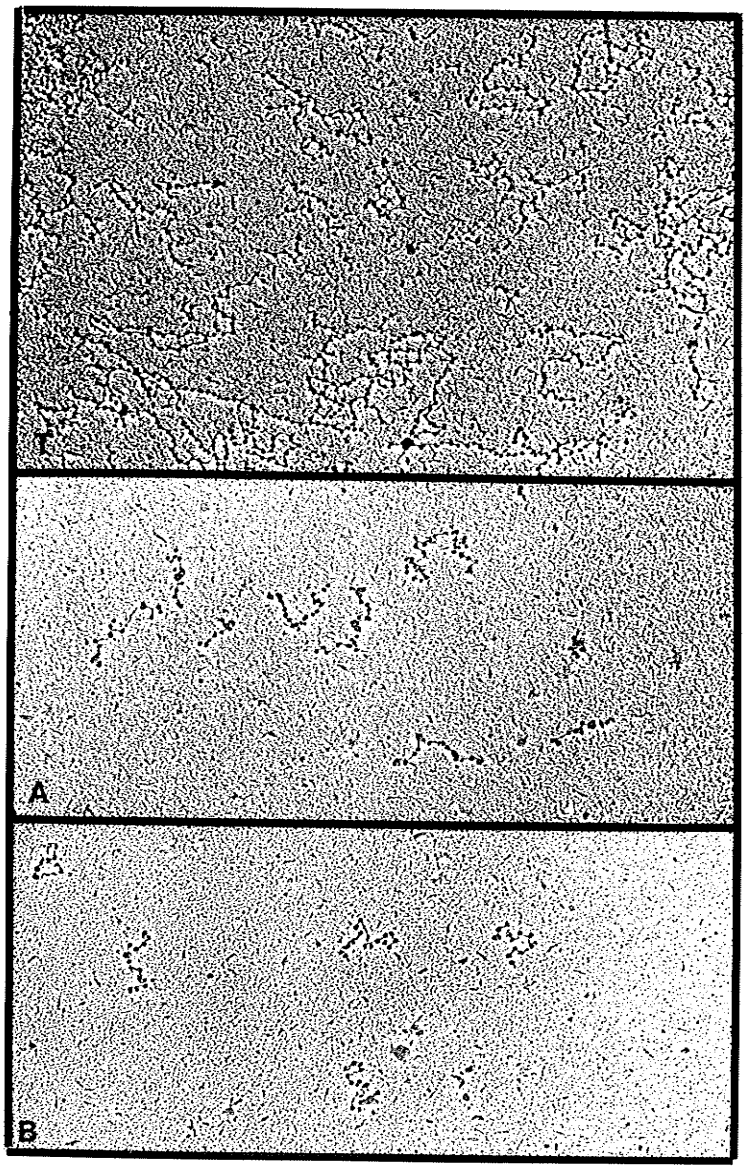

Figure 21

Electron micrographs of chromatin fragments. EDTA released chromatin fragments (T) and salt-soluble polynucleosomes isolated by gel exclusion chromatography on Bio-Gel A5m (A and B) were examined by electron microscopy. A and B are approximately equivalent to column fractions I and II (see part II).

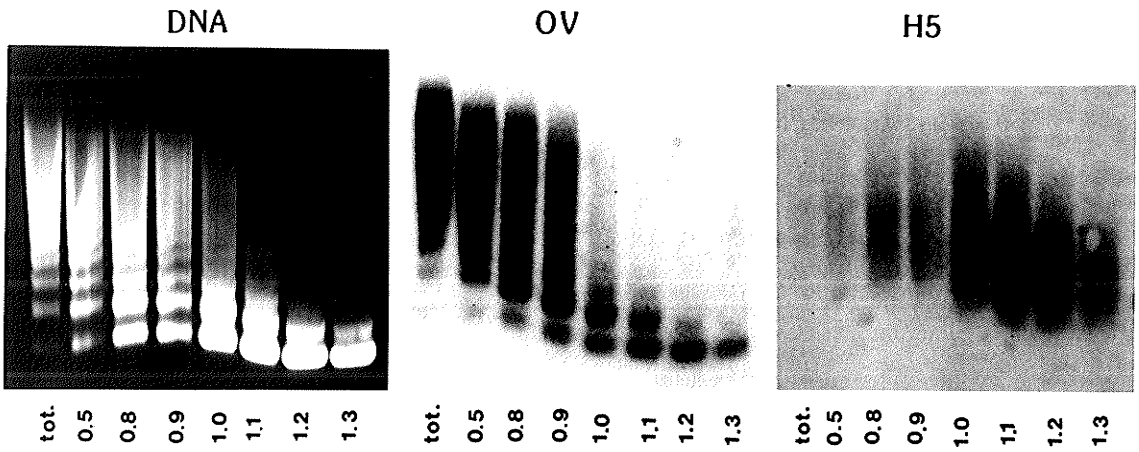

Figure 22

Hybridization of DNA fragments isolated from salt-soluble reconstituted chromatin. Chromatin, stripped of and reconstituted with H1 and H5 histones, was precipitated with 0.15 M NaCl. The total reconstituted material (tot.) and the soluble fraction from reconstitutions carried out with varying quantities of H1 and H5 (given as the fractions of H1 and H5 levels in EDTA soluble chromatin) were hybridized with DNA probes for histone H5 (H5) and ovalbumin (OV) genes.

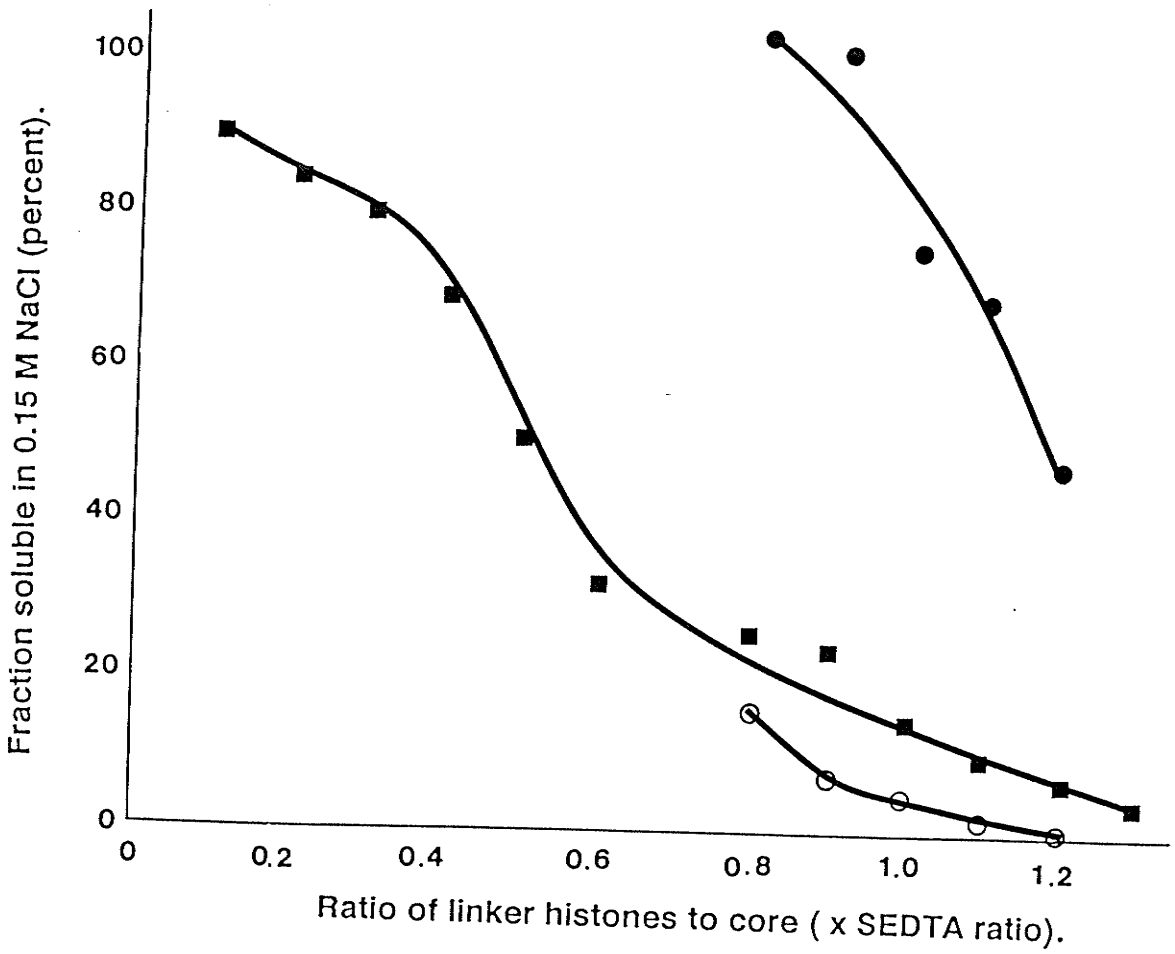

Figure 23

The relationship between solubility and histone H1 and H5 content of reconstituted chromatin. - A quantitative analysis of the hybridization of DNA from an experiment carried out as described in Figure 22. The percent of chromatin reconstituted with varying quantities of histones H1 and H5 that was soluble in 0.15 M NaCl from is shown for total (■) and for chromatin containing gene sequences from H5 (●) and ovalbumin (o) is shown. The fraction soluble of the gene chromatins for any given level of reconstituted linker histones is: hybridization of soluble material x percent of total soluble ÷ hybridization of EDTA soluble material (SEDTA)

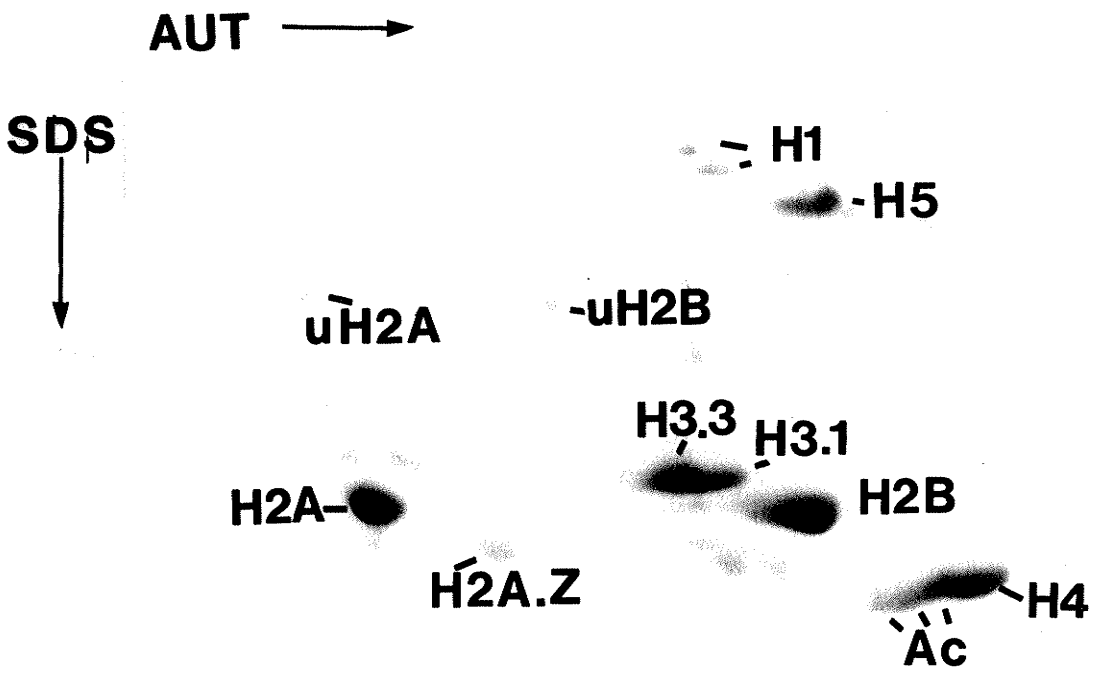

Figure 24

Proteins associated with the salt-soluble polynucleosomes of reconstituted chromatin. Proteins were resolved by two dimensional AUT into SDS polyacrylamide gel electrophoresis. Ac are acetylated species of histone H4. The prefix "u" indicates the ubiquitinated forms of H2A and H2B.

DISCUSSION OF PART III

DNase I sensitive genes are preferentially localized in salt-soluble chromatin.

The results presented here indicate that the chromatin fragments that are soluble as polynucleosomes in 0.15 M NaCl are from the same genomic chromatin domains which are sensitive to DNase I. Moreover, these chromatin fragments exclude the cooperative interaction amongst H1 histones which lead to the formation of higher-order structure.

Analysis of the DNase I sensitivity and salt solubility properties of various gene chromatins indicated the differential chromatin organization of erythroid-specific, housekeeping and repressed genes. Erythroid-specific gene sequences (H5 and β-globin) and histone H2A.F gene sequences are sensitive to DNase I and also found enriched in chromatin which is salt-soluble as polynucleosomes. The relationship between DNase I sensitivity and salt solubility indicates that the biochemical features of these gene chromatins may determine both properties. Our results suggest that the salt solubility properties of the chromatin of erythroid-specific genes are a consequence of an altered association with linker histones (H1/H5). This altered association, which may give rise to a reduction in the steady state

levels of these proteins complexed to the transcriptionally competent chromatin regions, would probably result in the destabilization of linker histone dependent higher-order structure. Also, the salt-soluble polynucleosomes are enriched in acetylated and ubiquitinated histones (Gross and Garrard, 1987; Ferenz and Nelson, 1985; Nelson et al., 1986; Alonso, et al., 1987). Since the N-terminal tails of the histones are thought to be involved in the formation of higher-order structures, the acetylation of the lysine residues within this region of the molecule may lead to the weakening of these interactions (Allan et al., 1982). The housekeeping genes (c-myc, thymidine kinase) also have, to some extent, an atypical chromatin structure. It is interesting to note that the chromatin structures of housekeeping and erythroid-specific genes can also be distinguished by in situ nuclear nick-translation. The labeled chromatin regions are localized at the borders of condensed chromatin masses along interchromatin channels communicating with the nuclear periphery (Hutchinson and Weintraub, 1985). Since the labeling of chromatin by this technique requires access by both DNase I and E. coli DNA polymerase I, our results predict that only regions with the atypical structure will be available to both enzymes. Indeed, erythroid-specific gene chromatin is preferentially labeled by this technique (Hutchinson and Weintraub, 1985). We expect that histone H2A.F gene chromatin would also be

selectively labeled. Housekeeping genes, such as thymidine kinase, have only a very limited solubility as polynucleosomes and are labeled to a considerably lower extent than erythroid-specific chromatin regions.

The atypical, open chromatin structure might be expected to facilitate the transcription process. It should be noted that although the mature erythrocyte has very low levels of mRNA, at least some of the erythroid-specific genes are in a transcriptionally ready state (Affolter et al., 1987).

Reconstitution of stripped chromatin with varying amounts of linker histone demonstrated that an inherent property of the erythroid-specific and histone H2A.F gene chromatins alters H1/H5 induced precipitability in 0.15 M NaCl. A simple explanation based on our results is that the chromatin of erythroid-specific (and H2A.F) genes has a reduced affinity or altered mode of binding to the linker histones, as opposed to the ovalbumin gene chromatin which avidly associates with H1 and H5. This hypothesis is in agreement with the observation by Weintraub (1984) that ovalbumin gene chromatin exchanges exogenously added histones H1 and H5 while β-globin gene chromatin does not.

The role of the H1 histones in chromatin condensation is well established (Caplan et al., 1987) and

the process probably occurs through the cooperative interactions of these histones (Renz et al., 1977, Allan et al., 1981). Our results suggest that linker histone binding and/or chromatin fibril condensation to the 30 nm form is a process involving cooperative interactions between linker histones. That erythroid-specific gene chromatin associates with the linker histones in forming salt precipitable structures in an apparently noncooperative manner does not imply that this chromatin type does not bind to linker histones. Indeed, the salt-soluble polynucleosomes from reconstituted chromatin contain linker histones (Figure 24). Cross-linking studies indicate that H1 histones interact with one another as well as nucleosome cores and linker DNA (McGhee et al., 1981) while other studies indicate that chromatin exists in structures which do not depend on the continuity of the DNA strand for their integrity (Weintraub, 1985; Ruiz-Carrillo et al., 1980). Some models of chromatin higher-order structure suggest that there is contiguous H1 molecules stabilizing the 30 nm fibril. Periodic perturbation of this type of interaction and consequent destabilization of the 30 nm fibril may be sufficient to account for the various properties of competent chromatin.

Caplan et al. (1987) observed that the sedimentation behaviour of EcoRI produced globin-gene-chromatin-fragments was consistent with an altered association with linker

histone. These authors argue that the sedimentation behaviour of this gene chromatin is due to the presence of the DNase I hypersensitive regions which are localized at the 3′ and 5′ ends of the gene. Such nuclease hypersensitive sites are probably not responsible for the solubility of the competent gene enriched polynucleosomes because examination of the highly competent gene enriched salt-soluble polynucleosome fraction by electron microscopy indicated a typical "beads-on-string" appearance and nucleosome density. This chromatin fraction maintained an extended appearance even in 0.15 M NaCl while total solubilized chromatin is highly aggregated under these conditions (not shown). It is likely that the observed extended configuration in NaCl of this chromatin fraction could account for any observed alterations in the hydrodynamic properties of β-globin chromatin. It is unlikely, however, that nucleosome free regions, in bulk, can account for the altered solubility of this chromatin fraction.

Both the stripped chromatin and linker histone preparation used for reconstitution were free of detectable nonhistone proteins. Therefore, the observed differences of the ovalbumin and H5 gene chromatins are most likely the result of differences in nucleosome core structure.

In summary, our results suggest that the competent chromatin state is due to an altered association of

chromatin domains with linker histones and that this altered association itself results from differences in the structure of the nucleosome core.

PART IV

In this section the relationship between histone acetylation and the capacity of H1 histones to cause the 0.15 M NaCl-induced aggregation/precipitation of transcriptionally active/competent gene chromatin fragments was investigated. We show that the degree of solubility of the active/competent gene chromatin fragments in 0.15 M NaCl is correlated with the level of acetylated histone species. Furthermore, the level of histone acetylation determines the resistance of active/competent gene chromatin fragments to H1/H5-induced salt precipitation. These results suggest that histone acetylation alters the capacity of linker histones to form higher-order chromatin structures such that transcriptionally active/competent gene chromatin is maintained in a less folded state than the bulk of chromatin.

RESULTS

Effect of Histone Acetylation on Solubility of Transcriptionally Active/Competent Gene Chromatin Fragments in 0.15 M NaCl.

In order to determine whether altered levels of acetylated histone species influence the salt solubility of the active/competent-gene-enriched chromatin fragments, immature erythrocytes were incubated in the presence or absence of sodium butyrate, an inhibitor of the histone deacetylase (Candido et al., 1978). In the presence of sodium butyrate, a small percentage of the nucleosomal histone population become hyperacetylated, while in the absence of sodium butyrate, this same population of histones have reduced levels of acetylated histones (Brotherton et al., 1981; Zhang and Nelson, 1986). Chromatin isolated from these cells was fractionated. The distribution of DNA among chromatin fractions PEDTA, SEDTA, P150, and S150 measured by the diphenylamine method was 25.9 ± 6.4, 74.4 ± 5.9, 66.3 ± 6.0, and 7.8 ± 0.8 (- butyrate; n=3) and 20.2 ± 2.6, 79.9 ± 2.6, 70.5 ± 3.0, and 9.5 ± 0.1 (+ butyrate; n=3), respectively. Thus, incubation of the cells with or without butyrate did not influence the fractionation of the bulk of the chromatin fragments. The partitioning of active and competent DNA with fraction PEDTA, which is not shown here, was not

affected by these incubations (Delcuve and Davie, 1989).

Figure 25 shows the protein content of fractions SEDTA and S150 isolated from cells incubated in the absence or presence of butyrate. Acetylation levels can be easily judged by examining the different acetylated forms of H4. As expected the levels of acetylated histone species do not change dramatically in the SEDTA fractions which contain the bulk of erythrocyte chromatin. However, the levels of acetylated histones in the salt-soluble chromatin (fraction S150) drop significantly as a result of the incubation in the absence of butyrate. Further separation of the proteins of the S150 fractions by two-dimensional PAGE demonstrates that incubating cells in the presence of butyrate results in elevated levels of acetylated species of histones H4, H2B and H3. The amount of ubiquitinated histones is not altered.

The chromatin fragments of fraction S150 were size-resolved by gel exclusion chromatography. Figure 26 demonstrates that incubation of cells in the absence of butyrate results in a major decline in the 260 nm-absorbing material in the polynucleosome fractions. This observation suggests that decreased levels of acetylated histone species reduce the amount of salt-soluble polynucleosomes in fraction S150. Note also that the loss of polynucleosomes, which are associated with linker histones H1 and H5 (Delcuve and Davie, 1989), results in a reduction

of the amount of linker histones in fraction S150 (- butyrate) (see Fig. 25, two dimensional gels).

DNA fragments, which were isolated from chromatin fractions SEDTA, S150 and P150, were electrophoretically separated on a 1% agarose gel. Figure 27 (DNA) shows that although the DNA fragment sizes of chromatin fractions SEDTA (+ or - butyrate) are similar, there is a striking difference in the size distribution of the DNA fragments present in fractions S150 (compare + and - butyrate). Chromatin fraction S150 (+ butyrate) clearly contains a greater abundance of longer DNA fragments than chromatin fraction S150 (- butyrate), as indicated by gel filtration (Fig. 26).

Southern blot analysis of the DNA shows that incubation of the cells in the absence or presence of butyrate results in different distributions of the competent DNA (ϵ-globin and vimentin) among the salt-soluble and salt-insoluble chromatin fractions (Fig. 27). There is a marked decrease in the amount of competent DNA located in the salt-soluble chromatin fragments (fraction S150), and an increase in the content of competent DNA found in the aggregation-prone, salt-insoluble chromatin fraction P150 as a result of cells being incubated in the absence of butyrate. Conversely, when cells are incubated in the presence of butyrate, the salt-soluble chromatin is highly enriched in competent DNA. Repressed gene chromatin

(vitellogenin) is not affected by incubation in the presence or absence of butyrate, and repressed DNA is salt-soluble primarily as mononucleosomes in 150 mM NaCl.

The distribution of DNA sequences among the salt-soluble and -insoluble chromatin fractions was quantified by densitometry of slot blots (Table IV). Incubation of cells in the absence of butyrate results in a loss of salt-soluble competent and active gene chromatin fragments. The partitioning of competent and active DNA into the salt-soluble chromatin fraction decreases 6.0 ± 0.7-fold (n=4; combined average for ϵ-globin and vimentin) and 3.0 ± 0.7-fold (n=3; combined average for β-globin and histone H5), respectively, when cells are incubated in the absence of butyrate.

Effect of Histone Acetylation on the 0.15 M NaCl Solubility of Active/Competent Gene Chromatin Fragments Reconstituted with Linker Histones

In Part III, H1/H5-stripped chromatin fragments of mature chicken erythrocytes were reconstituted with varying linker:nucleosomal histone ratios. The results suggested that linker histones acted cooperatively in the formation of aggregation-prone, NaCl-precipitable chromatin structures. Active/competent gene chromatin fragments were more resistant than bulk chromatin fragments to added-linker-histone-induced NaCl precipitability.

In this study we determined whether modulating the level of acetylated histone species would alter the ability of active/competent gene chromatin fragments to resist exogenously-added-linker-histone-induced NaCl precipitation. Histone H1/H5-stripped EDTA-soluble chromatin fragments, which were isolated from immature erythroid cells incubated in the presence or absence of butyrate, were reconstituted with varying amounts of linker histones. Linker-histone-reconstituted chromatin fragments were fractionated into 0.15 M NaCl-soluble and -insoluble fractions. As the amount of reconstituted linker histones approaches native chromatin levels (linker histone density = 1), there is a decrease in the percentage of chromatin fragments that are salt-soluble, a decrease in the concentration of salt-soluble polynucleosome fragments, and an increase in the level of mononucleosomes (see previous section). More polynucleosomes of + butyrate-treated cells remain soluble in 0.15 M NaCl than those of - butyrate-treated cells (Figure 28).

The histones, which were isolated from the salt-soluble chromatin fragments, were electrophoretically resolved on AUT 15% polyacrylamide gels (Fig. 29). As the amount of linker histones added increases to native levels (linker histone density = 1), there is an increase in the content of hyperacetylated histone H4 and histone H2B species associated with the salt-soluble chromatin

fragments which were isolated from butyrate-incubated cells. This increase in the level of acetylated histone species is not observed for salt-soluble chromatin fragments isolated from cells incubated in the absence of butyrate. Note that the salt-soluble chromatin fractions contain linker histones H1 and H5.

Figure 30 shows the solubility of reconstitued chromatin as a function of the amount of linker histone added. Butyrate incubation does not significantly alter the solublility of bulk chromatin. The sigmoid shape of the cure defined by this relation suggests a cooperative interaction amongst linker histones in giving rise to salt precipitable chromatin structures.

Figure 31 shows a quantitative assessment of the amount of competent ϵ-globin and repressed vitellogenin gene chromatin soluble in 0.15 M NaCl as a function of the amount of linker histone added. The same relationship is shown for total chromatin. Note that the total chromatin and vitellogenin curves are distinctly sigmoidal in shape, suggesting a cooperative interaction amongst the linker histones in inducing the formation of salt-precipitable chromatin structures. The ϵ-globin gene chromatin fragments isolated from cells incubated in the presence or absence of butyrate show markedly different degrees of solubility as a function of linker histone density. ϵ-globin gene chromatin fragments from butyrate-incubated

cells remain completely soluble until the amount of linker histones added is equivalent to the levels of these histones found in native chromatin fragments. Further increases in the amount of linker histones reconstituted onto the chromatin fragments result in a decline in the solubility of the globin gene chromatin fragments. However, ϵ-globin chromatin fragments, which were isolated from cells incubated in the absence of butyrate, have lost most of their ability to resist linker-histone-induced NaCl precipitation. Thus, when about 11% of the bulk chromatin fragments are soluble in 0.15 M NaCl, ϵ-globin gene chromatin fragments from cells incubated in the presence or absence of butyrate are 100% and 18% soluble, respectively. This represents a 5.6-fold difference in the solubility of competent gene chromatin fragments.

Active gene chromatin fragments, which are considerably shorter than competent gene chromatin fragments (Delcuve and Davie, 1989), also have this difference in salt solubility, with the ratio of histone H5 gene chromatin solubility (+ butyrate : - butyrate) at a nominal 1-fold linker histone density being 2.1 ± 0.2 (n = 4). [The solubility of bulk chromatin fragments at this linker histone density is 11.3 ± 1.5 for chromatin isolated from butyrate-treated cells and 11.0 ± 1.0 for chromatin isolated from cells incubated in the absence of butyrate (n = 4).] Within each experiment with

reconstituted chromatin (6 separate experiments), active/competent gene chromatin was always more enriched in the NaCl-soluble chromatin fragments from cells incubated in the presence of butyrate than those from cells incubated in its absence.

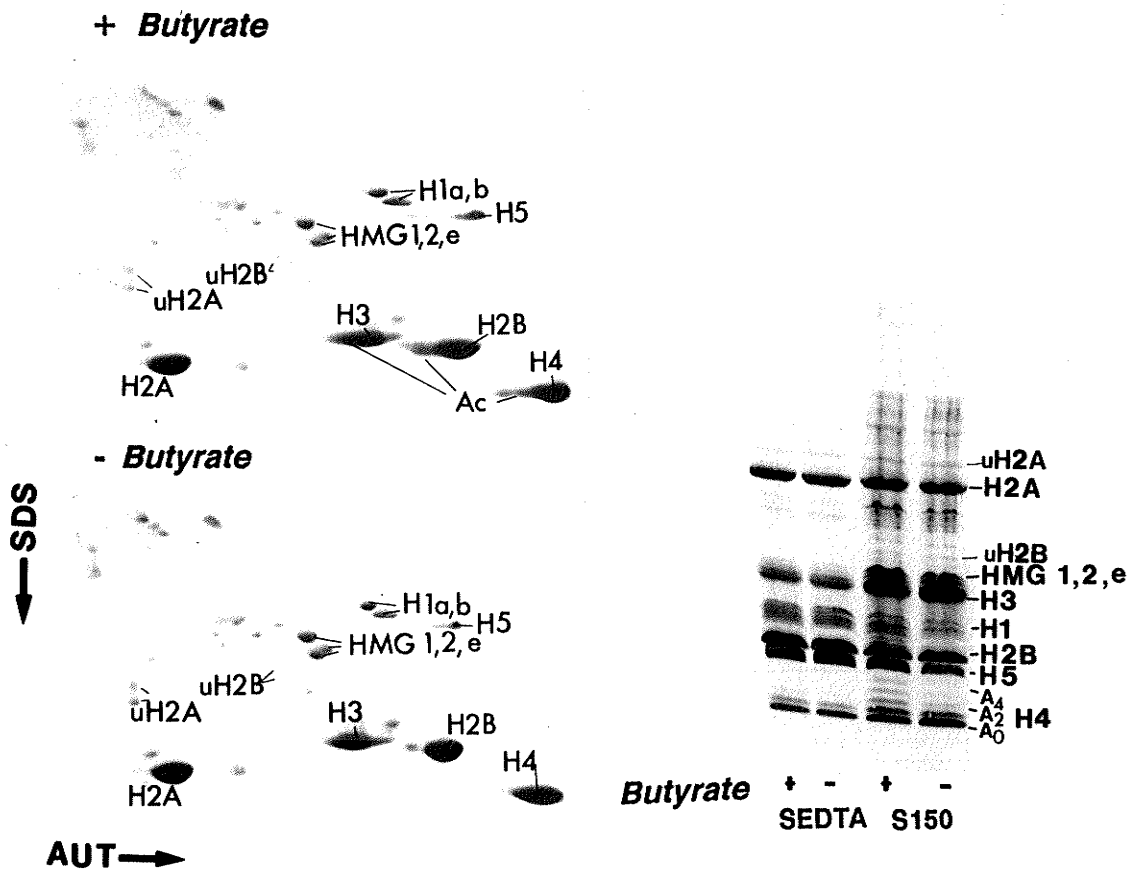

Figure 25

Protein content of 0.15 M NaCl-soluble chromatin fragments from cells incubated with or without butyrate. The right hand panel shows a Coomassie Blue stained AUT PAGE gel pattern containing proteins (10 ug) of chromatin fractions SEDTA (SEDTA) and S150 which were isolated from immature erythrocytes incubated in the presence (+) and absence(-) of butyrate. A_0, A_2 and A_4 indicate the non-, di- and tetraacetylated forms of histone H4, respectively. The left hand panels show the same S150 fractions further resolved by electrophoresis into a second-dimension SDS 15% polyacrylamide gel. Ac indicates acetylated forms of the nucleosomal histones. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. Note that on SDS gels the ubiquitinated histone species migrate as doublets (Nickel et al., 1989).

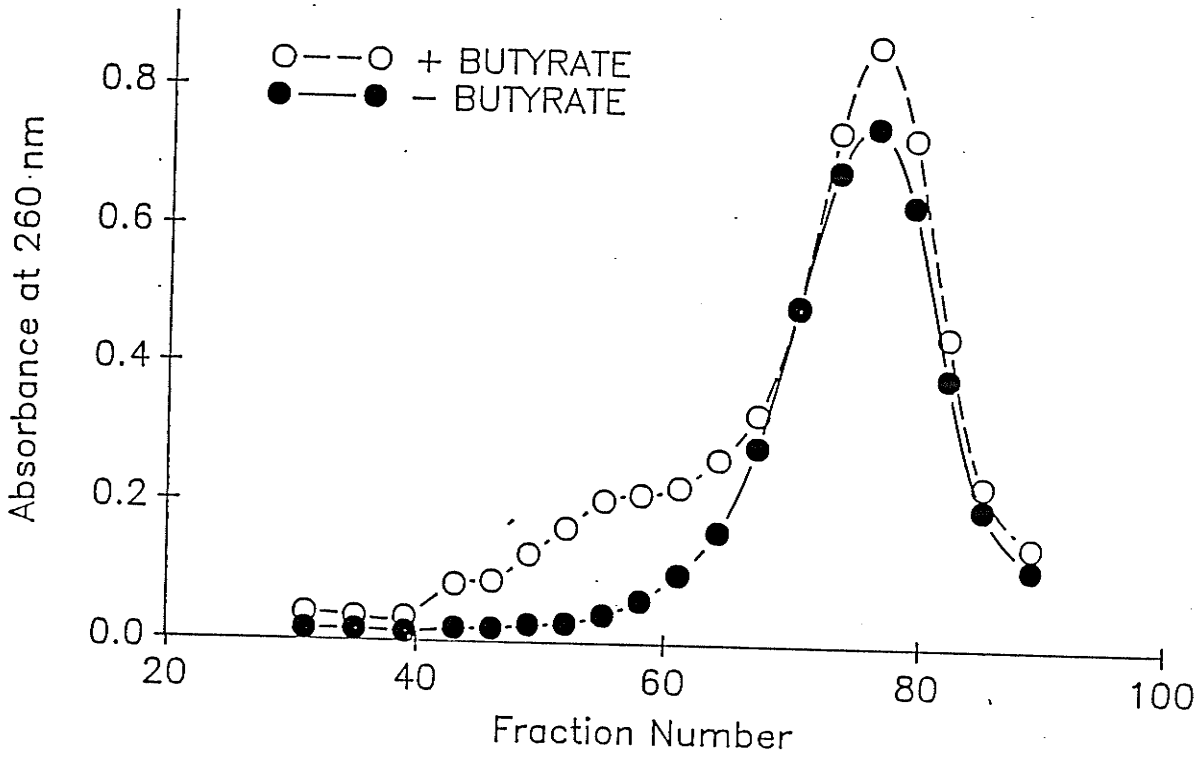

Figure 26

Size resolution of 0.15 M NaCl-soluble chromatin fragments isolated from cells incubated in the presence and absence of butyrate. 0.15 M NaCl-soluble chromatin fragments were size-resolved by gel filtration on a Bio-Gel A5m column as described under Materials and Methods. The major peak around fraction 75 consists primarily of mononucleosomes. The various sized polynucleosomes are contained in fractions 40 to 70.

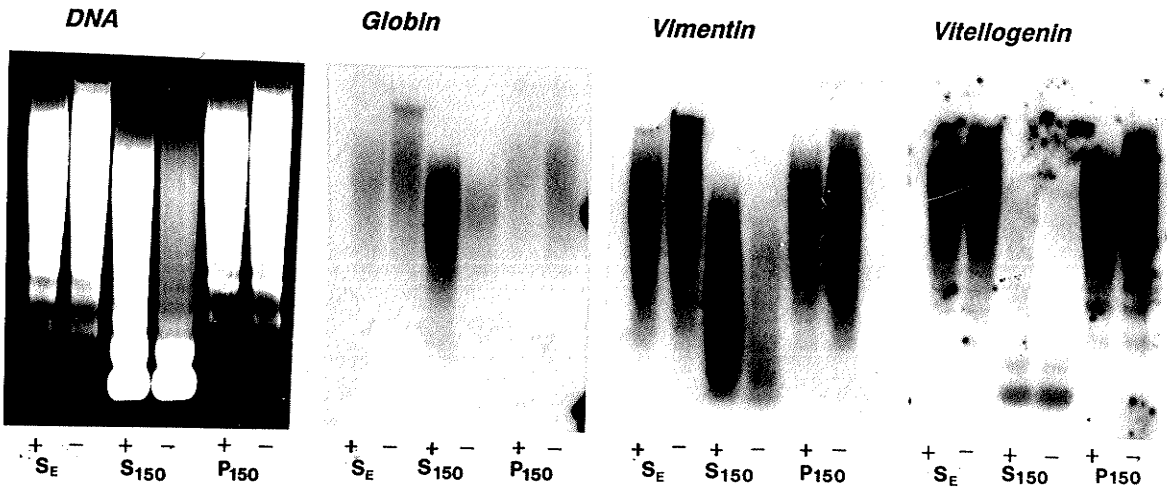

Figure 27

Southern blot analysis of DNA from chromatin fractions. Immature chicken erythrocyte nuclei, which were isolated from cells incubated in the presence (+) and absence (-) of butyrate, were digested with micrococcal nuclease for 10 min as described under Materials and Methods. DNA (10 ug) of the chromatin fractions was electrophoretically resolved on a 1% agarose gel, stained with ethidium bromide (DNA) or transferred onto nitrocellulose and hybridized to ^{32}P-labelled probes as indicated. ϵ-globin is shown as Globin.

TABLE IV

FRACTION	% DNA	% H5	% ∈-globin	% vimentin	% vitel'n
S150 (+Bu)	11.4	65	41	42	2
S150 (-Bu)	9.7	25	6	7	2
RATIO (+/-)	1.2	2.6	6.8	6.0	1.0

TABLE IV. The effect of butyrate treatment on the 0.15 M NaCl solubility of various gene chromatin fragments. Salt solubility of gene chromatin fragments isolated from immature chicken erythrocytes incubated in the presence (+ Bu) or absence (- Bu) of sodium butyrate was determined. The percentage of DNA in the EDTA released chromatin fragments (SEDTA) that is soluble in 0.15 M NaCl was determined by absorbance at 260 nm, and the percentage of each sequence in SEDTA that fractionates into S150 was determined by hybridization of slot blots and densitometric analysis of the autoradiograms. (Vitel'n = vitellogenin)

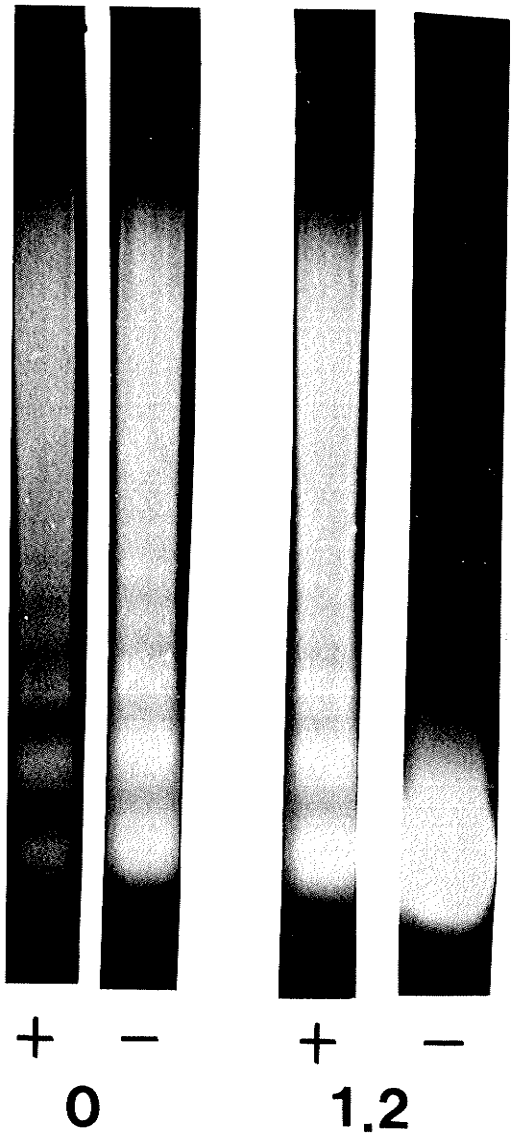

Figure 28

Polynucleosomes from reconstituted chromatin are more soluble in 0.15 M NaCl in chromatin from cells incubated in the presence of butyrate than from those incubated in its absence. Chromatin from cells incubated in the presence (+) or absence (-) of butyrate was used for reconstitution with linker histone. Linker histone was added to 0 or 1.2 times the levels of fraction SEDTA. DNA from chromatin soluble in 0.15 M NaCl was electrophoresed on 1% agarose gels.

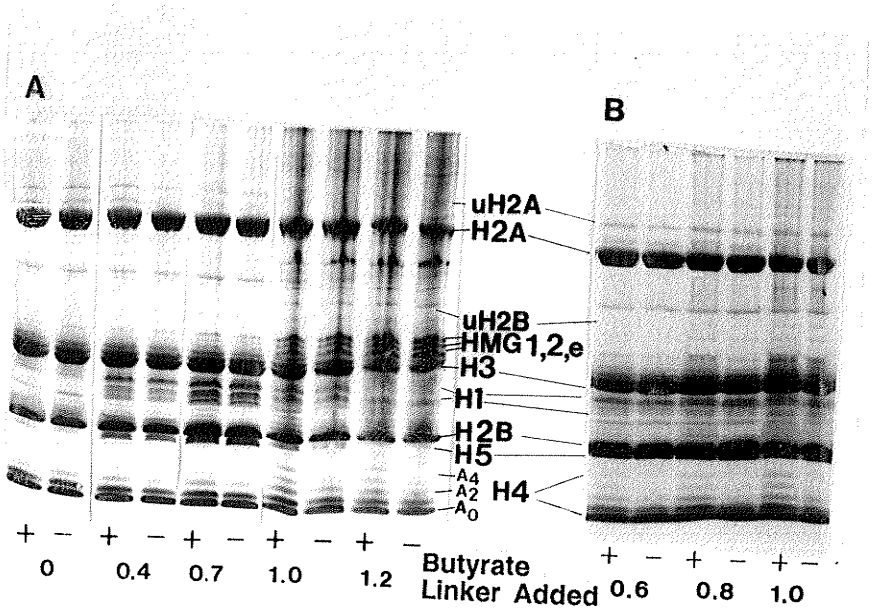

Figure 29

Proteins associated with the 0.15 M NaCl-soluble fractions of H1/H5-reconstituted chromatin fragments. H1/H5-stripped chromatin fragments of fraction SEDTA, which were isolated from cells incubated in the presence (+) and absence (-) of butyrate, were reconstituted with linker histones at various stoichiometric ratios. Proteins isolated from the salt-soluble chromatin fragments (10 ug) were analyzed by AUT PAGE, and Coomassie Blue stained gel is shown. A_0, A_2 and A_4 indicate the non-, di- and tetra-acetylated forms of H4, respectively. The ubiquitin adducts of histones H2A and H2B are denoted as uH2A and uH2B, respectively. In the last four lanes of A histone H5 migrates partially with H2B. B shows proteins from a different experiment where the H5 is more focused on the gel.

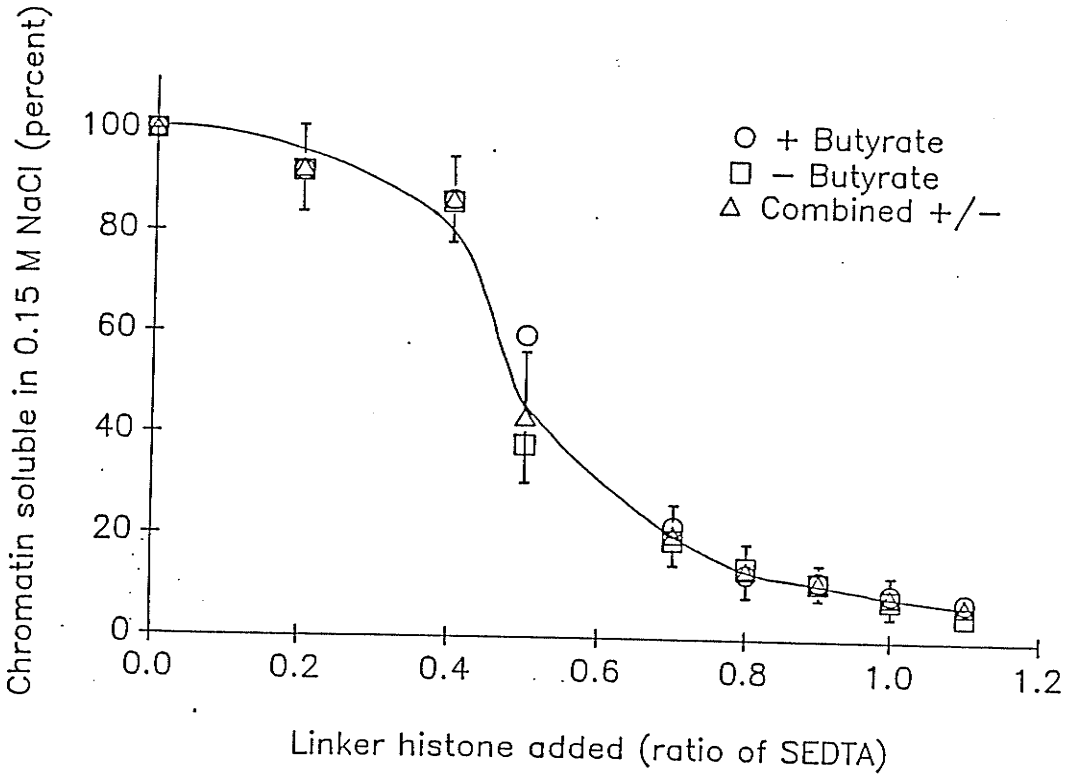

Figure 30

The solubility of chromatin fragments as a function of linker histone density. Chromatin fragments of fraction SE were isolated from cells incubatyed in the presence (o) or abscence (□) of butyrate. The values are means. As these values are similar for both + and - butryate treated cells, combined means (Δ) Standard deviations (error bars) are also shown. The number of different experiments for each linker density are: 0, n = 3; 0.2, n = 4; 0.4, n = 4; 0.5, n = 10; 0.7, n = 10, 0.8, n = 11; 0.9, n = 13; 1.0, n = 10; 1.1, n = 4.

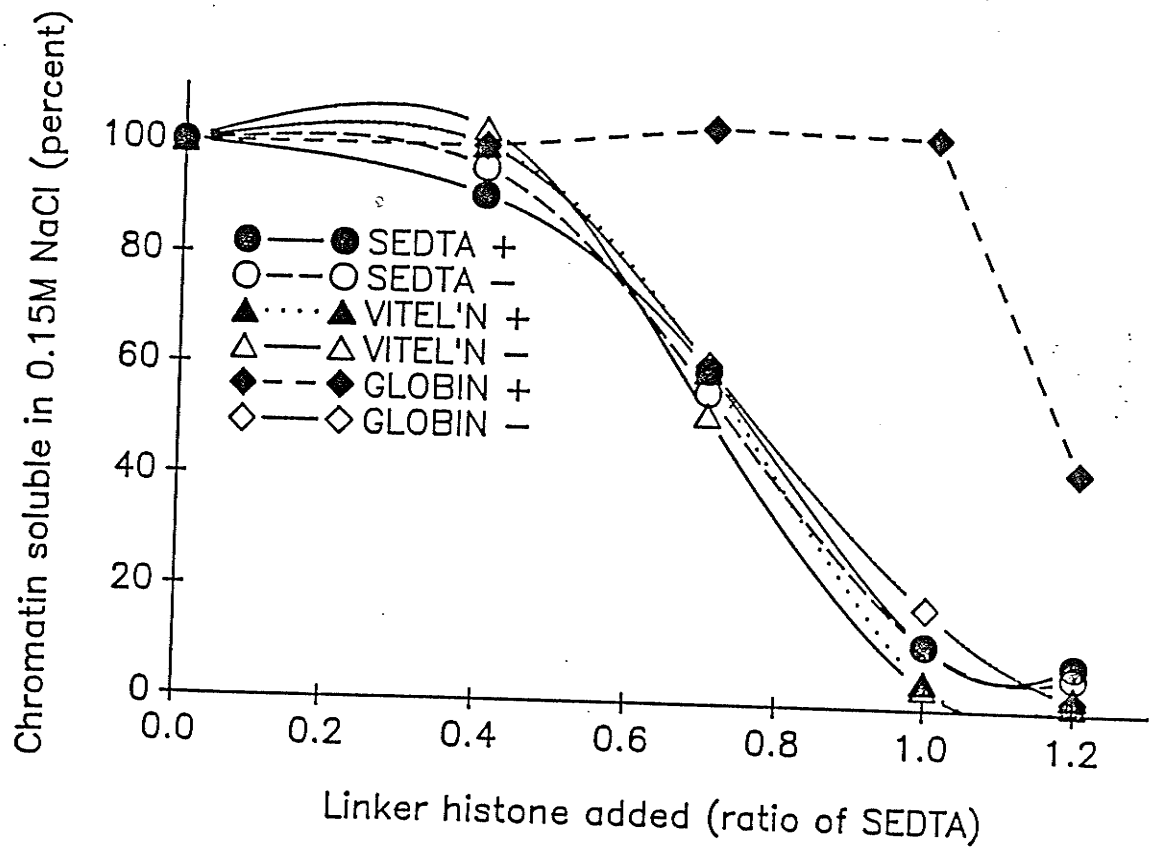

Figure 31

The solubility of gene chromatin fragments as a function of linker histone density and butyrate incubation. Chromatin fragments of fraction SEDTA (SEDTA) were isolated from cells incubated in the presence (filled symbols with "+") and absence (empty symbols with "-") of butyrate. DNA prepared from the 0.15 M NaCl-soluble fraction of chromatin reconstituted with varying quantities of linker histone was applied to nitrocellulose by slot blotting and hybridized against labelled gene probes for ϵ-globin (GLOBIN) and vitellogenin (VITEL'N). Hybridization was quantified by densitometric scanning of the autoradiograms as described under Materials and Methods. The results are expressed as the percentage of gene chromatin fragments in fraction SEDTA that are soluble in 0.15 M NaCl.

DISCUSSION OF PART IV

Linker histones are known to act in the salt-dependent formation of higher-order chromatin structures which are observable by electron microscopy (Thoma et al., 1979). The formation of aggregation-prone, NaCl-precipitable structures shows a similar histone dependency (this study; Allan et al., 1981). The results of Widom's (1986) study indicate that aggregation is a built-in property of the 30 nm fibre. Thus, the aggregation/precipitation property of a chromatin fragment in 0.15 M NaCl in vitro may correlate with the capacity of the chromatin fibre to form higher-order structures in vivo.

The results of this study provide evidence that histone acetylation has a role in altering the capacity of the linker histones to fold the chromatin fibre. The bulk of erythrocyte chromatin fragments aggregate and precipitate in 0.15 M NaCl. In contrast, active/competent-gene-enriched chromatin fragments, which are associated with linker histones, do not precipitate in 0.15 M NaCl and are probably in an unfolded conformation. Elevated levels of hyperacetylated nucleosomal histones increase the 0.15 M NaCl solubility of active/competent gene chromatin fragments, while lowered levels of acetylated histones decrease the 0.15 M NaCl solubility of these fragments.

Reconstitution experiments with linker histones demonstrate that lowered levels of the acetylated histones reduce the ability of active/competent gene chromatin fragments to resist linker-histone-induced salt precipitation. These observations suggest that the H1 histones associated with highly acetylated nucleosomes are not able to condense the active/competent gene chromatin fibre. Removal of the basic N-terminal "tails" of the nucleosomal histones, which contain the sites of acetylation, does not prevent the H1 histones from binding to the chromatin fibre (Allan et al., 1982). But removal of the tails does interfere with the capacity of the linker histones to condense the chromatin fibre (Allan et al., 1982). Thus, acetylation of lysyl residues located within the N-terminal basic domains of the nucleosomal histones may have the same effect on altering H1 histone action as does the removal of this basic domain.

The EDTA-soluble competent gene chromatin fragments isolated from micrococcal nuclease digested immature erythrocyte nuclei are considerably longer than the active gene chromatin fragments (Delcuve and Davie, 1989). This disparity in chromatin fragments sizes may account for the more noticeable transition of competent (6-fold) versus active (2 to 3-fold) gene chromatin to an aggregation-prone state (native or linker histone reconstituted) as a consequence of deacetylation. In studies of exchange of linker histones between chromatin fragments, Thomas and

Rees (1983) demonstrated that at an ionic strength of 0.75 M, histone H5 of short chromatin fragments preferred to associate with long chromatin fragments that had formed higher-order structures. Short active gene chromatin fragments may be more susceptible than long competent gene chromatin fragments to losing their linker histones. The loss of linker histones and the reduced ability to form higher-order structures would tend to decrease the salt-induced aggregation and precipitation of active gene chromatin fragments.

Recently, Norton et al. (1989) reported that histone acetylation reduces the amount of negative DNA supercoils constrained by the nucleosome. Removal of the nucleosomal histone tails also leads to the loss of DNA normally constrained by the nucleosome (Allan et al., 1982). The liberation of DNA from the nucleosome and/or change in nucleosome shape as a consequence of acetylation may alter the path of the DNA entering and leaving the nucleosome which in turn may alter the interaction between H1 histones and nucleosomal/linker DNA (Allan et al., 1980). These alterations in DNA path and linker histone-nucleosome interaction may prevent (or alter) the formation of compact higher-order structures.

In conclusion, our observations suggest that in immature chicken erythrocytes dynamic acetylation of nucleosomal histones complexed with transcriptionally

active/competent DNA prevents histone H1 and H5 (including newly-synthesized H5) interactions that allow for normal condensation of the chromatin fibre. Chan et al. (1988) presented evidence indicating that histone acetyltransferase is preferentially associated with the active/competent gene chromatin domains. This would ensure that the transcriptionally active gene chromatin is maintained in a less folded state than the bulk of chromatin.

CONCLUSIONS AND COMMENTS

The role of chromatin structure and, in particular, of linker histones in the regulation of gene expression may be thought of as follows: The primary function of the nucleosome is to define a path for the very long genomic DNA that permits its orderly folding and compaction. This compaction is stabilized by the linker histones. This stabilization of compaction results in the DNA becoming less accessible to the transcriptional machinery. To maintain transcriptional competence this stablization of the compacted structure is disrupted by ongoing processes which result in an altered association with linker histones. The main process which results in this destabilization is, probably, dynamic acetylation of the core histones.

These studies give evidence which suggest the following:

1. Competent chromatin is enriched in a number of modified and variant core histone types and is associated with linker histones.
2. This type of chromatin has an altered association with linker histones so that they do not interact in a cooperative manner in folding the fibril. This altered association is due to the differences in the nucleosome core.

3. Dynamic core histone acetylation is a key factor in determining the observed properties of competent chromatin.

Salt-soluble polynucleosomes and competent chromatin

Various chromatin fractionation procedures have been utilized in order to gain an insight into the structure of transcriptionally active/competent chromatin. One fairly common weakness of most procedures is an incomplete accounting of the DNA during the fractionation. In the fractionation used here, almost all of the chromatin except the shortest DNA products of the action of micrococcal nuclease on the linker regions are accounted for and analyzed. The EDTA released fraction (which is the starting point for the isolation of the salt-soluble polynucleosomes) contains about 95% of the DNA (as measured by the diphenylamine assay) present in the digested nuclei of mature cells.

The central point of this work is the isolation of salt-soluble polynucleosomes and their characterization as a highly-competent-gene-enriched chromatin class. The goal of this aspect of the project was to delineate some aspects of the biochemical basis for the differences between competent and repressed chromatin. In order to do this a reliable procedure to preparatively separate competent and repressed chromatin was needed.

The high degree of enrichment of competent gene sequences in this fraction makes it reasonable to suggest that its biochemical constitution is representative of the state of competent chromatin. A 50-fold enrichment would be the maximum possible for a population that made up two percent of the total. If two percent is a good estimate of the amount of competent chromatin in eukaryotic cells (it is estimated that 1 -2 % of the genome is expressed in any given cell, Lewin, 1986; and that euchromatin constitutes about 7 %, Watson et al. 1987), then the salt-soluble polynucleosomes can be said to contain all of it. Furthermore, the almost total absence of repressed gene sequences in this fraction also indicates that this fraction is essentially a "pure" representation of competent chromatin.

In fact, only about half of the sequences of competent genes fractionate into the salt-soluble polynucleosomes: some remain with the EDTA insoluble material and some are precipitated by NaCl. The sequence distribution of the material remaining with the EDTA insoluble pellet has been characterized (Delcuve and Davie, 1989). The bulk of sequences are not enriched in this fraction indicating that their presence in the pellet is of no functional significance. The average DNA size of the pellet associated material is large and its presence there may be explained by its having a high probability of random

trapping in aggregating material during nuclear lysis in EDTA. Only gene sequences that are within regions that were undergoing or poised for transcription are preferentially retained in the EDTA insoluble pellet. This probably represents a dynamic matrix association (Bodnar, 1988) which is due to the presence on those gene sequences of transcription-related complexes.

The presence of competent chromatin in the NaCl precipitate may be explained in two ways. The first is, again, random aggregation. An intuitive argument might suggest that figures 9 and 10 give evidence that this is occuring: These experiments indicate that, while more chromatin mass can be precipitated from chromatin solutions at high concentrations, the enrichments of active gene sequences does not seem to change significantly. The most likely explanation of this is that an aggregation phase accompanies precipitation. Some chromatin fragments that would be soluble in 0.15 M NaCl in a dilute solution are trapped by the forming aggregates after the addition of salt, and hence become insoluble.

In addition to random trapping, some competent chromatin fragments may not have a sufficient number of contiguous nucleosomes (that is, a run or string of nucleosomes) which are altered from the canonical form, to prevent the formation of salt precipitable structures. The mathematical model for chromatin fibre folding of Graziano

et al. (1988), for example, assumes that a string of a number of contiguous nucleosomes having normal association with linker histones is necessary for the transition to higher-order structure. Predictions from the model, when fitted to data, suggested that a run of five to seven contiguous nucleosomes are needed for the transition to higher-order structure.

The salt solubility property of competent chromatin may lie in periodic perturbations of a "foldable" chromatin structure. As long as too few contiguous nucleosomes are not of the canonical structure, which allows for normal transitions to higher-order structure, precipitation may only occur through random aggregation. In this way a given competent chromatin fragment may become insoluble if it contains sufficiently long runs of non-altered nucleosomes.

It is interesting in this regard to note the differing degrees of polynucleosome solubility that distinguish the housekeeping and tissue specific gene-chromatin classes. A simple explanation for this observed difference may be that it reflects the relative density of non-canonical nucleosomes. It follows from this that altered-nucleosome-density correlates with transcriptional rate. This makes sense in terms of the replication-expression model where changes in various aspects of chromatin structure are coupled to transcription related events.

The role of linker histone in determining chromatin structure

The sigmoid shape of the curves defined by chromatin solubility as a function of linker histone density strongly suggests that these histones interact cooperatively in the formation of salt precipitable chromatin structures.

That erythroid specific gene chromatins associate with the linker histones in forming salt precipitable structures in an apparently noncooperative manner does not imply that these chromatin types are depleted in linker histones. Indeed, the salt-soluble polynucleosomes from reconstituted chromatin contain significant levels of linker histones. It does suggest that the competent chromatin fragments do not permit the normal type of interaction amongst bound linker histones.

Perhaps the most economical way of explaining the role of linker histone in gene repression is to suggest that the nucleosome core is responsible for defining the path of the DNA in the 30 nm fibril, but, without linker histone, the structure is not stable. The possibility that the core particle structure alone is capable of folding the chromatin is indicated by the fact that linker stripped chromatin is precipitable by divalent cations. This suggests that higher-order structure is possible in the absence of linker histones. Furthermore, the EM

observations of Thoma et al. (1979) indicate that some form of salt dependent transition to a higher-order structure does occur in linker stripped chromatin; but that the form of the higher-order structure is different. In terms of the stabilizing role of linker histones, cross-linking studies indicate that H1 histones interact with one another as well as nucleosome cores and linker DNA (Thomas and Khabaza, 1980). Other studies indicate that chromatin exists in structures which do not depend on the continuity of the DNA strand for their integrity (Ruiz-Carrillo et al., 1980; Weintraub, 1984). Taking these two facts together some models of chromatin higher-order structure posit an interaction between contiguous H1 molecules in stabilizing the 30 nm fibril (Weintraub, 1985).

The apparent cooperativity amongst linker histones in the formation of higher-order structures may result from a structural nucleation effect where the transition to 30 nm type structure, amongst a limited number of contiguous nucleosomes, strongly favours the transition along the entire fragment. Such a process is akin to the "zipper" model of cooperativity B - Z DNA transitions as a function of helical density in topologically constrained molecules (Peck and Wang, 1983). As discussed above, periodic perturbation of this cooperative type of interaction and consequent destabilization of the 30 nm fibril may be sufficient to account for the various properties of

competent chromatin.

The role of acetylation

The current work provides evidence that histone acetylation is a major factor in determining the differences in chromatin structure that make competent gene enriched chromatin more resistant to exogenously added linker histone induced precipitation.

The data presented here support the conclusion that the acetylation of core histones is important in determining the NaCl-solubility characteristics of competent-gene-chromatin and that this occurs via an alteration of the interaction of this type of chromatin with the linker histones. We cannot, however, exclude the possibility that other properties other than the acetylation state of the core particle also contribute to the altered association of linker histone and increased solubility in salt of competent chromatin. Although there is a significant decline in the amount of competent gene enriched salt-soluble polynucleosomes from cell incubated in the absence of butyrate, some still remain, so that the hybridization of competent gene sequences to DNA from these fractions is of a greater extent than that of probes for repressed gene sequences. This may be due to residual acetylation that remains during the incubation, differences

in nucleosome structure which are due to other factors (such as variant histone content, ubiquitination etc.) or both.

To conclude, I will suggest that greatest value in the research described here does not lie in the demonstration of some novel phenomenon or effect; but rather in a certain clarifying function it may provide to the area of chromatin research. The essential contributions of the work are: 1) a delineation of the histone variants and modifications which constitute the structurally altered chromatin that is competent for gene expression; and 2) evidence that the function of these modified and variant histones is to prevent the stabilization of a repressed conformation which is brought about by an alteration of the association of competent chromatin with the linker histones. The results from this project has given fairly direct evidence of the basic correctness of the hypothesis delineated by Weintraub (1985) and others: that the essential structural differences between competent and repressed chromatin are those that give rise to an altered association with the linker histones.

REFERENCES CITED

Affolter, M., Cote, J., Renaud, J., and Ruiz-Carrillo, A. (1987) Regulation of histone and β^A-globin gene expression during differentiation of the chicken erythroid cells. Mol. Cell. Biol. 7, 3663-3672

Alvey, M.C. Tsai, M.-J., and O'Malley, B.W. (1984) DNase I sensitive domain of the gene coding for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Biochemistry 23, 2309-2314

Allan, J., Hartman, P.G., Crane-Robinson, C., and Aviles, F.X. (1980) The structure of histone H1 and its location in chromatin. Nature 288, 675-679

Allan, J., Cowling, G.J., Harborne, N., Cattini, P., Craigie, R., and Gould, H. (1981) Regulation of the higher-order structure of chromatin by histones H1 and H5. J. Cell Biol. 90, 279-288

Allan, J., Harborne, N., Rau, D.C., and Gould, H. (1982) Participation of core histone "tails" in the stabilization of the chromatin solenoid. J. Cell. Biol. 93, 285-297

Allegra, P., Sterner, R., Clayton, D.F., and Allfrey, V.G. (1987) Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. J. Mol. Biol. 196, 379-388

Alonso, W.R., Ferris, R.C., Zhang, D.-E., and Nelson, D.A. (1987) Chicken erythrocyte β-globin chromatin enhanced solubility is a direct consequence of induced histone hyperacetylation. Nucleic Acids Res. 15, 9325-9337

Annunziato, A.T., Frado, L.-L., Y., Seale, R.L., and Woodcock, C.L.F. (1988) Treatment with sodium butyrate inhibits the complete condensation of interphase chromatin. Chromosoma 96, 132-138

Ausio, J., Sasi, R., and Fasman, G.D. (1986) Biochemical and physiochemical characterization of chromatin fractions with different degrees of solubility isolated from chicken erythrocyte nuclei. Biochemistry 25, 1981-1988

Ausio, J., and van Holde, K.E.(1986) Histone hyperacetylation: its effects on nucleosome conformation and stability. Biochemistry 25, 1421-1428

Barsoum, J., and Varshavsky, A. (1985) Preferential localization of variant nucleosomes near the 5'-end of the

mouse dihydrofolate reductase gene. J, Biol. Chem. 260, 7688-7697

Bates, D.L., and Thomas, J.O. (1981) Histones H1 and H5: one or two molecules per nucleosome? Nucleic Acids Res. 9, 5883-5894

Bloom. K., and Anderson, J.N. (1979) Conformation of ovalbumin and globin genes in chromatin during differential gene expression. J. Biol. Chem. 254, 10532-10539

Bode, J., Henco,., and Wingender, E. (1980) Modulation of nucleosome structure by histone acetylation. Eur. J. Biochem. 110, 143-152

Bode, J., Gomez-Lira, M.M., and Schoter, H. (1983) Nucleosomal particles open as the histone core becomes hyperacetylated. Eur. J. Biochem. 130, 437-445

Bodnar, J.W. (1988) A domain model for eukaryotic DNA organization: a molecular basis for cell differentiation and chromosme evolution. J. Theor. Biol. 132, 479-507

Bouteille, M., Bouvier, D., and Seve, A.P. (1983) Heterogeneity and territorial organization of the nuclear matrix and related structures. Int. Rev. Cytol. 83, 135-182

Brotherton, T.W., Covault, T., Shires, A., and Chalkley, R. (1981) Only a small fraction of avian erythrocyte histone is involved in ongoing acetylation. Nucleic Acids Res. 9, 5061-5073

Brown, D.D. (1984) The role of stable complexes that repress and activate eucaryotic genes. Cell 37, 359-365

Burch, J.B.E., and Weintraub, H. (1983) Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. Cell 33, 65-76

Burlingame, R.W., Love, W.E., Wang, B.C., Hamlin, R., Xuong, N.-H., and Moudrianakis, E.N. (1985) Crystallographic structure of the octameric histone core of the nucleosome at 3.3 Å. Science 228, 546-553

Candido, E.P.M., Reeves, R., and Davie, J.R. (1978) Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14, 105-113

Caplan, A.T., Kimura, T., Gould, H., and Allan, J. (1987) Perturbation of chromatin structure in the region of the adult beta-globin gene in chicken erythrocyte chromatin. J.

Mol. Biol. 193, 57-70

Caron, F., and Thomas, J.O. (1981) Exchange of histone H1 between segments of chromatin. J. Mol. Biol. 146, 513-537

Cartwright, I.L., Abmayr, S.M., Fleischman, G., Lowenhaupt, K., Elgin, S.C.R., Keene, M.A., and Howard, G.C. (1982) C.R.C. Crit. Rev. Biochem. 13, 1-86

Chan, S., Attisano, L., and Lewis, P.N. (1988) Histone H3 thiol reactivity and acetyltransferases in chicken erythrocyte nuclei. J. Biol. Chem. 263, 15643-15651

Chicoine, L.G., Schulman, I.G., Richman, R., Cook, R.G., and Allis, C.D. (1986) Nonrandom utilization of acetylation sites in histones isolated from Tetrahymena: evidence for functionally distinct H4 acetylation sites. J. Biol. Chem. 261, 1071-1076

Cockerill, P.N., and Garrard, W.T. (1987) Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell 44, 273-282

Conklin, K.F., and Groudine, M. (1986) Varied interaction between proviruses and adjacent host chromatin. Mol. Cell. Biol. 6, 3999-4007

Darnell, J., Lodish, H., and Baltimore, D. (1986) Molecular Cell Biology. Scientific American Books: New York

Davie, J.R. (1982) Two dimensional gel systems for rapid histone analysis for use in minislab polyacrylamide gel electrophoresis. Anal. Biochem. 120, 276-281

Davie, J.R., Numerow, L., and Delcuve, G. (1986) The nonhistone chromosomal protein, H2A-specific protease, is selectively associated with nucleosomes containing histone H1. J. Biol. Chem. 261. 10410 -10416

De Boni, U and Mintz A. (1986) Curvilinear, three-dimensional motion of chromatin domains and nucleoli in neuronal interphase nuclei. Science 234, 863-866

De Murcia, G., Huletsky, A., and Poirier, G.G. (1988) Modulation of chromatin structure by poly(ADP-ribosyl)ation. Biochem. Cell Biol. 66, 626-635

Delcuve, G.P., and Davie, J.R. (1989) Biochem. J. in press

Eickbush, T., and Moudrianakis, E.N. (1978) The histone core complex: an octamer assembled by two sets of protein-

protein interactions. Biochemistry 17: 4955-63

Einstein, A. (1953) The Meaning of Relativity (Fifth Edition). Princeton University Press: Princeton N.J.

Ferenz, C.R., and Nelson, D.A. (1985) N-Butyrate incubation of immature chicken erythrocytes preferentially enhances the solubility of beta A chromatin. Nucleic Acids Res. 13, 1977-1995

Fey, E.G., Krochmalnic, G., and Penman, S. (1986) The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analysed by sequential fractionation and resinless section electron microscopy. J. Cell Biol. 102, 1654-1665

Finley, D., and Varshavsky, A., (1985) The ubiquitin system: functions and mechanisms. Trends Biochem. Sci. 18, 343-347

Gallinaro, H., Puvion, E., Kister, L., and Jacob, M. (1983) Nuclear matrix and hnRNP share a common structural constituant associated with premessenger RNA. EMBO J. 2, 953-960

Gasaryan, K.G. (1982) Genome activity and gene expression in avian erythroid cells. Int. Rev. Cytol. 74, 95-126

Giles, K.W., and Myers, A. (1965) An improved diphenylamine method for estimation of deoxyribonucleic acid. Nature 206, 93

Gomori, G. (1955) Preparation of buffers for use in enzyme studies. Meth. Enzymology 1, 138-146

Goring, D.R., Rossant, J., Clapoff, S., Breitman, M.L., and Tsui, L.-C. (1987) In situ detection of β-galactosidase in lenses of transgenic mice with gamma-crystallin/lac z gene. Science 235, 456-458

Graziano,V., Gerchman, S.E., and V. Ramakrishnan (1988) Reconstitutuion of chromatin higher-order structure from histone H5 and depleted chromatin. J. Mol. Biol. 203, 997-1007

Gross, D.S., and Garrard, W.T. (1987) Poising chromatin for transcription. Trends Biochem. Sci. 12, 293-297

Grosveld, F., van Assendelft, G.B., Greaves, D.R., and Kollias, G. (1987) Position independent high level expression of the human β-globin gene in transgenic mice. Cell 51, 975-985

Groudine, M., and Casmir, C. (1984) Post-transcriptional regulation of the chicken thymidine kinase gene. Nucleic Acids Res. 12, 1427-1445

Hannon, R., Bateman, E., Allan, J., Harborne, N., and Gould, H. (1984) Control of RNA polymerase binding to chromatin by variations in linker histone composition. J. Mol. Biol. 180: 131-149

Harvey, R.P., Whiting, J.A., Coles, L.S., Kreig, P.A., and Wells, J.R.E. (1983) H2A.F: an extreme variant of histone H2A sequence expressed in chicken embryo. Proc. Natl. Acad. Sci. 80, 2819-2823

Hebbes, T.R., Thorne, A.W., and Crane-Robinson, C. (1988) A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J. 7, 1395-1402

Hersko, A. (1983) Ubiquitin: roles in protein modification and breakdown. Cell 34, 11-12

Huang, H., and Cole, R.D. (1984) The distribution of H1 histone is nonuniform in chromatin and correlates with different degrees on condensation. J. Biol. Chem. 259, 14237-14242

Huang, S, -Y., Barnard, M.B., Xu, M., Matsui S.-I., Rose, S.M., and Garrard W.T. (1986) The active immunoglobin kappa chain gene is packaghed by non-ubiquitin-conjugated nucleosomes. Proc. Natl. Acad. Sci. 83, 3738-3742

Hutchison, N., and Weintraub, H. (1985) Localization of DNase I-sensitive sequences to specific regions of interphase nuclei. Cell 43, 471-482

Imai, B.S., Yau, P., Baldwin, J.P., Ibel, K., May, R.P., and Bradbury, E.M. (1986) Hyperacetylation of core histones does not cause unfolding of nucleosomes. J. Biol. Chem. 261, 8784-8792

Jackson, D.A., and Cook, P.R. (1985) Transcription occurs at a nucleoskeleton. EMBO J. 4, 919-925

Jin, Y., and Cole, R.D. (1986) H1 histone exchange is limited to particular regions of chromatin that differ in aggregation properties. J. Biol. Chem. 246, 3420-3427

Kimura, T., Mills, F.C., Allan, J., and Gould, H. (1983) Selective unfolding of erythroid chromatin in the region of the active ß-globin gene. Nature 306, 709-712

Komaiko, W., and Felsenfeld, G. (1985) Solubility and structure of domains of chicken erythrocyte chromatin containing transcriptionally competent and inactive genes. Biochemistry 24, 1186-1193.

Lawson, M., and Cole, R.D. (1979) Selective displacement of histone H1 from whole HeLa nuclei: effect on chromatin structure in situ as probed by micrococcal nuclease. Biochemistry 18, 2160-2166

Lewin, B. (1986) Genes II John Wiley: New York. pg. 305

Libertini, L.J., and Small, E.W. (1980) Salt induced transition of chromatin core particles sudied by tyrosine fluorescence anisotropy. Nucleic Acids Res. 8, 3517-3522

Loidl, P. (1988) Towards an understanding of the biological function of histone acetylation. FEBS Lett. 227, 91-95

Losa, R., and Brown, D.D. (1987) A bacteriophage RNA polymerase transcribes in vitro through a nucleosome core without displacing it. Cell 50, 801-808

Lorch, Y., LaPointe, J.W., and Kornberg, R.D. (1987) Nucleosomes inhibit initiation of transcription but allow chain elongation with the displacement of histones. Cell 49, 203-210

Louters, L., and Chalkley, R. (1985) Exchange of histones H1, H2A and H2B in vivo. Biochemistry 24, 3080-3085

Maniatis T., Fritsch, E.F., and Sambrook. J. (1982) Molecular Cloning. Cold Spring Harbor Lab.: Cold Spring Harbor, N.Y.

Maniatis, T., Goodburn, S., and Fisher, J.A. (1987) Regulation of inducible and tissue specific gene expression. Science 236, 1237-1245

Marzluff, W.F., and Huang, R.C.C. (1984) Transcription of RNA in isolated nuclei. In Transription and translation: a practical approach. IRL Press: Oxford. pp 89-129

Mazen. A., De Murcia, G., Bernard, S., Pouyet, J and Champagne, M. (1982) Localization of histone H5 in the subunit organization of chromatin using immonoelectron microsopy. Eur. J. Biochem. 127, 169-176

McGhee, J.D., Wood, W.I., Dolan, M., Dolan, J.D., and Felsenfeld, G. (1981) A 200 base pair region at the 5′ end of the chicken adult β-globin gene is accessible to nuclease digestion. Cell 27, 45-55

McMurray, C.T., and van Holde, K.E. (1986) Binding of ethidium bromide causes dissociation of the nucleosome core particle. Proc. Natl. Acad. Sci. 83, 8472-8476

Mirkovitch, J., Spierer, P., and Laemmli, U. (1986) Genes and loops in 320,000 base-pairs of the Drosophila melanogaster chromosome. J. Mol. Biol. 190, 255-258

Nelson, D.A., Ferris, R.C., Zhang, D.-E., and Ferenz, C.R. (1986) The β-globin domain in immature chicken erythrocytes: enhanced solubility is coincident with histone hyperacetylation. Nucleic Acids Res. 14, 1667-1682

Nelson, P.P., Albright, S.C., Wiseman, J.M., and Garrard, W.T. (1979) Reassociation of histone H1 with nucleosomes. J. Biol. Chem. 254, 11751-11760

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

Nickel, B.E. and Davie, J.R. (1989) The protamine gene chromatin in developing trout testis exists in an altered state. Biochim. Biophys. Acta 1007, 23-29

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

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

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

Oliva, R., Bazett-Jones, D., Mezquita, C., and Dixon, G.H. (1987) Factors affecting nucleosome disassembly by protamines in vitro. J. Biol. Chem. 262, 17016-17025

Pantazis, P., and Bonner, W.M. (1981) Quantitative determination of histone modification: H2A acetylation and phosphorylation J. Biol. Chem. 256, 4669-4675

Park, K., and Fasman, G.D. (1987) The histone octamer, a conformationally flexible structure. Biochemistry 26, 8042-8045

Paulson, J.R., and Laemmli, U.K. (1977) The structure of histone-depleted metaphase chromosomes. Cell 12, 817-828

Peck, L.J., and Wang, J.C. (1983) Energetics of B-to-Z transition in DNA. Proc. Natl. Acad. Sci. 80, 6206-6210

Pederson, D.S., Thoma, F., and R.T. Simpson (1986) Core particle, fiber and transcriptionally active chromatin structure Ann. Rev. Cell Biol. 2, 117-147

Perry, M., and Chalkley, R. (1981) The effect of histone hyperacetylation on the nuclease sensitivity and solubility of chromatin. J. Biol. Chem. 256, 3313-3318

Perry, M., and Chalkley, R. (1982) Histone acetylation increases the solubility of chromatin and occurs sequentially over most of the chromatin. J. Biol. Chem. 257, 7336-7347

Phi-Van, L and Stratling, W.H, (1988) The matrix attachment region of the chicken lysosyme gene co-map with the boundries of the chromatin domain. EMBO J. 7, 655-664

Prigogine, I. (1980) From Being to Becoming: Time and Complexity in the Physical Sciences. W.H. Freeman and Co.: New York

Quaife, C.J., Pinkert, C.A., Ornitz, D.M., Palmiter, R.D., and Brinster, R.L. (1987) Pancreatic neoplasia induced by ras expression in acinar cells of transgenic mice. Cell 48,1023-1034

Rammal, R. Toulouse, G., and Virasoro, M.A. (1986) Ultrametricity for physicists. Rev. Mod. Phys. 58, 765-788.

Rattner, J.B., and Hamkalo, B.A. (1978) Higher-order structure in metaphase chromosome. I. The 250 Å fiber. Chromosoma (Berl.) 69, 363-372

Razin, S.V., Yarovaya, O.V. (1985) Initiated complexes of RNA polymerase II are concentrated in the nuclear skeleton associated DNA. Exp. Cell. Res. 158, 2739-2755

Razin, S.V., Yarovaya, O.V., and Georgiev, G.P. (1985) Low ionic strength extraction of nuclease-treated nuclei destroys the attachment of transcriptionally active DNA to the nuclear skeleton. Nucleic Acids Res. 13, 7427-7444

Reeves, R. (1984) Transcriptionally active chromatin. Biochim Biophys. Acta 782, 343-393

Renz, M., Nehl, P., and Hozier, J. (1977) Involvement of histone H1 in the organization of the chromatin fiber. Proc. Natl. Acad. Sci. 74, 1879-1882

Rhodes, D. (1985) Structural analysis of a triple complex between the histone octamer a Xenopus gene for 5S RNA and transcription factor IIIA. EMBO J. 4, 3473-8233

Richards, R.G., and Shaw, B.R. (1982) In situ protamine release: a versatile sample preparation method for the electrophoretic analysis of nuclear proteins on acid/urea-based gels. Anal. Biochem. 121, 69 -82

Richmond, T.J., Finch, T., Rushton, B., Rhodes, D., and Klug, A. (1984) Structure of the nucleosome core particle at 7 Å resolution. Nature 311, 532-537

Rocha, E., Davie, J.R., van Holde, K.E., and Weintraub, H. (1984) Differential salt fractionation of active and inactive genomic domains in chicken erythrocyte. J. Biol. Chem. 259, 8558-8563

Ruiz-Carillo, A., Puigdomenech, P., Eder, G., and Lurz, R. (1980) Stability and reversibiltity of higher ordered structured of chromatin: continuity of DNA is not required for folded structure. Biochemistry 19, 2544-2554

Ruiz-Carrillo, A., Affolter, M., and Renaud, J. (1983) Genomic organization of the genes coding for the six main histones of the chicken: complete sequence of the H5 gene. J. Mol. Biol. 170, 843-859

Sanders, M.M. (1978) Fractionation of nucleosomes by salt elution from micrococcal nuclease digested nuclei. J. Cell Biol. 79, 97-109.

Schlissel, M.S. Brown, D.D. (1984) The transcriptional regulation of Xenopus 5s RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. Cell 37, 903-913

Schroder, H.C., Bachman, M., Diehl-Seifert, B., and Muller, W.E.G. (1987) Transport of mRNA from nucleus to cytoplasm. Prog. Nuc. Acids. Res. Mol. Biol. 34, 89-142.

Schubach, W., and Groudine, M. (1984) Alteration of c-myc chromatin structure by avian leukosis virus integration. Nature 307, 702-708

Seale, R.L. (1981) Rapid turnover of the ubiquitin conjugate, protein A24. Nucleic Acids Res. 9, 3151-3158

Simpson, R.T. (1978) Structure of chromatin containing extensively acetylated H3 and H4. Cell 13, 691-699.

Sun Y.L., Xu, Y.Z., Bellard, M., and Chambon, P. (1986) Digestion of chicken β-globin gene with micrococcal nuclease reveals the presence of altered nucleosomal array characterized by an atypical ladder of DNA fragments. EMBO J. 5, 293-300

Sung, M.T., Harford, J., Bundman, M., and Vidalakas, G. (1977) Metabolism of histones in avian erythroid cells. Biochemistry 16, 279-285

Thoma, F., Koller, T., and Klug, A. (1979) Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. J. Cell Biol. 83, 403-427

Thomas, J.O., and Khabaza, A. (1980) Cross-linking of H1 in chromatin. Eur. J. Biochem. 112, 501-511

Thomas, J.O., Rees, C., and Pearson, E.C. (1985) Histone H5 promotes the association of condensed chromatin fragments to give pseudo-higher-order structures. Eur. J. Biochem. 147, 143-151.

Thomas, J.O., and Rees, C. (1983) Exchange of H1 and H5 between chromatin fragments. Eur. J. Biochem. 134, 109-115

Thomas, P.S. (1979) Hybridization of denatured RNA and small DNA fragments transfered to nitrocellulose. Proc. Natl. Acad. Sci. 77, 5201-5205

Thorne, A.W., Sautiere, P., Briand, G.M., and Crane-Robinsion, C. (1987) The strucutre of ubiquitinated histone H2B. EMBO J. 6, 1005-1010

Uberbacher, E.C., Harp, J.M., Wilkinson-Singley, E., and Bunick G.J.(1986) Shape analysis of the histone octamer in solution. Science 232, 1247-1249

Urban, M.K., Franklin, S.G., and Zweidler, A. (1979) Isolation and characterization of histone variants in chicken erythrocytes. Biochemistry 18, 3952-3960

Verheijen, R., Van Venrooij, W., and Ramaekers, F. (1988) The nuclear matrix: structure and composition. J. Cell Science 90, 11-36

Vidali, G., Ferrari, N., and Pfeffer, U. (1988) Histone

acetylation: a step in gene activation. Adv. Exp. Med. Biol. 231, 583-596

Villeponteau, B., Landes, G.M., Pankratz, M.J., and Martinson, H.G. (1982) The chicken β-globin gene region: delineation of transcription units and developmental regulation of interspersed DNA repeats. J. Biol. Chem. 257, 11015-11023

Villeponteau, B., Lundell, M., and Martinson, H. (1984) Torsional stress promotes the DNase I sensitivity of active genes. Cell 39, 469-478.

Vogelstein, B., Pardoll, D.M., and Coffey D.S. (1980) Supercoiled loops and eucaryotic DNA replication. Cell 22, 79-85

Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A., and Weiner, A.M. (1987) Molecular Biology of the Gene (Fourth Edition). Benjamin/Cummings: Menlo Park, CA.

Weintraub, H. (1984) Histone-H1-dependent chromatin superstructures and the supression of gene activity. Cell 38, 17-27

Weintraub, H. (1985) Assembly and propagation of repressed and derepressed chromosomal states. Cell 42, 705-711

Weintraub, H., and Groudine, M. (1976) Chromosomal subunits in active genes have an altered conformatin. Science 93, 848-858

Widom, J. (1986) Physicocheimical studies on the folding of the 100 Å nucleosome filament into the 300 Å filament: cation dependence. J. Mol. Biol. 190, 411-424

Williams, A.F. (1972) DNA synthesis in purified populations of avian erythroid cells. J. Cell Sci. 10, 27-46

Williams, S.P., Athey, B.D., Muglia, L.J., Schappe, S., Gough, A.H., and Langmore, J.P. (1986) Chromatin fibers are left-handed double helices with diameter and mass per unit length that depend on linker length. Biophys. J. 49, 233-248

Wilson, K. (1983) The renormalization group and critical phenomena. Rev. Mod Phys. 55, 583-600.

Woo, S.L.C., Dugaiczyk, A., Tsai, M.-J., Lai, E.C., Catterall, J.F., and O'Malley, B.W, (1978) The ovalbumin gene: cloning of the natural gene. Proc. Natl. Acad. Sci. 75, 3688-3692

Wong, N.T.N., and Candido, E.P.M. (1978) Histone H3 thiol reactivity as a probe of nucleosome structure. J. Biol. Chem. 253, 8263-8268

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

Wu, R.S., and Bonner, W.M. (1981) Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell 27, 321-330.

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

Wu, R.S., Tsai, S., and Bonner, W.M. (1982) Patterns of histone variant synthesis can distinguish G0 from G1 cells. Cell 31, 367-378

Wu, R.S., Tsai, S., and Bonner, W.M. (1983) Changes in histone H3 composition and pattern during lymphocyte activation. Biochemistry 22, 3868-3880

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

Yaniv, M., and Cereghini, S. (1986) Structure of transcriptionally active chromatin. C.R.C. Crit. Rev. Biochem. 21, 1-25

Zehner, Z.E., and Paterson, B.M. (1983) Characterization of the chicken vimentin gene: single copy gene producing multiple mRNAs. Proc. Natl. Acad. Sci. 80, 911-915

Zeitlin, S., Parent, A., Silverstein, S., and Efstratiadis, A (1987). Pre-mRNA splicing and the nuclear matrix. Mol Cell Biol 7, 1111-1120

Zhang, D.-E., and Nelson, D.A. (1986) Histone acetylation in chicken erythrocytes. Estimation of the percentage of sites actively modified. Biochem. J. 240, 857-862

Zhang, D.-E., and Nelson, D.A. (1988a) Histone acetylation in chicken erythrocytes: rates of acetylation and evidence tht histones in both active and potentially active chromatin are rapidly modified. Biochem. J. 250, 233-240

Zhang, D.-E., and Nelson, D.A. (1988b) Histone acetylation in chicken erythrocytes: rates of deacetylation in immature

and mature red blood cells. Biochem. J. 250, 241-245