CHROMATIN STRUCTURE AND GENE ACTIVITY: THE ROLE OF NONHISTONE CHROMOSOMAL PROTEINS

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I. INTRODUCTION

The basic chromatin fiber for all DNA sequences is currently believed to consist of a chain of repeating subunits, the ν bodies or nucleosomes, constructed by wrapping the DNA around histone cores. Obviously this fiber must be folded or organized into higher order structures, culminating ultimately in the metaphase chromosome. It is thought that the basic chromatin fiber is wound further into a solenoid, using approximately six nucleosomes per turn to generate a 300-Å fiber. Data from genetics and cytology would suggest that the chromosome must be organized in units of some 10 to 200 kb of DNA, representing the cis-dominant complementation units or Mendelian genes. The structural basis of this latter organization remains unknown.

Given this broad outline of the organization of chromatin, one will next wish to address the question of specificity within this organization. To what extent can one detect specific chromatin structures correlated with DNA sequence maps which are now becoming available through recombinant DNA technology? With what specificity are nucleosomes distributed along the DNA sequence? What is the structural organization and degree of specificity of the 300-Å fiber? Can genes be defined in terms of some specific structure or set of boundary conditions that delineate the unit? The transcription unit and the metaphase chromosomes, the initial and final units of organization, appear to have well-defined boundaries, but what is the intermediate organization, if any?

While it appears that both active and inactive chromatin possess to some degree the fundamental structure dictated by DNA association with histones, distinct structural differences between these states have been detected. Active genes have a more open configuration in general. A specific chromatin structure, detected as a site hypersensitive to cleavage by DNase I, appears to be necessary, but not sufficient for gene activity. In a gene being transcribed, the histone-DNA interaction has been altered. A determination of the boundaries of these perturbations of chromatin structure in relation to the region

of transcription may help to define functional units within chromatin. One would like to know what changes in the macromolecules of chromatin are involved (i.e., additions, removals, and modifications). One would like to know the causality of the events, what is necessary and what is sufficient, for commitment as well as for gene expression.

Given the apparent constancy of the histone-DNA interaction in nucleosome structure, it has been attractive to approach some of these problems through analysis of the nonhistone chromosomal proteins (NHC proteins). These proteins may play particular roles in establishing domains, i.e., in defining boundaries and/or in the general packaging of the chromatin fiber into different types of structures, e.g., heterochromatin or euchromatin. It is also reasonable to speculate that such proteins are involved in the changes of chromatin structure that are related to gene expression, both as specific effectors and as components of the necessary "machinery". During the last several years, a method for analyzing the distribution patterns of nonhistone chromosomal proteins on the polytene chromosomes of *Drosophila* has been developed. Use of this material allows resolution at the level of the chromomere, or gene, and allows interpretation of the data obtained in terms of the extensive genetic and molecular knowledge of the *Drosophila* genome. Results to date suggest that one can identify several distinct classes of the nonhistone chromosomal proteins: those present in a very broad general distribution, in that regard analogous to the histones, which might play some role in the general chromatin fiber structure; those limited to a major subset of chromatin such as heterochromatin or euchromatin; those associated primarily with the loci which can be active in a given cell type; and those associated primarily with the loci which are active at the moment of analysis. This classification in itself suggests that many of the nonhistone chromosomal proteins play a key role in determining the general chromatin structure of subsets of genes related to their activity state. More recently, techniques have been developed which are useful in determining the specific interactions between DNA and chromosomal proteins that must ultimately be identified to explain specificity in chromatin structure. These methods and others have been used to analyze changes in chromatin structure that occur during gene activation.

The goal in this review will be to examine present knowledge of chromatin structure, the evidence for specificity in the structural organization, and the results available concerning the process of gene activation. The authors will report in particular detail relevant work on the nonhistone chromosomal proteins. They believe that an understanding of the role of these proteins is critical for a resolution of the questions of interest.

II. LEVELS OF CHROMATIN ORGANIZATION

In this section the authors attempt to assess critically the myriad studies performed to analyze chromatin structure at each level from the simplest association of DNA with histone through the complex packaging of chromatin in the metaphase chromosome. Recent reviews pertinent to this area have been published by Felsenfeld,[1] Mirzabekov[2] and McGhee and Felsenfeld.[3] It is becoming abundantly clear that the organization of chromatin in the nucleus of eukaryotes plays an important role in the functional aspects of gene expression. At the purely structural level, the nucleosome and its hierarchy of higher order structures must be involved. In addition, the association of NHC proteins with chromatin can modulate such structures and also play more direct roles in the regulation of genes.

A. The 100-Å Fiber

The basic repetitive unit of chromatin organization is the nucleosome, a structure originally revealed both biochemically, by nuclease digestion of chromatin,[4-7] and

cytologically, by visualization of chromatin in the electron microscope.[8-10] Since then, increasingly sophisticated studies have led to a refined view of the nucleosome core as a deoxyribonucleoprotein (DNP) particle consisting of a flat disk of protein composed of two each of histones H2A, H2B, H3, and H4 associated with 145 base pairs (bp) of B-form DNA wound around the protein core in 1 3/4 left-handed superhelical turns with 80 bp per turn.[11,12] Implicit in this model is the core octamer of histones. Much effort has been devoted to investigating the histone-histone interactions that occur in solution and in chromatin itself (e.g., References 13 to 15). It was shown conclusively that the four core histones are present in equimolar amounts in nucleosomes,[16,17] a result absolutely required by the current model. It appears that histones display a precise and conserved interaction.[2,18,19]

In the electron microscope, this level of organization is revealed as a 100-Å diameter nucleofilament consisting of the nucleosomal particles connected by short stretches of DNA (the "linker" or "spacer") giving the familiar "beads-on-a-string" picture. The linker DNA is thus relatively exposed to the action of nucleases leading to the production of oligo- and mononucleosomal particles on digestion. Digestion studies with micrococcal nuclease show that at relatively early stages of digestion, mononucleosome particles containing approximately 200 bp of DNA are released. These are in addition associated with one molecule of histone H1 and with NHC proteins. At later stages of digestion a relatively resistant particle containing 165 bp of DNA (the "chromatosome") is produced, leading ultimately to the resistant nucleosome core particle with 145 bp of DNA.[20-22]

In addition to all four species of core histone the chromatosome contains one molecule of histone H1; the interaction of H1 with the DNA of this particle and with linker DNA between nucleosomes is of critical interest. Simpson noted that H1 appeared to protect 10 bp of DNA at each end of the core particle from digestion and thus stabilized a DNA fragment capable of two complete turns around the histone core.[22] This interpretation has received support from studies of cross linking of H1 to DNA,[23,24] as well as from electron microscopy (EM). In each nucleosome of H1-containing chromatin, the DNA appears to enter and exit at the same point, while in H1-depleted chromatin the DNA shows a random entry-exit pattern.[25] However, it seems likely that the interaction of H1 with nucleosomes may not be identical when comparing the monomer with oligonucleosomes.

Chromatin from virtually all species and tissues examined can be digested to a 145 bp core particle with concomitant loss of H1. It has been suggested that the 165 bp particle may also be a distinct structural component of all eukaryotic chromatin fibers.[26] The distance of one nucleosome from another can vary widely, with approximately 165 to 250 bp of DNA per nucleosome as produced by light nuclease digestion.[27] The difference is in the length of linker DNA. The linker DNA appears to be located in a symmetrical fashion at each side of the core, indicating that in vivo the linker is packaged between cores nonrandomly.[28] Recent evidence has shown that the property of variable linker length can be detected within a given cell type as well as in chromatin from different tissues of an organism. This aspect of nucleosome organization and its functional implications will be discussed further.

The reconstitution of the basic repeating nucleosomal pattern structure in vitro from DNA and the histone octamer has been a subject of much investigation.[29] It soon became clear that a reversal of the chromatin dissociation process (i.e., a slow dialysis from high salt and/or urea solution) could reassemble the histone octamer with 145 bp of DNA into a genuine nucleosome core particle.[30] Positive results can also be obtained using high molecular weight DNA.[31] More recent studies have shown that even at physiological ionic strengths, nucleosome cores can reassemble from dispersed histones and DNA.[32] This is facilitated by polyglutamic acid.[33] However, in all of these studies the 200 bp

spacing of native chromatin is not reproduced; digestion with micrococcal nuclease produces only 145 bp-containing cores. Addition of H1 to the reconstitution mixture does not alter the spacing and often leads to problems of precipitation. Two different proteins have been implicated in assembling DNA and histone octamers into nucleosomes in vitro. Germond et al. reported that a nicking-closing enzyme from *Drosophila* (DNA topoisomerase I) assembled closely spaced nucleosome cores from relaxed closed circular DNA, introducing in the process the correct change in winding number.[34] However, more recent studies indicate that topoisomerase I is not sufficient for assembly.[35] An acidic, thermostable protein has been isolated by Laskey et al. from *Xenopus* eggs which assembles nucleosome cores onto DNA at physiological ionic strength.[36] This protein probably acts as a molecular "chaperone" by first binding to the assembled histones and thus allowing only the correct interactions with DNA for nucleosome assembly to occur. There is no topoisomerase activity associated with this protein, termed "nucleoplasmin"; it cannot introduce the correct supercoiling into closed circular DNA in the absence of added enzyme. Again, the system does not introduce correct 200 bp spacing into the nucleosomal array. It has, however, proved possible to assemble chromatin on naked DNA with approximately 200 bp spacing using unfractionated cell homogenates from both *Xenopus* eggs[37] and *Drosophila* embryos.[38] The two proteins previously described are probably involved, but obviously other factors, which remain to be characterized, are required. Since the precise nature of histone-DNA interactions has not yet been determined, the accuracy of the system cannot yet be assessed. That the "nucleoplasmin" factor is of relevance to in vivo assembly is borne out by studies which show that in *Xenopus* oocytes, with their large histone pools, it represents up to 10% of the soluble nuclear-associated protein.[39,40] In fact, it is found in the nuclei of most cell types examined, although not in erythrocytes or spermatocytes which lack chromatin replication.[39,41]

Some rather interesting points about nucleosome placement are raised by these studies and others. It has been suggested that in the *Xenopus* cell-free assembly system, the nucleosomes are placed on DNA randomly, and the correct spacing is achieved by sliding of nucleosomes along the DNA.[29] Certainly there is ample precedent for sliding of nucleosomes, not only in studies at high ionic strengh where the chromatin has been depleted of H1,[31,42-44] but also in studies using more physiological conditions.[45] It is, however, not necessary that depletion of H1 from chromatin inevitably leads to rearrangements; milder methods of depletion have shown that the nucleosomal repeat can remain intact at approximately 200 bp,[26,46,47] and that reconstitution of such gently stripped chromatin with H1 (or H5) can reestablish the 165-bp chromatosome (see preceding) if not the same 200-bp repeat.[47-50] It would seem that some cellular factor(s) is absolutely required to establish the correct spacing or to limit sliding of nucleosomes.

Reconstitution studies have been carried out using prokaryotic DNA as substrate,[38,51] and it is clear that nothing in the sequence of eukaryotic DNA is necessary to achieve nucleosome assembly and spacing. This is not to say, however, that there is no underlying sequence information to direct specific placement of nucleosome cores relative to one another under appropriate conditions and so achieve a "positioned" nucleosomal arrangement. The topic of positioning will be discussed in Section III.B. However, it is worth pointing out that a specific placement of histone cores has been detected in reconstitution experiments using 203- and 144-bp fragments from the *Escherichia coli* lac control region.[52] Although the 144-bp fragment was derived from the 203-bp fragment by a restriction cut within the latter, there was no correlation between the two preferred positions for the larger fragment and the three preferred placements for the shorter fragment. Random sequence calf thymus DNA of 145 ± 10 bp gave a random reconstitution pattern with respect to the 5′ end in this study, in contrast to the results of Tatchell and van Holde[53] who found distinct "end effects" when reconstitutions with

random sequence DNA fragments up to 161 bp were performed. The inference from the latter work was that the 5′ ends of such fragments were preferentially located at a given position on the histone core, leading to a nonrandom placement. However, fragments of 177 bp were placed randomly, since the length of the DNA was sufficient to cover both "end" binding sites on the histone core. The discrepancy between these reports may be due to a poor degree of size determination of the random sequence DNA in the former case. Clearly precise location of nucleosomes in vivo would not rely on end effects although single-stranded nicks or other specific boundaries in genomic DNA might have the same organizational effect. Further studies on sequence regularities and positioning of nucleosomes will be described in Section III.B.

The accessibility of the major and minor grooves of the DNA double helix to solvent or other molecules could be affected by winding around the histone core. This has been directly tested in experiments where isolated nucleosomes or chromatin were reacted with dimethyl sulfate. The patterns of methylation of N-7 of guanine in the major groove and N-3 of adenine in the minor groove were sufficiently similar to that found in free DNA for it to be concluded that both grooves were probably highly accessible in the nucleosome structure.[54-56] Such an observation is of importance when one considers that hypotheses of gene regulation by protein effectors would necessarily involve highly specific interaction with DNA sequences. That specific sequences are recognizable in nucleosome structures was shown particularly effectively by Chao et al.[57,58] when they demonstrated the tight binding of *lac* repressor protein to *lac* operator DNA previously reconstituted with core histones into nucleosomes. This, of course, utilizes a sequence recognition phenomenon from a prokaryotic system normally devoid of histones.

B. 300-Å Fiber

Early investigations of chromatin in nuclei using the electron microscope revealed the existence and generality of a level of packaging producing thick fibers with a diameter of 250 to 300 Å (see References 59 and 60 for review). This represents the next level of DNA packaging above the nucleosome. It has been readily demonstrated in many studies that the conversion of the thick 300-Å fiber to the well-known 100-Å diameter nucleosomal filament (or vice versa) is sensitive to the monovalent and/or divalent cation concentration (e.g., references 25 and 61 to 63). The question as to how the nucleosomes themselves are organized relative to one another in this thick fiber has been the subject of much experimentation in recent years using a variety of methods and producing a variety of results. Many differences are apparent in the nuclear and/or chromatin preparative procedures adopted by different laboratories and the conditions under which observations utilizing the same basic methodology are made. In particular much (and sometimes not so much) attention has been paid to observations made under different ionic conditions with a range of chromatin substrates ranging from short oligonucleosomes up to intact chromosomes and nuclei. Typical among the problems to be encountered when working with chromatin are the very limited solubility at moderate ionic strengths (e.g., 0.15 *M* salt), easy extraction of NHC proteins and histone H1, and dissociation of nucleosomes at low DNA concentration.[64]

Two main schools of thought have developed. In one, arising from the original electron microscope observations of Finch and Klug, the nucleosomes are envisaged as coiling into a regular superhelix or solenoid of relatively fixed dimensions.[61] Each turn of the solenoid is suggested to contain 6 to 7 nucleosomes and have a pitch of approximately 100 Å with a diameter of 250 to 300 Å. The alternative hypothesis is a form of supranucleosomal packaging entitled the "superbead."[65] This cluster of nucleosomes (varying in number from 7 up to 20 or more) is presumed to have a distinct free energy minimum generating a stable intermediate. The arrangement of nucleosomes within the superbead is not clear, but the superbead is reported to be stabilized as a unit by H1 (and

NHC proteins). In vivo, such superbeads are thought to be arranged tandemly to give the 300-Å fiber, which thus must possess additional features for stabilizing superbead interaction. While less attractive, a model of a 300-Å fiber which is neither of the preceding but oscillates between them also seems plausible.

The early electron microscope study of Finch and Klug showed a condensation of the 100-Å nucleofilament into what was interpreted as a regular solenoidal structure.[61] This interpretation received support from X-ray studies of chromatin fibers.[66] Newer data from other groups have confirmed and extended the model to a rather sophisticated level. Neutron scattering affords the ability to obtain structural data from chromatin in solution. Suau and his colleagues concluded that chromatin prepared from gently lysed nuclei was arrayed in the regular helical structure of a solenoid.[67] It appeared that the individual nucleosome disks were packed edge to edge in the 100-Å filament (confirmed by Thoma et al.[25] in their EM study), and that this was further wound into a superhelix with the disk faces perpendicular to the solenoidal axis and containing six nucleosomes per turn, with a seventh constrained in the central hole thereby formed. The precise arrangement of nucleosome disks within the solenoid has been challenged by a study using electric dichroism by McGhee and his colleagues.[68] While confirming the edge-to-edge arrangement in the 100-Å fiber, their data implied that in the solenoidal configuration induced in chicken erythrocyte chromatin by addition of divalent ion, the chromatosome faces are arranged radially and, assuming supercoiled spacer DNA, with a tilt of 20 to 25° from the solenoid axis. A rather detailed model of the 300-Å solenoid was proposed by these workers, but discussion of this will be deferred in order to compare it with other recent proposals. A further study has also used the electric dichroism technique (among others) to confirm the solenoid in cross-linked fragments of calf thymus chromatin, but estimating that the disk faces are tilted 39° from the solenoid axis,[69] a small difference from the work previously cited. All of these studies have used isolated chromatin, in most cases solubilized by varying degrees of micrococcal nuclease digestion, and as such cannot be said to represent chromatin in the in vivo state, since there may well be a loss of structural information upon breaking the nuclei and cutting the fiber (certainly a loss of torsional constraint) as well as inevitable losses of NHC proteins. It is likely, however, that the unit of six nucleosomes has relevance to higher-order packaging. A study of oligonucleosomes by electric birefringence gave a sharp transition in the optical properties at the level of the hexanucleosome. The structure formed was reported to be a symmetrical helix with a diameter of 250 Å[70]. A sedimentation study of oligonucleosomes over a range of sizes and under a variety of salt conditions showed a sharp step function at the six to seven nucleosome level at moderate monovalent ionic strengths (25 m*M*), suggesting a particularly stable conformation.[71] No other obvious changes with higher oligomers were observed other than could be explained by a flexing of a long helical fiber.[72] No discontinuities in the sedimentation behavior at different salt concentrations could be detected at the level of hexameric multimers (e.g., 12-mer, 18-mer), or at any other intermediate value at which a superbead might be concluded to have stability.

Physical studies such as neutron and X-ray scattering are beginning to be performed on nuclei or whole cells.[73,74] This certainly represents the ideal solution to the problems inherent in chromatin preparation, although the data at present are difficult to interpret as obviously concordant with the solenoidal model. In general, however, it would appear that the overall predictions of such a model are satisfied, together with a suggestion that the 300-Å fibers are close-packed, side-by-side as it were, as previously seen in thin-sections of cells.[75] Interestingly, the so-called 300-Å fibers appeared to be closer to 370 to 400 Å homogeneous cylinders in vivo.[74]

On the other hand, a number of other studies using EM have provided evidence for the existence of "superbeads" in the spread chromatin preparations, at ionic strengths

intermediate between those considered to be physiological and those at which the "string of beads" configuration is observed (e.g., References 62 and 65). These globular clusters of chromatin, the nucleosomal content of which seem to vary somewhat depending on the tissue studied and the method of preparation (e.g., References 62, 65, 76, and 77), have been presumed to be a stable form of nucleosomal packaging since they are observed with some regularity in the preparations. Much of the early work on the isolation and biochemistry of superbeads has been called into question, however, by the observation that the DNP clusters isolated on sucrose gradients after light micrococcal nuclease digestion of nuclei (and presumed to represent the intact superbeads[78,79]) were in many cases likely to have been ribonucleoprotein (RNP) particles.[80,81]

In addition, other groups carrying out careful analyses under controlled conditions have failed to find convincing proof for the significance of these nucleosome clusters other than as metastable aggregates in a subnormal ionic strength range. Thus, Thoma et al.[25] investigated the folding of chromatin fragments (released by micrococcal nuclease from nuclei) under a great variety of monovalent and divalent salt conditions and compared native chromatin with H1-depleted chromatin. At low sodium chloride concentrations, H1-containing chromatin appeared as the expected beaded structure with two full turns of DNA per nucleosome (see preceding). Slight elevation of the salt concentration resulted in chromatin compacted into a tighter structure characterized by an obvious zigzag disposition of nucleosomes one to the other (Figure 1). This arrangement has been noted by other authors and will be discussed. Between 20 and 60 m*M* salt, clusters that could well have been interpreted as superbeads, were occasionally seen, but these appeared random, and a smooth transition to a regular thick 250- to 300-Å fiber occurred at elevated salt concentrations. The relatively smooth geometry of this fiber together with its apparent cross striations led Thoma et al. to propose that a regular solenoidal arrangement was in evidence, with the nucleosome disk faces lying near parallel to the fiber axis.[25] Later physical studies have supported the apparent disposition of the core particles both in the 100- and 250- to 300-Å fibers.[68] Slight discontinuities in the 250- to 300-Å fiber were, however, apparent,[25] giving a slightly knobby appearance, and this observation is confirmed by Hamkalo's group in their studies of gently lysed nuclei.[63] Thoma and his colleagues have extended the preceding studies to look at the effects of salt-induced condensation on chromatin depleted either of a substantial fraction of its NHC proteins or of both its NHC proteins and H1.[82,83] NHC proteins extracted at 0.35 *M* NaCl were found to have little effect on the folding of chromatin into higher order structures, but could prevent unraveling of nucleosomes at very low ionic strength. The presence of H1 was critical in allowing the closed zigzag fibers to form, and subsequently to condense into continuous compact 300-Å fibers at elevated salt concentrations. Virtually identical results were obtained using depleted chromatin reconstituted with NHC proteins and/or H1. Partial removal of H1 resulted in a series of tangled structures, and it was concluded that an H1 polymer of some type was essential for higher-order stabilization.

All of the studies employing EM cited previously have used preparations of chromatin isolated from nuclei, generally by nuclease digestion, and therefore can hardly be considered a perfect model for the in vivo situation. Rattner and Hamkalo have gently lysed both metaphase cells[84,63] and interphase nuclei[85] and studied the unfolding of the chromosome as a function of time using the electron microsope. Mechanical lysis with glass beads and subsequent unfolding in solutions at physiological ionic strengh gave loops of 300-Å fibers with no observable superbead-type clusters. Negative shadowing allows the individual nucleosomes to be visualized. These are usually present two to three deep in the fiber, often have a zigzag type of configuration,[84,85] and can be broadly interpreted as existing as solenoids. However, the figures display a mosaicism, in that there are irregular features present which could represent short stretches of solenoid or

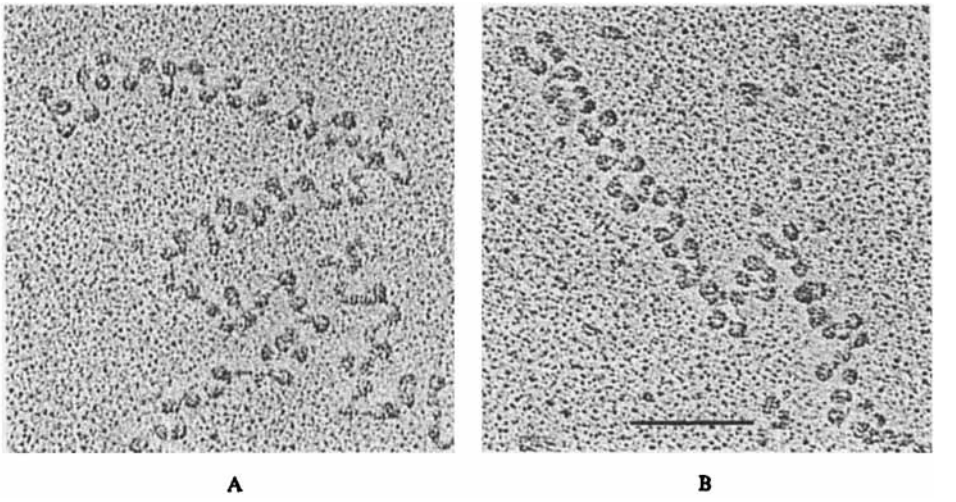

FIGURE 1. Electron micrographs of rat liver chromatin fibers fixed at two different ionic strengths and rotary shadowed with carbon platinum. Fixation was performed in: (A) 1 m*M* triethanolamine chloride (TEA Cl), pH 7.0, 0.2 m*M* EDTA; (B) 5m*M* TEA Cl, pH 7.0, 0.2 m*M* EDTA Bar, 0.1 μm. (Magnification × 160,000.) (From Thoma, F., Koller, Th., and Klug, A., *J. Cell Biol.*, 83, 403, 1979. With permission.)

other arrangements of nucleosomes, and might be precursors to the superbead-type clusters seen by others. It was suggested that such structural mosaicism of the 300-Å fiber might indicate different functional states of the genome and therefore would require sequence-specific elements.[86] When lysis was performed with detergents and/or formalin was used in the chromatin preparation, artifactual unfolding of chromatin (apparently due to loss of H1) was observed; included in the resulting substructures were apparent superbeads of irregular size.

While this latter work and that of Thoma et al.[25,82] would seem to have granted superbeads only a limited existence at best, new evidence from both nuclease digestion and EM studies is rather harder to refute, and it in fact seems likely that superbeads represent the disruption of local short-range interactions between solenoidal segments of limited length that may well have a functional basis. Two rather careful EM studies on the dispersal of chromatin from intact nuclei by swelling in buffers of varying ionic strength have recently been published. Zentgraf et al. have dispersed chicken erythrocyte nuclei into a buffer of low ionic strength and observed the unfolding (as a function of time) of the 300-Å fiber to the 100-Å fiber with intermediate discrete granular particles of mean diameter 290 Å.[87] These particles can be regenerated rapidly by exposure of the 100-Å fiber to higher concentrations of monovalent cations or to very low concentrations of divalent ions. The material can be recycled from cluster to monomer form repeatedly. The special importance of divalent cations to the integrity of the structure was emphasized in this study. This has been previously noted[61,25] and reemphasized by Pruitt and Grainger[88] in their extensive EM study of chromatin unfolding from chick erythrocyte nuclei. The results here in many ways complement those of Zentgraf et al.[87] Clustered structures of cylindrical shape (275 Å × 300 Å) were regularly seen when nuclei were prepared under physiological salt conditions and lysed into buffers of progressively lower ionic strength (in the range 8 to 40 m*M* monovalent cation, 0.07 to 0.2 m*M* divalent cation). In the presence of divalent cations, such structures were consistently observed and were statistically significant in size homogeneity. In the absence of divalent cations, the clusters were random in size and frequently dissociated to nucleosome monomers. In the absence of monovalent cations, regular 330-Å long cylinders could be observed down to 10^{-6} *M* divalent cation. Of particular interest was the observation that these same

regularly sized clusters were observed at physiological ionic strength when H1 (and some NHC proteins) was removed by a procedure utilizing tRNA, previously developed by Ilyin et al.[89] Clearly H1 was intimately involved in the higher-order packaging. It was emphasized that such regularly sized clusters were also seen from nuclei from other tissues including *Xenopus* and *Drosophila* embryos.

Stratling and Klingholz have continued to investigate the discrete DNP particles produced by nuclease action on rat liver and chicken erythrocyte nuclei.[90] Using the rat liver Ca^{++}/Mg^{++}-dependent endonuclease to digest nuclei, they have isolated the DNP particles on sucrose gradients, demonstrating specifically that RNP contamination is not a problem. The DNA purified from these particles shows a normal oligonucleosome pattern when displayed on agarose gels. In addition, when H1 is extracted from the DNP particles prior to the sucrose gradient run, no discrete size class is seen. It appears that H1 is of paramount importance in holding the particle together. Apparently the nuclease does not preferentially recognize the boundaries between particles in the 300 Å fiber, but produces the expected array of DNA fragments at 200-bp intervals by the normal mode of linker cleavage. The particles themselves are of 12 to 14 nucleosomes in size. It may be, of course, that the disruption of some protein-DNA contacts during the digestion of DNA by the nuclease leads to a situation where a folding readjustment between nucleosomes and H1 gives rise to clustered particles with a minimum free energy. The specificity or biological significance of the particles has not been demonstrated.

All the evidence together points to an interpretation that chromatin in the 300-Å fiber is arrayed in a helical manner, with local irregularity in both diameter and pitch which may or may not have functional implications. There is nothing in the superbead model to suggest that the clusters are not themselves short solenoidal stretches of nucleosomes. It may be that there are discrete, longer-range stabilizing interactions, mediated by H1 and/or NHC proteins, that represent a level of organization above the discrete six to seven nucleosome stability of a presumed complete solenoidal turn,[70,71] and that these regular foci can be visualized under certain conditions of unfolding. Note that the concept of superbead production by nuclease nicking along one face of a regular solenoid[25,71] seems not to be tenable in view of the regular oligonucleosomal pattern of DNA fragments from clusters as described by Stratling and Klingholz.[90] In any hypothesized correlation between chromatin function and discontinuous stretches of chromatin fiber, it is to be expected that there would be coincidence between such structures and the underlying DNA sequence. This should be demonstrable in a precise and reproducible fashion. Unfortunately, EM can in this instance offer no information at the sequence level, and until such a correlation is drawn, the superbead is likely to remain an enigma.

A further observation in nucleosome folding, as briefly mentioned previously, is the visualization of a zigzag arrangement of nucleosomes in compacted 100-Å fibers. Such a form has often been seen in electron microscope pictures of isolated chromatin,[25,82,91] as well as in structures unfolding from lysed nuclei.[84,85,87] That these structures may represent something meaningful in the packing array of nucleosomes is reinforced by recent observations of a dinucleosomal repeating unit released by DNase I digestion of various nuclei.[92–94] Worcel et al. have proposed a model for the 300-Å fiber based on the zigzag repeating unit of a dinucleosome.[95] The zigzag ribbon contains 1 3/4 turns of DNA per nucleosome, but the relative orientation of nonsupercoiled spacer DNA between units allows the linking number change per dinucleosome to be −2, or −1 per nucleosome. This is an attractive feature, as it resolves the so-called linking number paradox. Examination of the SV40 minichromosome, possessing closed circular DNA, indicates a linking number change of −1 per nucleosome,[96,97] while two complete turns of left-handed superhelix around the nucleosome core, as required by other data, represent a linking number change of −2. An average superhelical density of approximately

−1/200 bp has also been obtained in studies of nuclear DNA, but it is difficult to generate an accurate number.[98,99] Thus there has been a problem in constructing simple models where the predicted change corresponds to that observed experimentally. Generation of a coiled 300-Å fiber might be achieved by twisting of the flat ribbon with concomitant stacking of the nucleosomes.[95] The model allows unequal spacing between nucleosomes, i.e., variable length linker regions, as commonly observed,[27] and even nucleosome-free regions could be accommodated, generating a knobby fiber as observed.[62]

While this model has been generated to accommodate observations reported by a number of investigators on the morphology of unfolded chromatin seen in the electron microscope, McGhee et al. have constructed a model of the 300-Å solenoid based on their observations of the physical properties of chromatin during refolding of the 100-Å filament to a 300-Å fiber in the presence of divalent cations (see Figure 2).[68] This model is different in several respects from that of Worcel et al.[95] The electric dichroism data placed severe restraints on the orientation of the chromatosome faces. The radial arrangement of chromatosomes with the faces lying roughly parallel to the fiber axis is central to the model. This contrasts with the stacked nucleosomes (for which there is no evidence in intact nuclei) and nonparallel orientation of nucleosome faces of the twisted-ribbon model. The evidence for stacked nucleosomes comes mainly from in vitro work with cores (e.g., Reference 100). Interestingly, there is a dinucleosome-based symmetry in the radial model. However, owing to the particular flexibility of the 100-Å filament, it was concluded that there is nothing inherent in the structure of the 100-Å filament that is necessarily conserved in the 300-Å fiber. The radial model shows the spacer DNA following a similar supercoiled course to that in the chromatosome. In this regard the model appears to disregard the linking number requirements previously detailed. However, it seems possible that a suitable alteration in the hypothesized path of the spacer DNA might more easily accommodate the experimental observations on linking number. Other solutions to the linking number problem are possible. For example, analyses using DNase I and DNase II cleavage patterns suggest that the helical periodicity of B-form DNA on the nucleosome is 10.0 bp per turn, while the average in solution is 10.5 bp per turn; this difference in twist will resolve the winding problem.[101,102] All things considered, the model shown in Figure 2 for the 300-Å fiber is the most attractive at the moment.

The question of location and relative disposition of H1 within the 300-Å fiber is not entirely resolved. That H1 is of vital importance to the integrity of the higher-order structure has been amply demonstrated in studies with H1-depleted chromatin.[25,62,82,88,103] The location of H1 in the 165-bp chromatosome has been the subject of several reports.[23,24,49] It is generally thought that H1 binds at the point of DNA entry and exit, stabilizing two complete turns of DNA around the histone core. Protein cross-linking studies have produced evidence for a close proximity of H1 to H3.[104] H1 is an unusual protein in that it contains a globular central region, which alone helps protect the 165-bp chromatosome from digestion to 145-bp core particles,[49] flanked by highly basic and hydrophilic C- and N-terminal tails. Only the intact H1 protein is able to confer the degree of protection to linker DNA against micrococcal nuclease digestion that is seen with native chromatin.[21,49] In particular, the C-terminal region is expected to be of major importance in binding to linker DNA between adjacent nucleosomes and perhaps between one turn of the fiber and the next.[49] Although H1 shows very little tendency towards self-aggregation,[105] cross-linking studies have shown that H1 homopolymers can be isolated from both unfolded[106] and folded (i.e., in nuclei) chromatin.[107] These need not, of course, originate in precisely the same way. In the latter study, oligomers containing 12 histones and multiples thereof were found to be the most common form of poly-H1. This correlates well with the structural unit of 12 nucleosomes identified by Stratling and Klingholz.[90] Thoma et al. had previously suggested the existence of H1

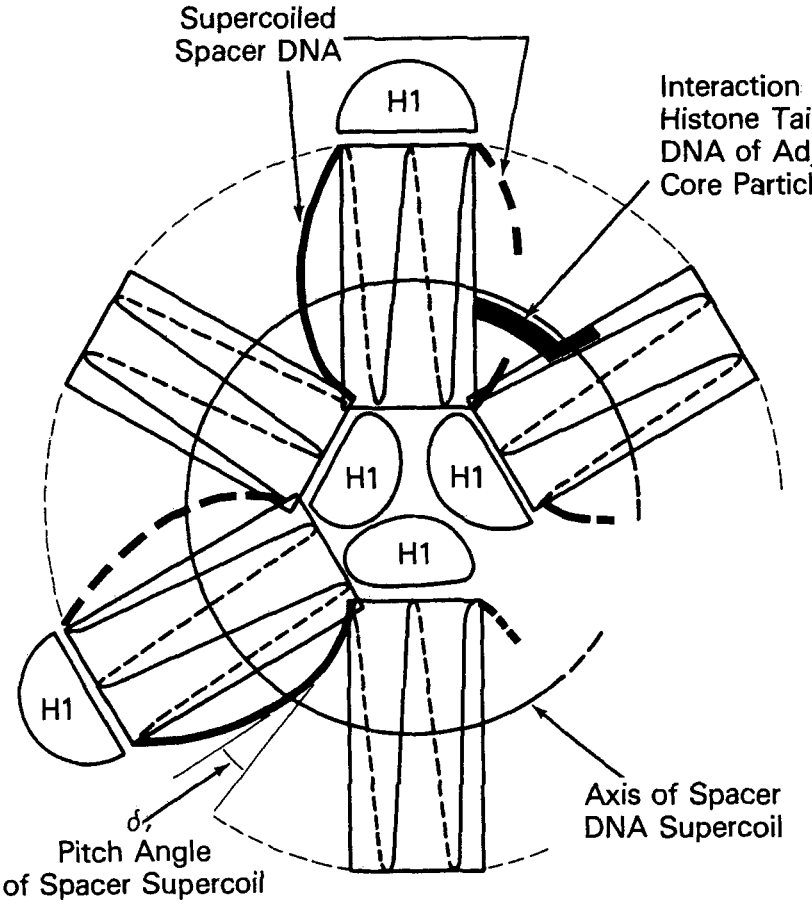

FIGURE 2. A model for the 300-Å solenoid. The chromatosome faces are arranged radially, and tilted 20 to 25° from the solenoid axis. There are six chromatosomes (with dimensions of 100 Å diameter and 55 Å thick) per turn. The spacer DNA is arranged as segments of a uniform left-hand (toroidal) supercoil wound around a helical axis which passes through the chromatosome centers, and which has a diameter of 300 − (2) (110/2) = 190 Å. This is indicated on the figure as "axis of spacer DNA supercoil". It seems most reasonable to construct a model in which the spacer supercoil has a diameter roughly the same as that of an individual chromatosome. Specifying the diameter of the spacer supercoil fixes the number of spacer superhelical turns per solenoid turn, and it is calculated that a diameter of 90 to 100 Å would lead to a total of three superhelical turns of the spacer DNA per solenoid turn, or approximately one half of a superhelical turn per chromatosome. A dimer repeating unit is illustrated for an arrangement in which all spacer segments would be equally accessible to nucleases. The spacer DNA loops up between one pair of neighboring chromatosomes (thick solid lines), and loops down between the adjacent pair (thick dashed lines). If histone H1 (or H5) is placed at the point of entry and exit of the DNA from the chromatosomes, then on one chromatosome H1 is in the middle of the solenoid, on its neighbor it is on the outside, and on its next nearest neighbor H1 is back in the solenoid center again. (Adapted from McGhee, J. D., Rau, D. C., Charney, E., and Felsenfeld, G., *Cell*, 22, 87, 1980. With permission.)

polymers to stabilize the supercoiled 300-Å fiber seen under the electron microscope.[25] The precise location of H1 within the coiled fiber has not been identified, but its inaccessibility to an antiserum prepared against H1 in chromatin fibers at 80 m*M* NaCl, compared to obvious accessibility in extended chains at 5 m*M* NaCl[108] suggests that it may reside within the solenoidal configuration as envisaged in the models of Worcel and Benyajati[91] and McGhee et al.[68] In this latter study (see Figure 2) one turn of the solenoid might derive some of its stabilization from H1 interaction in the central hole where 50% of the H1 molecules were expected to reside. Extraction of H1 from chromatin certainly

leads to a less compact and organized structure as determined by EM (see preceding) and sedimentation analysis.[21,103] There is, however, little direct evidence regarding the relative disposition of H1 in the 300-Å fiber.

It has been demonstrated that transcribing chromatin has a different, probably more open, structure than does inactive chromatin.[109,110] It is possible that in active regions H1 is removed, modified, or replaced, altering associations between nucleosomes and destabilizing the 300-Å fiber structure (for a review see Reference 111). It is suggested by McGhee et al. that the 300-Å fiber may derive at least some of its stabilization by interchromatosome contacts mediated by histone: DNA interactions.[68] In particular, the basic NH_2-terminal tails of the core histones appear to be unnecessary for folding of DNA into a nucleosome structure[112,113] and might well interact stably with DNA in an adjacent chromatosome. Acetylation of histones has been correlated with gene transcription (see Section V.B). The major sites of this acetylation are in the basic NH_2-terminal tails.[18,114] Therefore it is not unreasonable to propose that such acetylation weakens the interchromatosome stabilization (as previously outlined) and allows the 300-Å fiber to adopt a more open conformation.

C. Higher-Order Structures

A consideration of the level of packaging above the 300-Å fiber takes one into a realm where little that is definitive is known. As an extension of the solenoid model, it might be considered that a super-solenoid existed as the next level of packaging. Indeed such structures, referred to as unit fibers, have been reported for both human and *Drosophila* chromosomes.[115-117] In contrast, the unfolding of metaphase chromosomes under physiological conditions as studied by Rattner and Hamkalo, while giving no indication of a helical arrangement, does show the 300-Å fibers as loops of chromatin emanating from the condensed central region of the euchromatic arms.[84] This is in accord with previous reports of looped structures observed in the electron microscope.[118,119] In a careful study of Chinese hamster mitotic chromosomes using the electron microscope, Okada and Comings observed rosettes, i.e., condensed centers, with many loops emanating from these points in a radial fashion.[120] Each rosette was connected by a chromatin fiber to another rosette. The uniformity of the structures and their defined and regular positioning one to another certainly gave the impression of domains of DNA condensing in a nonrandom fashion. Such observations have received strong support from the elegant work of Rattner et al., who have studied the meiotic prophase condensation of chromatids in *Bombyx mori*.[121,124] Again, as condensation proceeds, 300-Å fibers are seen to organize as loops along a fiber which coalesce to form rosette structures. In later prophase the rosettes themselves fuse to form the central element of the synaptonemal complex (Figure 3). Following metaphase I, the chromosomes appear to shorten lengthwise while the loop lengths remain the same. This suggests that this particular pattern of folding applies to metaphase as well as meiotic chromosomes. These studies clearly indicate organization using loop structures as have studies on mitotic chromosomes by Yunis and Bahr[122] and Mullinger and Johnson.[123] The question of specificity of the anchorage points is of great interest.

Two systems which have been of significance in this area because of their obvious amenity to detailed observation are the Balbiani rings of *Chironomus tentans* polytene chromosomes and the transcription loops of lampbrush chromosomes from amphibian oocytes. In both systems loop structures are readily identified, and it is clear that such loops can be the sites of intense transcriptional activity. Two giant puffs on the fourth chromosome of *Chironomus*, known as Balbiani rings 1 and 2, are the sites of synthesis of large 75S RNAs which are the precursors for mRNA for salivary polypeptides. Recent EM studies by Daneholt's group have shown that in both Miller spreads and serial

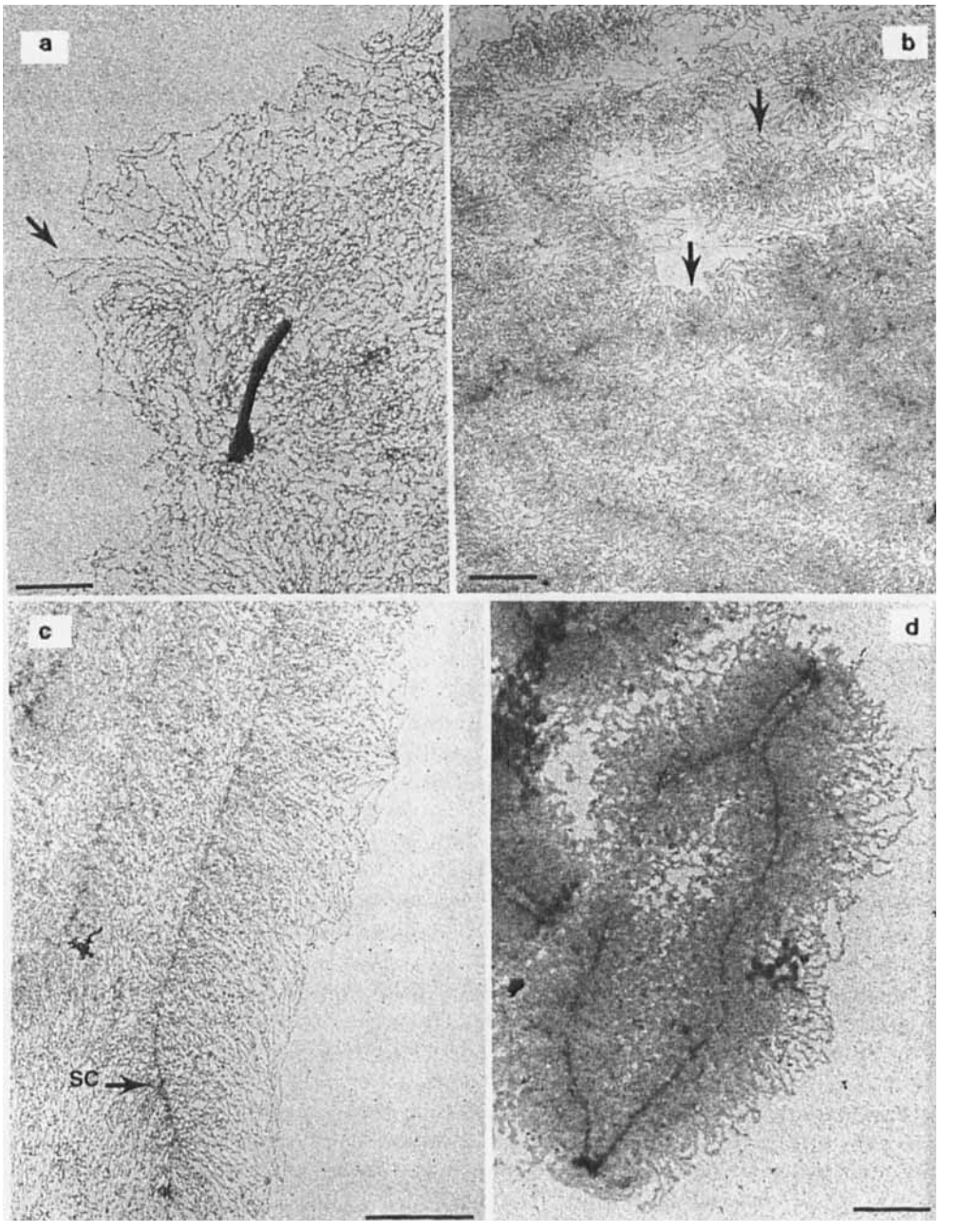

FIGURE 3. Electron micrographs showing sequence of chromatin organization during early meiotic prophase of *Bombyx mori:* (A) margin of early meiotic prophase nucleus in which loops of chromatin can be identified (arrow) (the dense black material is cellular debris); (B) early meiotic prophase nucleus displaying prominent chromatin fiber loops which are arranged in a linear array, and frequently aggregated into discrete foci (arrows); (C) meiotic chromosome in which axial elements of the synaptonemal complex (SC) can be identified; the chromatin loops extend radially from the elements, and appear homogeneously distributed along the length of the chromosome; (D) a minimally dispersed chromosome illustrating the condensation of the chromatin loops along the synaptonemal complex. Bar: (A) 0 to 0.5 μm; (B), (C), and (D) 1 μm. (From Rattner, J. B., Goldsmith, M., and Hamalko, B. A., *Chromosoma (Berl.).* 79, 215, 1980. With permission.)

sections through the rings, looped structures, each with single long transcriptional units, are present.[125,126]

The lampbrush chromosomes of amphibians are formally similar in that they present the aspect of long central organizing elements with loops of DNA extending in a lateral fashion. The DNA is, of course, continuous. The loops are often the site of intense RNA synthesis, with packed RNP fibrils which can readily be observed (see Reference 127 for review). It has been suggested that each loop represents a functional transcription unit. Recently, studies of the transcription of the genes of the newt *Notophthalmus* have revealed a more complex story. The histone genes are clustered in a 9 kb piece of DNA; this repeating unit is separated by 50 to 100 kb of a tandemly repeated 222 bp satellite DNA. The long transcripts observed arise from transcription not only of the histone genes, but also of the interspersed satellite DNA. Transcription can take place on both strands since one histone gene is of opposite polarity. However, it appears that transcription in both directions on a given stretch of DNA at the same time does not occur. Frequently more than one transcription unit per loop is observed; these appear to have independent promoters.[128,129] In their studies of meiotic chromosome condensation in *B. mori,* Rattner et al.[121] have observed active rDNA genes. In this case, one transcription unit was seen per loop. These results as well as others have reinforced the concept of a domain of DNA organization, in these cases a loop, which represents a functional entity.

Biochemically, such structures are rather ill-defined. Benyajati and Worcel demonstrated the presence of supercoiled domains in *Drosophila* interphase chromatin.[98] These domains could be relaxed by ethidium bromide intercalation or by DNase I nicking of the DNA. Such an observation implies that DNA is restrained at points within the nuclear superstructure. The size of the domain was about 85 kb of DNA on average. Protein, RNA, or both were implicated as stabilizing components at the domain boundaries since treatment of the chromosomes with ribonuclease or pronase led to complete relaxation. Subsequently, Igo-Kemenes and Zachau[130] arrived at a similar result by limited micrococcal nuclease and restriction digestions of rat liver nuclei. Their domain model visualized cross links between strands or between DNA and a supporting structure in the nucleus with an average loop size of 34 kb. Such domain sizes are larger but of the same order of magnitude as the functional or transcriptional units described previously and by others (e.g., References 131 and 132).

In any consideration of the domain organization of chromatin and its significance, the organization of the polytene chromosomes of *Drosophila* and the associated genetic and cytological correlates are of paramount importance. The constancy and precision of chromatin folding, inherent in any domain model of functional significance, is well illustrated by such chromosomes which appear identical in their banding patterns from cell type to cell type.[133-135] Polytene chromosomes are constructed of many identical DNA strands arranged in a parallel fashion and apparently precisely aligned. The dark bands are due to condensation of DNA resulting in an increased packaging ratio. The band-interband pattern of polytene chromosomes presents a visual counterpart to the domain hypothesis. Does the pattern reflect the organization of functional genetic units? This has been previously interpreted as asking, in the simplest terms, whether one gene is represented by one band. It had been shown by Rudkin that, on average, each band contained about 30 kb of DNA,[136] and in general this seems more than enough to encompass one transcription unit. Classical genetic studies using complementation analysis of crosses with deletions in the chromomeric pattern allowed the concept of one gene (or at least one cis-dominant complementation unit) per band (or chromomere) to be formulated and approximately confirmed.[137,138] The chromomere was seen as a structural gene with associated regulatory sequences, the whole unit acting as a cis-dominant complementation unit. This implied that any single mutation arising would

affect the functioning of the whole unit. Such a stringent view of one gene per chromomere has since been modified in the light of findings of both genetics and molecular biology. Young and Judd, in an exhaustive search for new mutations in the 3A to 3C region of the *Drosophila* X-chromosome, concluded that the limit of essential genes at one per chromomere was reached.[139] However, nonessential genes (i.e., nonlethal if mutated) could coexist in a chromomere with these essential genes. In a study aimed at probing the complexity of polysomal RNA in *Drosophila,* approximately 16,000 different sequences were found to be present in larvae, pupae, or adults.[140] This is considerably more than the best estimate of approximately 5000 separate polytene bands (see Reference 141). Interestingly though, the number of polyadenylated mRNA sequences found was approximately one third of the total polysomal population (5000 to 6000).[140]

Molecular biology is now beginning to complement the findings of genetics regarding the boundaries of genetic units and their relative organization. In a functional sense, one of the more interesting discoveries has been the disposition of developmentally regulated gene clusters. The chorion of the silkworm *Antheraea polyphemus* is composed of many different polypeptides that can be classified according to their size and sequence homology. At the genomic level it has been shown that genes from different families of polypeptides are interspersed one with another in a short region of DNA that is physically separated from another cluster of genes that code for very similar peptides, but which are expressed at a later developmental stage.[142] Hence there is clearly more than one gene per domain (or chromomere), but these may be clustered according to their temporal expression. This suggests that local chromatin unfolding could be used as one level of control. This is not a sufficient model, however, as cases are known of closely spaced genes expressed differentially. For example, the locus 67B in *Drosophila* contains the genes for four small heat shock proteins. Located between these closely spaced inducible genes, however, is a developmentally regulated gene.[143] Furthermore, many coordinately expressed genes are widely dispersed in the genome, e.g., the heat shock genes of *Drosophila.*[144,145] Additional levels of control must clearly be involved here.

Gene transcription in Diptera has been correlated cytologically with the puffing patterns of polytene chromosomes,[146,147] although visible puffs are not present at all loci where RNA synthesis is occurring. Recent careful observations on the giant puff Balbiani ring 2 of *Chironomus* polytene chromosomes have allowed an unequivocal assignment of a particular band as the site of origin of the puff.[148] It was also shown that the RNA associated with dissected Balbiani rings hybridized exclusively with the same band. Mott et al. have shown, using a microdissection technique that eliminates the acid fixation step inherent in traditional *Drosophila* polytene preparations, that loops can be seen at the periphery of puffed regions.[149] This represents the first direct observation of transcriptionally active loops from *Drosophila* polytene chromosomes, something which, as previously mentioned, has been well visualized in the *Chironomus* polytene chromosome. It is reported that the *Drosophila* loops contain certain associated RNP particles arranged in a symmetrical structure around a 100-Å nucleofilament. While there is no doubt that puffs are sites of intense RNA synthesis, there is considerable controversy over whether biologically significant low level transcription occurs in interbands. (See Reference 150 for a review of this problem.)

It has now been possible for the first time to delimit biochemically the boundaries for full expression of a gene in vivo. A series of deletion and translocation mutants of *Drosophila* that had been previously mapped close to the cytological locus of a salivary gland glue protein was used. With recombinant clones for the transcribed and surrounding regions,[151] it was possible to determine, by restriction and/or Southern analysis of DNA from suitable crosses, that a maximum region of 19 kb spanning the transcribed region was required for full expression of the protein.[152] This is clearly on the

order of a *Drosophila* chromomere. It should be noted, of course, that much smaller fragments have been transcribed in vitro or used successfully in transformation.

What could define the boundaries of such functional domains? In the case of *Drosophila,* the prevailing evidence would imply that boundaries occur at or within the interband regions of the polytene chromosomes. While there have been many hypotheses concerning satellite DNA, snapback or cruciform structures derived from palindromic or polypurine-polypyrimidine sequences as boundaries, these have not been established. Recently a different and intriguing possibility has come to light. It has been shown that antibodies generated against the left-handed helical form of DNA, Z-DNA,[153] bind specifically and highly reproducibly to the interband regions of polytene chromosomes as assayed by indirect immunofluorescence.[154] This is a finding of some importance since the existence of left-handed Z-DNA in vivo had previously been a subject of speculation. Synthetic, alternating deoxypurine-deoxypyrimidine sequences (e.g., poly [dG-dC]) have been found to convert to the Z-form at high salt concentrations,[155] and can do so even when these segments are inserted into normal B-form DNA sequences.[156] Z-form DNA has a very different helical repeat than does B-form DNA (13.6 vs. 10.5 bp per turn),[157] suggesting that its interaction with various proteins and its short-range flexibility might be very different. Methylation at the five-position of cytosine in such polymers allowed the conversion to Z-DNA at low concentrations of divalent cation and at micromolar concentrations of polyamine;[158] methylation at the seven-position of guanine has a similar affect.[159] Hence the possibility of the occurrence of Z-DNA in vivo appeared much more likely. However, it has proved difficult to detect 5-methylcytosine (m^5Cyt) in *D. melanogaster* larval or adult tissue by chemical or biochemical methods,[160-162] although an antiserum to 5-methylcytosine appeared to cross react with polytene chromosomes.[163] The consistent observation of an antigen to Z-form DNA antisera in *Drosophila* interbands (the antigen presumably being a segment(s) of preexisting or induced Z-DNA) is a significant observation in the search for unique structural features at chromomeric boundaries. Whether the role of Z-DNA in vivo is to define functional domains or whether it plays more direct roles in transcriptional control or other areas will no doubt be the subject of intense investigation. Additional forms of DNA are known (see review, Reference 164) and are observed under appropriate conditions.[165] An unusual (and unconfirmed) form, left-handed B-DNA, has been suggested in a model of catabolite gene activator protein interaction with DNA.[166] The DNA spanning several adjacent bands of *Drosophila* polytene chromosomes has been cloned using recombinant DNA techniques. Hence such boundary regions, as discussed, are available for study, and these might be investigated in regard to cytological correlation with regions of unusual base composition or structure.

D. Metaphase Chromosomes

The condensation of interphase chromatin during mitosis to form metaphase chromosomes represents the packaging of DNA to its greatest extent. The ability of chromosomes to attain this condensation reproducibly during each cell division coupled with the accuracy of the division process and of somatic recombination indicates a closely regulated mechanism. A serious problem in the study of metaphase chromosomes is the possible generation of artifacts during preparation for microscopic examination. Although many preparations appear "normal" in the microscope, some procedures are known to cause significant loss of proteins and damage to the DNA. Nevertheless, there are some results which serve to place constraints on any proposed model.

One model of current interest centers on possible domain organization through the anchorage of chromatin loops to a central proteinaceous skeleton. Paulson and Laemmli observed that, after removal of histones with heparin and dextran sulfate, HeLa

metaphase chromosomes consisted of a large number of DNA loops projecting from a central mass which they termed the "scaffold".[167] The loops ranged in size from 10 to 30 μm (30 to 90 kb). This is in good agreement with the loop sizes reported earlier using physical analyses for interphase nuclei.[99,98] Further support for a radial loop model has been provided by EM examination of metaphase chromosomes isolated under different conditions (e.g., in the presence of chelating agents or more physiological buffer conditions) before fixation.[168,169] A number of light microscopic studies have reported the existence of a central chromosomal element in metaphase chromosomes.[170-172] Examination of salt-extracted, isolated metaphase chromosomes from Chinese hamster cells using scanning EM revealed a longitudinal array of fibers consistent with the existence of some form of a central chromosomal element.[173] Adolph et al. have further characterized the scaffold as resistant to 2*M* NaCl, micrococcal nuclease, and RNase, but not resistant to trypsin, 4*M* urea, or SDS.[174,175] The scaffold retained its morphology as determined by fluorescence and EM after isolation on sucrose gradients. Analysis by SDS polyacrylamide gel electrophoresis showed the scaffold material to contain approximately 30 proteins.

Despite considerable work, the existence of a defined, specific scaffold remains controversial. A number of serious objections must be dealt with. First, because of the tendency of chromosomal proteins to aggregate, it could be argued that aggregation alone is sufficient to account for the observations. Examination of spread chromosomes after removal of the histones indicated considerable variation in the extent of unfolding. Hadlaczky et al. have demonstrated that the spreading technique itself is at least as great a variable in the procedure as the conditions of salt extraction.[176] Extraction of the chromosomes in the presence of sucrose (which is said to reduce protein aggregation) results in looser structures. Structures isolated by this method and treated with DNase after their attachment to EM grids revealed only protein particles and no scaffold.[177] Goyanes et al. examined Chinese hamster chromosomes by EM after formaldehyde cross linking and subsequent trypsin digestion. At concentrations of formaldehyde which will preferentially fix histones to DNA, only a 250-Å fiber was seen. There was no suggestion of a protected proteinaceous core.[178] However, as previously described (Section II.C), Rattner et al.[121,124] have observed a temporal sequence in the condensation of chromatids during meiotic prophase in the silkmoth *B. mori* which suggests formation of a core; toward the end of the observed process a central element becomes visible (see Figure 3). It is, however, not yet clear if this element is analogous to the scaffold reported by Paulson and Laemmli.[167] It is possible that the organization implicit in the scaffold model is correct, but that the material recovered for biochemical analysis includes aggregated material not relevant to higher order organization.

Implicit in the existence of a core, as suggested by Paulson and Laemmli[167] or by Rattner et al.,[124] is the requirement for specific DNA sequences which would act as attachment points of the looped DNA. Although some work in this direction has begun, no such sequences have yet been identified. Razin et al. deproteinized metaphase chromosomes of mouse L-cells with salt, digested the DNA with restriction enzymes, and examined the remaining DNA by analytical centrifugation. This was found to be enriched for satellite sequences. Analysis of the NHC proteins in the fraction revealed only a few high molecular weight proteins.[179] Jeppersen and Bankier examined the 0.1 to 0.5% of the total DNA remaining after acid or salt extraction of the histones from chromatin of CHO cells and subsequent nuclease digestion. Using a melting and reannealing assay, the DNA remaining was again shown to be enriched in highly repeated sequences.[180] In an investigation of HeLa chromosomes, Bowen found preparations of protected DNA generated using a restriction enzyme to be enriched for satellite DNA, while that generated using micrococcal nuclease appeared general by reassociation

kinetics.[181] Nelkin et al., investigating SV40 infected 3T3 cells, reported that the DNA remaining in the chromosomal core after nuclease digestion was enriched three to seven times over total cellular DNA for SV40 DNA sequences. Globin DNA sequences showed no such enrichment.[182] These results suggest that the organization of sequences in the loop itself are potentially variable and may be related to the activity of the genes. However, no well-defined set of core sequences has been defined.

Some evidence exists for an ordering of chromosomes in the interphase nuclei of several organisms.[183,184] Reports from the laboratory of Comings have suggested that the chromosomes might be attached to the nuclear matrix (Section IV.A) and/or nuclear membrane by particular DNA sequences.[185,186] Mayfield and Ellison demonstrated by *in situ* hybridization of radioactively labeled satellite DNA to *Drosophila* interphase nuclei that these sequences are all contained in the heterochromatic mass attached to the nuclear membrane. This occurs despite the fact that these sequences are found on more than one of the chromosomes.[187] Such an organization of the satellite sequences into one or a few heterochromatic masses was also shown in mouse.[188]

The significance of this aspect of interphase chromosomal organization is not yet understood, but it would seem likely that some elements of the metaphase structure must remain during interphase. The evidence for a domain organization in interphase chromatin was discussed in Section II.C (e.g., Reference 98). The condensation of these domains presumably occurs in a specific fashion with preservation of the relative aspect of one domain with respect to another. Such an assessment would seem to be in agreement with the condensation sequences observed by Rattner et al.,[121,124] although it must be remembered that these studies refer to meiosis. Comings and Riggs suggested that some chromatin-associated proteins might undergo allosteric changes at the onset of mitosis to allow them to self-associate or bind to other chromosomal proteins.[189] The data on the relative constancy of chromosomal proteins between interphase and metaphase states (see following) support this idea.

One approach attempting to analyze the molecular basis of chromosome condensation has been to examine the proteins of chromatin from each of these states with the hope of establishing a correlation between condensation and the presence, absence, or level of modification of a particular protein. In a recent experiment, Wray et al. used one- and two-dimensional gel electrophoresis to compare the chromosomal proteins of HeLa and CHO cells in both the mitotic and interphase states. Protein bands of 65,000 and 68,000 daltons were enriched in interphase chromatin and bands of 50,000, 90,000 to 100,000, and 200,000 daltons were enriched at metaphase for both cell types.[190] A comparison of the acid soluble proteins of the DON Chinese hamster cell line demonstrated that protein A24 was one of the few proteins found in interphase chromatin, but not in metaphase chromosomes.[191] An increase in the H2A:DNA ratio during mitosis indicated that the ubiquitin moiety had been released to a large extent from H2A at that time and, with or without protein synthesis, recombined with H2A to generate A24 in the G1 stage. H2A remained bound to the DNA throughout the cell cycle. As a mechanism of chromosome condensation, Matsui et al. suggested that the removal of ubiquitin renders the exposed C-terminal tail of H2A available for interaction with the phosphorylated H1 and H3 of other chromatin fibers.[191] A correlation of H1 phosphorylation with mitosis has been reported for *Physarum polycephalum,*[192,193] CHO cells,[194] and Novikoff ascites cells.[195] (For a review of the involvement of histone phosphorylation in chromosome condensation, see Reference 196). Further support for this model came from Matsumoto et al.[197] and Yasuda et al.,[198] who isolated a temperature-sensitive mutant of a C3H mouse mammary carcinoma cell line. This cell line showed abnormally condensed chromosomes and reduced H1 phosphorylation at the nonpermissive temperature.

The interactions of phosphorylated H1 in condensed chromosomes at the molecular level are unknown. Histone H1-DNA interactions have been shown by proton magnetic

resonance to change after H1 phosphorylation.[199] Analysis of turbidity and other studies with phosphorylated histone H1-DNA complexes have shown the interaction is dependent on the salt concentration.[200,201] A specific, nonhormonally mediated kinase has been implicated in the cell cycle-dependent phosphorylation of histone H1.[195] It phosphorylates H1 in the C-terminal region[201] and has been shown to be activated during the G2 phase without *de novo* synthesis.[202]

The state of knowledge of metaphase chromosome structure and condensation remains as an accumulation of rather disjointed observations. Despite the very interesting results from the laboratories of Laemmli, Hamkalo, and others, a consensus has not yet emerged. This is certain to be an area of increasing activity, and it seems likely that experiments utilizing conditional lethal mutants that affect chromosome morphology will be rewarding.

III. SPECIFICITY IN CHROMATIN ORGANIZATION

Evidence from a variety of cytological and genetic studies has implied that a substantial degree of specificity exists in the organization of chromatin structure. Biochemical analyses are capable of describing such specificity at the level of the DNA sequence. The only clear examples of such chromatin structures detected to date are the sites hypersensitive to cleavage by DNase I. Although biochemical studies have in some cases indicated a consistent positioning of nucleosomes on specific DNA sequences, these findings remain to be confirmed.

A. DNase I-Hypersensitive Sites

The discovery of sequence-specific chromosomal loci hypersensitive to cleavage by DNase I was preceded by observations of aberrant nucleosome distribution in papovaviruses. The DNA of these viruses is associated with histones in lytically infected cells; the "minichromosome" of Simian virus 40 (SV40) has become a paradigm for the basic packaging of DNA into chromatin. Varshavsky et al. noted the preferential exposure to restriction enzymes of a region within the SV40 minichromosome which included the origin of replication, the T-antigen binding site, and the promoter region for "late" viral mRNAs.[203,204] Experiments involving other nucleases as probes of the SV40 structure supported this finding. Both Waldeck et al.,[205] using an endogenous endonuclease associated with SV40 and polyoma complexes, and Scott and Wigmore,[206] working with DNase I, found that up to 30% of a population of viruses was specifically cut within a region spanning the origin of replication. The latter failed to detect preferential cleavage of SV40 by micrococcal nuclease. Sundin and Varshavsky, however, while confirming micrococcal nuclease did not discriminate between sites in the SV40 minichromosome under similar conditions (digestion at 37°C), found that a majority of the double-stranded cuts induced by incubation with this enzyme at 4° C were again located near the viral origin[207] (but note the results of Reference 208 discussed in Section III.B).

The minichromosome was directly observed using EM, and specific features mapped using denaturation or restriction markers. Jakobovits et al. observed that 25% of an SV40 minichromosome population lacked a beaded structure in the region at 0.67 to 0.75 map units.[209] Saragosti et al. found that 15 to 20% of the minichromosomes had such a gap, and that this feature was not correlated with the number of nucleosomes on a molecule as a whole (average: 24 nucleosomes).[210] The gap measured 250 bp on average and mapped asymmetrically around the origin of replication. Although it was not established and, in fact, seems unlikely that this aberrant region is devoid of associated proteins in vivo, it seems clear that specific accessibility of these sequences is an integral feature of the minichromosome during at least part of the viral life cycle.

It was known through prior experience that when eukaryotic nuclei were digested with pancreatic DNase I, the resultant DNA fragments formed a continuum of sizes perceived as a uniform smear in electrophoretic sizing gels. Nonetheless, work with DNase I was of continuing interest due to the finding of Weintraub and Groudine[109] that active chromatin is preferentially digested by this enzyme. Wu et al., using Southern blot analysis with specific cloned DNA fragments, first discovered that a discretely sized, reproducible set of fragments was generated at an individual locus at early times of digestion. The pattern of bands obtained was unique for each of five cloned *Drosophila* loci examined, suggesting that specific sites which could be recognized by DNase I were nonuniformly distributed along the chromatin fiber.[211] One can map these cleavage sites relative to known restriction sites, and this has now been done for a number of *Drosophila* genes. The sites tend to occur at the 5′ end of regions of transcription. Wu could clearly show a series of hypersensitive sites at the 5′ end of the gene encoding the 83,000 dalton heat shock protein (hsp 83).[212] It was also evident that such hypersensitive sites were present near the 5′ ends of at least some and possibly all copies of the hsp 70 gene at 87A and 87C. A cluster of sites downstream from the 3′ terminus of hsp 83 have now been shown to map at the end of a nonheat shock transcript.[213] Keene et al. found that for each of four heat shock genes (hsp 22, 23, 26, and 28), a DNase I hypersensitive site was located within 200 bp of the 5′ end and that a lesser site was positioned 200 to 300 bp upstream.[214] A pair of minor sites within this locus (67B), previously unexplained, have now been shown to fall at the 5′ end of a developmentally regulated gene.[215]

Examination of the chromatin structure of the constitutively expressed ribosomal protein 49 gene of *Drosophila* revealed a series of five DNase I-hypersensitive sites, about 240 bp apart, at the 5′ end.[216] In the case of the *Drosophila* histone genes, hypersensitive sites could also be found near the 5′ ends of transcribed regions.[217] The extrachromosomal rRNA genes of *Tetrahymena pyriformis,* arranged as inverse repeats exhibit a single hypersensitive site in the central spacer region between the 5′ ends of the transcribed regions.[218]

Whereas the experiments previously cited have dealt with universally inducible or constitutive genes using whole embryonic tissues or cultured cells, several investigators have now demonstrated that an accessible region at the 5′ temini of genes expressed in a tissue specific pattern is observed in those specific cell types. Wu and Gilbert found a hypersensitive site at the 5′ end of the rat preproinsulin II gene in chromatin from a pancreatic β-cell tumor tissue, but not in chromatin from rat liver, spleen, kidney or brain.[219] A hypersensitive site within the gene near its 3′ end was seen in liver chromatin. Stalder et al., studying the globin system in chick red blood cells, have produced most compelling evidence for the specificity of the phenomenon.[220] Hypersensitive sites specific for red blood cell chromatin were found for the β-globin genes, different in the embryonic and adult cells. In cells producing only fetal hemoglobin, a site was found near the 5′ end of the embryonic, but not the adult β-globin gene. In cells producing adult hemoglobin, two sites upstream from the 5′ end of the adult gene had appeared while the site adjacent to the embryonic gene had disappeared. Analogous results have been obtained for the α-globin gene cluster.[221] These results have been summarized in a recent minireview.[222]

McGhee et al. have made a detailed study of such a hypersensitive site located at the 5′ end of a second adult chicken β-globin gene.[223] The site is an accessible region extending from approximately 60 to 260 bp upstream from the start of transcription; this includes the CCAAT sequence but not the "TATA box" of the gene. An average base pair within the region is cleaved roughly 100-fold faster than in bulk chromatin by DNase I, and a variety of endonucleases produce cleavages throughout the 200-bp region. In particular, a 115-bp DNA fragment contained within this sequence is released in high yield from nuclei digested with Msp I, and roughly one third of the recovered material behaves as

protein-free DNA. It seems highly unlikely that the sensitive DNA is packaged in association with histones; it may now be possible to show whether this sequence is normally complexed with specific proteins.

The information at hand suggests that the presence of a DNase I-hypersensitive site in chromatin is necessary although not sufficient for gene transcription in vivo. This hypothesis has received additional support from the combined work of Beckendorf and Shermoen[224] and Muskavitch and Hogness[151] on the glue protein locus Sgs4 of *Drosophila.* This gene is normally expressed in the salivary gland in early third instar larvae. A major DNase I-hypersensitive site approximately 400 bp upstream from the start of transcription is observed in the polytene chromatin of that tissue and not in chromatin from embryo nuclei.[224] It has recently been found that the BER-1 strain, which fails to express this gene, has a small (ca. 100 bp) deletion covering this DNase-I hypersensitive site, but does contain all other gene sequences from 300 bp upstream of the 5′ end through the normal transcript.[151,225] It is now generally recognized that while the canonical TATAA box at −30 is dispensable in vivo, certain sequences located further upstream are not (see Section V.B).

While in two instances the position of regions of transcription have been successfully predicted from the finding of DNase-I hypersensitive sites, one would not argue that all such sites are initiation sites. In fact, Nasmyth[226] has recently observed such a site in yeast chromatin at a point involved in switching of the mating type locus. The existence of the DNase-I hypersensitive site is under control of the *mar* locus, and is correlated with the position of the site within the genome rather than solely with DNA sequence per se. Another interesting case is the hypersensitivity of the terminal inverted repeat sequences in the macronucleus of the ciliate *Stylonychia mytilus.*[227] Macronuclear development involves the formation of polytene chromosomes, the elimination of greater than 90% of the genomic DNA, and the precise fragmentation of the rest into gene-sized, presumably functional subunits. All such fragments have identical 20-bp inverted terminal repeats, and it is these sequences which are hypersensitive to cleavage by DNase I. One might surmise that DNase I-hypersensitive sites mark regions where special accessibility of the DNA is required.

Although the EM studies of SV40 previously mentioned suggested an absence of nucleosomes in regions hypersensitive to cleavage by DNase I as an explanation for that sensitivity, other possibilities can be entertained. The presence of single-stranded regions in eukaryotic DNA has been reported.[228] Mouse L-cells and CHO cells were found to have gaps detectable by single-strand specific nucleases; these were spaced 10^5 bp apart on the average in G1 phase cells and, so, were presumably not related to replication forks. DNase I is thought to generate single-stranded gaps when digesting chromatin,[229] so single-stranded regions in the genome might be preferentially cleaved during digestion. Control experiments involving DNase I-digestion of purified genomic DNA have generally failed to produce analogous cleavage patterns, however, and since single-stranded gaps present in vivo would presumably persist in vitro, it seems unlikely that this could explain at least most DNase I-hypersensitive sites. It has also been shown that local denaturation of DNA can be a consequence of supercoiling under normal conditions.[230] Experiments using supercoiled plasmids containing cloned DNA known to span DNase I-hypersensitive sites in vivo failed to demonstrate such sensitivity in the absence of chromosomal proteins.[231]

With regard to the biological relevance of the hypersensitive site, one need not belabor the prokaryotic paradigms for the interaction of regulatory molecules with appropriate DNA sequences. The concept of an open "window" for effector proteins in an otherwise compact and relatively unavailable structure is attractive in view of the regulatory problem presented by the sheer quantity of eukaryotic DNA. If the effective sample size is reduced in this fashion, then the probability of correct binding is vastly increased, and

both the required titer of regulatory molecules and the probability of error are decreased. This problem has been treated at length elsewhere.[232,233] The finding that purified *lac* repressor could bind to its operator sequences when they were packaged in nucleosomal form[57] is intriguing, but leaves several questions unanswered. What is the possibility that in a different configuration (for instance with the entire sequence rotated ½ turn due to a different start site on the nucleosome) the appropriate binding will occur? (The *lac* repressor is known to bind predominantly on one side of the DNA.[234]) What is the effect of oligonucleosome and higher order structure? Too little is known of the interactions of nucleosomal DNA with other proteins to speculate on the importance of this phenomenon as yet. An alternative model which deserves attention is that the DNase I-hypersensitive site is involved in a general activator function of some sort. The modulation of torsional constraint or change in winding number remains an attractive model for the control of the activity state of a relatively large domain by action at a small site and has been proposed more than once.

B. Nucleosome Arrangement in Chromatin

Since the discovery of the basic nucleosome structure in eukaryotes, it has become evident that the bulk of the genome of all eukaryotes is organized in this manner.[27] It is of interest to note at the outset, however, that specific sections of repetitive sequence associated with the extrachromosomal rDNA genes in *Tetrahymena* have been shown to be devoid of nucleosomes, although clearly associated with proteins.[235] The central, nontranscribed spacer of this palindromic DNA also appears to be packaged in a nonstandard fashion.[236] Mitochondrial DNA is thought to be lacking in nucleosomes, although nucleosome-like structures have been claimed for both *Xenopus*[237] and *Paramecium* mitochondria.[238] The question as to whether nucleosomal packaging of DNA serves merely as the primary, essentially random means of condensation, or whether differential function can be expressed at some level through the structure imposed by the nucleosome is not resolved. At present this question has been posed most frequently in terms of whether or not the placement of nucleosomes is, in any way, in any part of the genome, specific with respect to the DNA sequence of that section of the genome. Such a specific placement (sometimes termed "phasing", but more properly called "positioning") necessarily implies that in cells of identical lineage, the nucleosome placement is identical with respect to the sequence. This is not to imply, however, any necessity that the whole genome be so organized. Numerous models are possible. A considerable amount of experimental work is currently being done on this question.

The degree of control which precisely positioned nucleosomes might allow in the regulation of complex processes is an interesting question. Specific DNA sequences will probably be relatively more or less accessible to a variety of macromolecules depending on their location as either core or linker DNA and on their orientation on the nucleosome surface (facing in or out). In particular, transcriptional control via exposed 5′ ends of genes or internal control regions (e.g., References 239 and 240) might be effected by a "positioned" conformation. As previously discussed, DNase I-hypersensitive sites, which may indicate an absence of nucleosomes, may be involved in regulatory events. Further, replication origins and "hot spots" for rearrangement events might require a relatively nucleosome-free region of DNA. However, it also appears that the case for functional importance of positioned nucleosomes can be overstated. As was discussed at greater length in Section II, DNA folded by histone cores appears to be very accessible to external molecules of both small and large dimensions. Hence it remains possible that nucleosomes have little impact on control mechanisms.

It would appear that histones show no sequence specificity per se in their binding to DNA.[241] On the other hand, it is also clear that overall nucleosome spacings do vary

between species and between tissues within species.[27] The factors involved in determining spacing have not been conclusively identified as yet. An obvious candidate in this regard is histone H1/H5, which is known to be involved in binding to the linker region of DNA (see Section II). Thus Weintraub found that in chick erythrocyte cell lineages, the nucleosome repeat-length increases concomitant with the stage of cell differentiation from primitive erythroblast through to adult erythrocyte.[242] Paralleling this is an increase in the H5 content of the nucleosomes; the H1 remains relatively constant. Evidence for divergent DNA binding characteristics of histone H1 subtypes has come from a circular dichroism study of bovine H1 variants where differences in their interactions with both linear and superhelical DNA were evident.[243] Different H1 subfractions also have different effects in condensing dinucleosomes.[244] However, other studies indicate that it is unlikely that H1 is the sole determinant. Zalenskaya et al. compared the nucleosomal repeat in sea urchin and starfish sperm nuclei; the histones involved are all very similar, except for H2B.[245] The longer repeat (sea urchin) was observed in the cell with the larger form of H2B, this having an extended N-terminal region which could be involved in linker interactions. The rather elegant study of Hsiung and Kucherlapati involved investigation of nucleosome repeat lengths in mouse-human and mouse-mouse cell hybrids.[246] In the mouse-human hybrids, all the nucleosomes were of the mouse repeat length, and all were associated with mouse H1. However, in the mouse-mouse hybrids, the nucleosomal repeat lengths were characteristic of one parent, while the bulk of the histone H1 was characteristic of the other. It was hypothesized that other (nonhistone) proteins must be involved with H1 in determination of the linker length. In the *Lytechinus pictus* sea urchin system, the nucleosome repeat-length increases in relatively defined steps over a period of a few hours during embryogenesis.[247] This takes place, however, against a background of major switches in the synthesis of histone subtypes from a large multigene family. In particular, there are obvious variants of H1, H2A, and H2B present in embryonic chromatin from later stages (blastula and gastrula) compared to early cleavage embryos. The increase in nucleosome repeat length also parallels a general decrease in transcriptional activity (as well as mitotic activity). The accessibility of chromatin to micrococcal nuclease cleavage also drops significantly over this period.

Such correlations with transcriptional activity and/or cellular maturity had been noticed previously,[248,249] although there are clear exceptions.[250] It has been found that cells in culture have a much more restricted range of nucleosome repeat lengths (approximately 180 to 190 bp[27]) which do not necessarily change when the cells are induced to differentiate or alter transcription patterns.[251] Of course the value of such studies of average properties of chromatin structure can be questioned given the complexity of the genome.

Several studies of the nucleosome repeat length of certain sets of genes have been carried out. Sequences involved in active transcription have been found in micrococcal nuclease-generated 11S particles by several investigators (e.g., References 252 and 253), although one does not anticipate that the actual conformation of histones and DNA is of the type present in bulk chromatin (see Section V.A). In an investigation of rat liver nuclei, the average nucleosomal repeat of transcribed chromatin was observed to be the same as that of the bulk chromatin.[254] This study, however, did not investigate specific genes, but rather the mass of genes giving rise to polyadenylated RNA. The 5S DNA genes of *Xenopus* have been studied intensively with regard to their chromatin structure.[255,256] In a comparison between different cell types it was observed that the nucleosomal repeat length of the 5S gene differed (the DNA sequence of course remains constant); the 5S gene chromatin repeat generally differed from that of the bulk genome. In a study of a nontranscribed satellite DNA from rat liver, a variety of nucleases were found to cleave the DNA with a different nucleosomal repeat from that of bulk DNA.[257] In both cases

cited, complications due to the sequence preference of the enzymes used appear to have been ruled out (as will be seen). These studies suggest that nucleosome spacing is a defined property for certain regions of the genome.

Extensive DNase I digestion of nuclei produces a sharp ladder of fragments that are regularly spaced at approximately 10-bp intervals. It was observed that on gels with high resolution this ladder extends all the way to 300 bp and above,[258] implying that not only does the enzyme recognize a regularly repeating structure in the nucleosome core, but that adjacent nucleosome cores (at a minimum, pairs) must be spaced at integral multiples of the 10-bp repeat. The repeating unit is presumably fixed by the known pitch of DNA; more precisely this is 10.4 bp on average,[259] although this is affected by the DNA sequence itself.[260,261] This result implies that the linker region is quantized, and to this extent at least, populations of nucleosomes are spaced with respect to each other. This type of phasing (which is to be carefully distinguished from the sequence-specific type of positioning to be discussed further) has been confirmed using exonuclease III.[262] It appears to cover a high proportion of the genome, at least in the three systems studied.[263] Interestingly, while HeLa cell and chicken erythrocyte linker lengths are based on integral multiples of 10 bp, that in yeast displays a (10n +5) bp periodicity,[263] implying a switch in the DNA-histone interface. One point that should be emphasized from all these studies is that in every case of nucleosome structure investigated, a resistant core involving 145 bp of DNA appears to be present.

Early investigations regarding the possibility of sequence-specific positioning of nucleosomes focused on the minichromosome of circular SV40 molecules produced during infection of monkey cells.[264] Initial studies on the location of nucleosomes involving comparisons of restriction endonuclease site accessibility in the SV40 chromatin led to a conclusion that the placement was random.[265,266] The rationale behind this approach lies in the expectation that a sequence-specific arrangement of nucleosomes will either mask a restriction site (which always falls within the core) or allow it to remain accessible (when it always lies in a linker region). Conversely, with random nucleosomal location, the accessibility of restriction sites will be lower than in naked DNA, but all fragments should be produced in equivalent amounts. Additional recent studies have supported this report, observing no preferential cutting by a number of restriction enzymes on SV40 minichromosomes prepared by a variety of methods.[267,268] However, as previously detailed (Section III.A), nuclease sensitive sites have been reported around the origin of replication by other investigators, and a nucleosome-free stretch of DNA in this region has been observed by electron microscopy. That populations of SV40 minichromosomes are heterogeneous in this regard is made clear by the electron microscopy results, and this may explain the discrepancy in the data concerning nuclease sensitivity. The occurrence of a nucleosome-free region in SV40 would appear to place restrictions on the placement of nucleosomes, at least in the immediate vicinity of this region.

In vitro reconstitution experiments with naked SV40 DNA and core histones have been performed, again with conflicting results. Examination using the electron microscope suggested nonrandom binding, since regions around the origin of replication especially were statistically lacking in nucleosomes.[269] A few sites (those where RNA polymerase II preferentially binds to the naked DNA) were also seen to be major sites of initial nucleosome formation. No obvious correlation between location of G-C- or A-T-rich sequences and nucleosome placement was observed. Using restriction enzymes to probe the reconstituted structure, Hiwasa et al. also reported that a region around the origin appeared to be somewhat resistant to nucleosome formation.[270] Of course, this study does not reflect the in vivo

situation, in that histone H1 and other chromosomal proteins were not present in the reconstitution mixture. Nucleosomes were clearly placed in close apposition, with no linker, as shown by mild micrococcal nuclease digestion. In contrast, no such nucleosome-free regions were detected by Simpson and Stein in a similar in vitro reconstitution study (using much lower histone:DNA ratios).[271] To date no naturally occurring sequence has been identified in viral or genomic DNA which appears able to dictate a nucleosome-resistant B conformation in the absence of other macromolecules. Further work is clearly needed.

The more commonly used approach to look for precise nucleosome placement has made use of micrococcal nuclease digestion followed by restriction analysis to map the micrococcal nuclease cutting sites relative to the DNA sequence. This approach was used by Ponder and Crawford, who found nonrandom placement of nucleosomes on both polyoma and SV40 chromatin when analyzed at the level of the core particle.[272] Fine structure mapping of cleavage sites revealed that the nucleosome placement agreed with the 10 bp periodic spacing previously described, but did not appear to have any absolute sequence-specific component. Interestingly, even though cores were placed at 10-bp intervals, the micrococcal nuclease also appeared to cleave in the linker at similar intervals. This investigation showed very clearly, however, that as a probe of nucleosome placement with respect to DNA sequence, micrococcal nuclease leaves much to be desired, since it very obviously had marked sequence preferences for cleavage on both purified polyoma and SV40 DNA. This aspect of micrococcal nuclease surprisingly received very little attention until recently, and a number of nucleosome positioning studies have been performed with few, if any, DNA control experiments.

Recent papers from Georgiev's lab have been careful to include such controls. Using a short cloned probe which contains the sequence adjacent to a restriction site used as a reference point to map the micrococcal nuclease cuts, it is possible to define unambiguously such sites of cleavage in one direction along the genome.[208,212] To obtain unambiguous results, samples are examined at relatively early times of micrococcal nuclease digestion when only one or a few cuts have been made in the region of interest. Even though the naked DNA has preferential cleavage sites in some places, it was considered by the authors that the data were sufficient to show nucleosome positioning in the "early" region of the genome. The number of sites mapped corresponded closely to the number of nucleosomes expected to cover the length of DNA investigated. No particularly strong cuts by micrococcal nuclease were detected in the origin region of the genome (in contrast to earlier results in Reference 207); such regions clearly appeared to be associated with nucleosomes in these preparations.[273]

The seemingly simple and therefore attractive system of SV40 minichromosomes has proved to be rather complex if the variety of conflicting results given above are any indication. It appears likely that populations of minichromosomes as routinely isolated are quite heterogeneous. The stages of maturity in the assembly of SV40 virions are not synchronized for all molecules in a given cell population, and this fact may be at the heart of the observed variation. It seems clear, however, that at some stage(s) nucleosome location is not completely random. Whether such positioning is imposed by binding of other (sequence-specific) proteins (e.g., T-antigen) or by adoption of some supranucleosomal structure is still unknown.

A number of other eukaryotic systems have been investigated, but the final resolution of this rather complex problem of nucleosome positioning is not immediately apparent. Some rather general studies were initiated by Prunell and Kornberg, who investigated the chromatin of rat liver nuclei.[274] They interpreted the results of their reassociation experiments with exonuclease III-treated core

particle DNA in terms of a random location of nucleosomes. However, the experiments were based on the premise of a precise positioning of nucleosomes throughout the complete genome in every cell of the rat liver, an unlikely occurrence. Prunell further investigated this question using DNase I digestion followed by reassociation and S1 digestion of single-stranded tails.[275] However, the arguments used in claiming the results showed random positioning depend on a number of assumptions about DNase I-cutting preferences.

Other investigations have used similar approaches to those used with varying success on SV40, and can be classified as studies on the repetitive sequence class of DNA (both transcribed and nontranscribed) or studies on unique regions of the genome. The nomenclature used in this review reserves the term "phasing" exclusively for a precise alignment of nucleosomes on tandemly repeating sequences, a situation which truly allows DNA sequences and nucleosomes to be in phase with respect to one another. In other cases the term "positioning" seems a more correct usage.[697]

In the former class, 5S genes and various satellite sequences have been intensively investigated. A problem with the 5S DNA studies as a whole is that in no case is it known in a given cell type whether all or just some of the tandemly repeated genes are being transcribed. Thus it is difficult to correlate any structural result with function in a direct and unambiguous fashion, nor can one anticipate a single packaging mode.

Gottesfeld and Bloomer[276] working with *Xenopus,* and Louis et al.[277] working with *Drosophila,* used digestion of chromatin with micrococcal nuclease to generate core particles with subsequent restriction analysis (albeit by somewhat different techniques) to locate sites of cutting in the 5S genes. Both groups claimed a nonrandom placement of nucleosomes. In the *Xenopus* oocyte 5S genes this took the form of at least four different phasing frames. Use of dimer- and trimer-length DNA confirmed some of the data. The results were obtained using a line of cultured cells in which the oocyte-specific 5S genes were expressed at a low level. No specific data were reported on the nucleosome arrangement in these genes in the inactive state, found in somatic cells. In both *Drosophila*-cultured cells and embryos two phasing frames were reported for 5S genes. No data on the transcriptional state were included. While both groups reported some preferential cutting by the enzyme on naked DNA, this was said to be unrelated to the chromatin structure pattern obtained, and no data were shown. Conversely, Baer and Kornberg reannealed radioactively labeled 5S RNA to core DNA generated from rat liver and found, after RNase treatment of the hybrids, a smear of radioactivity on gels, indicating a random sequence arrangement of 5S DNA on the core fragments.[278] However, the displayed gels did show an indication of banding patterns. These were discounted by the authors by comparison with broadly similar patterns produced on naked DNA. The core DNA was prepared after a long digestion with micrococcal nuclease; such conditions are rather suspect as regards the possibility of intranucleosomal cutting and/or nucleosomal sliding, an occurrence which could clearly result in randomized positions (as will be seen). None of the work reported seems conclusive.

Investigations into the nucleosomal organization of satellite sequences have been no less fraught with conflicting results. For example, the α-satellite of African green monkey cells was reported to be in perfect phase, one tandemly repeated DNA sequence unit associated with one nucleosome.[279,280] Singer disputed these claims by showing that reassociation of core particle DNA did not give rise to the parent 145 bp double-stranded fragments expected for a phased arrangement.[281] Such reassociation experiments as performed are, however, likely to be uninformative if

several frames of phasing are present. Fittler and Zachau reinvestigated the same sequence and found that the apparent phasing reported was almost certainly due to a strong sequence-specific cutting of the naked DNA by micrococcal nuclease, once per sequence repeat.[282] Using DNase II, the α-satellite nucleosomal structure conformed much more closely to that of bulk chromatin, and was distinct from the DNA sequence repeat. Hence a simple-phased relationship was ruled out, although more complex phasing patterns could not be discounted. Such complex phasing patterns certainly appeared to be present in the case of rat satellite I DNA.[283] Restriction digestion of nuclei (in the manner of some of the previous SV40 experiments) gave a defined subset of fragments, which, if one accepts that the enzymes could only cut the appropriate recognition sites when they were accessible in nucleosomal linkers, were apparently members of a phasing hierarchy which comprised at least three separate frames. A significant proportion of satellite I chromatin resisted restriction, and it was suggested that organization into phases that placed recognition sites in core locations was the cause.

The use of restriction enzymes for detecting nucleosomal location has been applied to a study of the C region of the kappa light chain gene in both mouse liver nuclei (where the gene is not transcribed) and a mouse myeloma T-cell line (where the gene is transcribed).[284] In the liver nuclei, restriction sites in the vicinity of the gene showed a distinct pattern of either relative accessibility or inaccessibility leading to an interpretation of nonrandom nucleosome placement. Comparison to restriction digests of naked DNA allowed a tentative assignment of location of nucleosomes along the gene. In the myeloma cell, all the sites were cleaved much more rapidly and with relatively equal intensity, implying an altered chromatin structure (lack of usual nucleosomes perhaps) extending not just over the transcribed region, but into flanking regions as well. As a control, the nonexpressed globin gene showed the same unequal pattern of accessibility of restriction sites in both cell types.

Upon light digestion of *Drosophila* nuclei with micrococcal nuclease and restriction digestion of the DNA, the indirect end-labeling method previously mentioned[208,212] has been used in a study of nucleosomes at the gene encoding the 83,000 dalton heat shock protein[212] and at the histone gene cluster.[217] In both cases, definite positioning of nucleosomes was reported, on the one hand covering the nontranscribed regions upstream of the 5′ end of the heat shock gene, and on the other covering the spacer DNA between the histone coding regions. In the latter case, only the long spacer between H1 and H3 appeared to show positioning above the level of two nucleosomes. In both studies the nucleosome positions appeared ill-defined in the transcribed region. Controls were reported in which purified DNA was subjected to the same digestion conditions. Clear and extensive cutting preferences of micrococcal nuclease within the DNA sequence were seen, although the results for chromatin itself appeared somewhat different, and were interpreted as being due to nucleosome-imposed structure. In both studies the location of the nucleosomes appeared to involve variable spacing between adjacent units. In neither case was the reported positioning observed to extend beyond seven to eight nucleosomes from the 5′ end of a transcribed region.

As a last example, a rather careful study of tRNA genes in chicken embryos was performed by Wittig and Wittig.[285] Using secondary restriction analysis they investigated isolated tetramers and higher oligomers released by micrococcal nuclease digestion, and concluded that all the tRNA genes in the study were represented in the genome in organized structures. Of some interest was the observation that in no case was the specific nucleosome placement determined to extend beyond five or six nucleosomes around each gene. Furthermore, on a section of chromatin in which two tRNA genes

were separated by only a few hundred base pairs, the placement associated with one gene abruptly changed and gave way to a second and different placement for the neighboring gene. There were, however, no naked DNA controls quoted in this study.

In summary, while the first studies appeared to have discounted positioning with respect to sequence as a possibility, many of the recent studies cited have swung the pendulum so that to some, positioning is now acceptable as a general phenomenon. It is certainly of interest to examine the basis on which such judgements might be made. Of most immediate importance are the indications contained in some of the quoted work that micrococcal nuclease shows significant sequence specificity in its mode of cleavage. This has now been amply confirmed. Thus preferential cleavage sites in chromatin observed in the vicinity of the four small heat shock genes of *Drosophila* at 67B have been found to be more or less present in the naked DNA purified from embryos.[286] The relevant sequences in the cloned recombinant plasmid itself displayed identical cuts to those in naked genomic DNA when digested briefly with micrococcal nuclease, thus precluding any contribution from *Drosophila* nuclear proteins remaining attached to the DNA or any effect of in vivo modification, nicking, or cutting. However, an interesting observation was that very few sequence preferential cuts in naked DNA appeared in the transcribed regions compared to the flanking DNA. Furthermore, in *Drosophila* 5S DNA, clearly observed sequence-specific preferred cutting sites occur in each DNA repeat, giving a pattern of DNA fragments identical to an oligonucleosomal ladder when displayed on agarose gels.[698]

It has recently been reported that when DNA was reconstituted into chromatin using homogenates of *Xenopus* eggs, the cleavage pattern on digestion with the enzyme was identical to that of the naked DNA used, showing that sequence preference could still be expressed at a high level in the face of nucleosomal organization.[287] This observation cannot yet be generalized; the protection of micrococcal nuclease DNA sites by chromatin structure has been observed in several studies. More work is needed to clarify the situation. The actual sequences involved in this recognition phenomenon have been delineated in some detail in a study of a satellite DNA, with CATA and CTA being preferred sites.[288] It is interesting to note that digestion of DNA by micrococcal nuclease has been found to proceed by endonucleolytic cleavage of the preferred sites, followed by exonucleolytic attack on the new ends;[288] hence the pattern of DNA digestion fragments is very persistent.[286] It is thus apparent that micrococcal nuclease is not the reagent of choice for investigating positioning of nucleosomes and, in fact, can lead to considerable confusion. It has been pointed out, for example, that a naked DNA pattern is likely to be rather more complex than a chromatin pattern, since protein removal will presumably open up sites unavailable in chromatin. It may not be obvious that the two patterns are related unless extremely careful mapping is performed.[288] In the light of this knowledge, a number of studies must be reassessed. Certainly it seems that a number should be reperformed.

A further problem concerns possible artifactual results from other causes. For example, although the experiments which used brief digestion of chromatin followed by mapping of cleavage sites[208,212,217] were criticized on the grounds that such brief digestion would positively select for sequence-specific cutting,[289] it seems that conversely, those experiments which used very prolonged digestion of chromatin with high concentrations of enzyme to produce core particles should be treated as suspect also. Sliding of nucleosomes (see Section II.A) has been documented, and such sliding is found to be facilitated under certain conditions in the presence of micrococcal nuclease.[290] This is all the more likely, since H1 is released from chromatin under conditions of extensive digestion.[21] Such sliding would of course give rise to a random histone-DNA association.

In examining the reports of specific nucleosome positioning, one notes that position

assignments have generally been made only seven to eight nucleosomes away from a reference site. There has been no evidence that regular positioning of nucleosomes exists over exceedingly long stretches of the genome. A further point concerns the irregular spacing of nucleosomes detected in several studies.[208,212,217] This is certainly an unexpected result, and must be questioned to some extent until more convincing control experiments are performed. However, the possibility of sequence preference in histone-DNA interactions should be reconsidered. A low level preference might be observed only in the presence of additional constraints, i.e., boundary conditions defining a starting point for a nucleosome array. The occurrence of relatively short-range positioning might be dictated by primary binding of sequence-specific proteins. Alternatively, a shift in the DNA configuration to one with an altered probability of forming nucleosomes could serve as a boundary. The fact that sequence-specific chromatin structures (DNase I-hypersensitive sites) are observed practically requires specific placement of the adjacent nucleosomes. A model of the immediate neighborhood of such sites can be generated from statistical considerations given a few of the constraints mentioned (see Reference 289). One problem which awaits resolution however, concerns the often used assumption that nucleosomes would find their spacing based on the bulk repeat prevailing in the cell. It is apparent (already mentioned) that this spacing is not constant throughout the genome. An alternative view of nucleosome positioning can be derived if one considers the suggested regular sequence-dependent deformational anisotropy found in eukaryotic DNA sequences.[291,292] Such a sequence-based correlation has been used successfully to make predictions concerning parts of the genome which are likely to have regularly positioned nucleosomes and those in which nucleosome formation may not be particularly favored. This view of the underlying sequence symmetries has recently been seriously challenged, however.[293]

The ultimate resolution of such questions will undoubtedly require more sophisticated probes of chromatin structure. For analysis of nucleosome organization, other enzymes, such as DNase I, DNase II, or endogenous nucleases (e.g., that from rat liver) could be considered. (Note that DNase I also has some sequence preferences in cutting DNA,[287,294] although this has generally not proved to be a problem.[214]) A method of sequence-independent chemical cleavage of DNA capable of detecting nucleosomes would be very useful. Ideally, an in vitro reconstitution system might be able to answer some of the questions posed in this section. However, at present, there appears to be little chance that a well-defined system, capable of mimicking in vivo nucleosome assembly, will be shortly available. If binding of NHC proteins or other stage-specific factors is important for accurate placement of nucleosomes, then it will not be possible to obtain information this way unless one can monitor reconstitution in a variety of in vitro systems that represent cell and developmental-specific stages. Lack of information on possible boundary conditions and the mechanisms by which these are established further hampers such attempts.

IV. NONHISTONE CHROMOSOMAL PROTEINS

Although NHC proteins are assumed to play a crucial role in the mechanisms for gene expression, specific proteins with defined roles have not been easily identified. Two general approaches have been used. In one, as will be discussed in Section A, an attempt is made to determine the role of isolated proteins. This has been done for several enzymes and cofactors as well as for a number of chromosomal proteins with no known enzymatic or enzyme-associated function. These latter proteins have been resolved from the bulk of the chromosomal proteins by electrophoresis or other techniques; most are present in high concentrations (approximately 10^5 to 10^6 molecules per nucleus). The lack of known

enzymatic functions and the large amounts of each suggest that these proteins are involved in the chromatin structure of a subset of the genome. Proteins in this category include the high mobility group (HMG) proteins,[295] ubiquitin and A24,[296] and H1° and its variants.[297]

Discussions of these proteins are complicated by a lack of consistent terminology and the lack of correlation between studies. Different groups of investigators have used different names in reporting studies on what appear to be the same proteins. It is difficult to distinguish which proteins are unique and which are species or cell type variants. Even the term "nonhistone" can be ambiguous, since many of the structural proteins characterized to date are lysine-rich, low-molecular weight proteins, several of which appear to play roles in the nucleus similar to those assigned to the histones (e.g., within the nucleosome core). Proteins which appear to be similar will be grouped together in the following discussion.

An alternative approach to the study of NHC proteins is to characterize proteins by their distribution with respect to the genome or by their pattern of interaction with DNA. For example, a number of proteins have been found associated with active genes, suggesting a necessary function in gene activation or transcription. This approach and the results obtained will be discussed in Section B.

A. Enzymes and Structural Proteins

1. Enzymes

A number of enzymes required for the metabolism of DNA or of the structural proteins of chromatin have been isolated from, or localized in, chromatin. These include enzymes involved in RNA, DNA, histone, and NHC protein metabolism or modification. Examples are presented in Table 1. The chromosomal distribution of some of these enzymes will be described in greater detail in Section IV.B.

Many enzymes are known to act upon chromosomal proteins, but have not been localized specifically on chromatin. Two reviews (References 114 and 350) give an appreciation of the large number of enzymes that produce modifications of chromosomal proteins (especially the histones) and the possible effects of these modifications. Some of these modifications are likely pertinent to gene activation and will be discussed in Section V.

2. Effectors of Transcription

A number of protein factors which are involved with the function of RNA polymerases have been reported. The most elegant studies utilize in vitro transcription systems with purified DNA as a template. In these studies, a number of cofactors have been identified and characterized.

The factors associated with RNA polymerase III have been studied in the laboratories of Roeder and of Brown. Segall et al., working with human KB cells, have purified two factors necessary for transcription of tRNA and VA RNA genes and a third factor which, with the other two, is required for transcription of the 5S RNA genes.[351] A factor analogous to the third KB factor has been isolated from *Xenopus*.[352,353] This 40,000 dalton protein has been shown to bind to 5S DNA at a site within the gene which falls within the region required for initiation of transcription.[656] It has also been found to form complexes with 5S RNA, which serve as storage particles in previtellogenic oocytes.[353,354]

Matsui et al. have identified four components (TF-IIA, TF-IIB, TF-IIC, TF-IID) from human KB cells which are required for the accurate initiation of transcription at the major late promoter of adenovirus 2.[355] TF-IIA stimulated the overall transcription rate when the other factors were present. TF-IIC suppressed random transcription. TF-IIB, TF-IIC, and TF-IID were chromatographically distinct from the factors involved with transcription by RNA polymerase III.

Table 1
ENZYMES ASSOCIATED WITH CHROMATIN

Enzyme	Organism and tissue	Ref.
RNA polymerase	Rat liver	298, 299
	Mouse myeloma	300
	Hen oviduct	301
	Coconut	302, 303
	Drosophila salivary gland	304
Poly-A polymerase	Wheat	305
DNA polymerase	Rat liver	306—309
	Rat ascites hepatoma	310, 311
	Sea urchin	312
	Calf thymus	313
	Mouse ascites	314
	Bovine lymphocytes	314
DNA endonuclease	HeLa cells	315
	Rat liver	316
	Papovavirus	317
DNA ligase	Rabbit bone marrow	318
DNase	Rat liver	319
Terminal DNA-nucleotidyltransferase	Calf thymus	320
	Tobacco	321
Poly ADP-ribose polymerase	Rat liver	322—324
	Calf thymus	325, 326
	HeLa cells	327, 328
Poly ADP-ribose glycohydrolase	Rat liver	329
Histone acetyltransferase	Rat thymus	330—332
	Bovine lymphocytes	333
	Calf liver	334
	Calf thymus	335
Histone methylase	Calf thymus	336
Histone kinase	Rat Walker 256	337
	Rat liver	338
	Calf thymus	339, 340
Histone protease	Rat liver	341—343
	Calf thymus	344
	Mouse testis	345
	Bull testis	345
Histone lysine methyltransferase	Rat brain	346
NHC protein kinases	Rat liver	347
Protease	Rat tumor cells	348
	Rat thymus, spleen, kidney, liver, brain	348
Nicotinamide mononucleotide adenyltransferase	Chick erythrocytes	349
Spermidine acetyltransferase	Calf liver	334

Using a less well-characterized assay, Natori et al. partially purified a protein from Ehrlich ascites tumor cells which stimulates the activity of RNA polymerase II.[356] The factor, S-II, a basic protein of approximately 40,500 daltons, stimulated the initiation of RNA synthesis from homologous DNA.[357,358] An antibody prepared in response to the factor inhibited the α-aminitin sensitive incorporation of ^{3}H-UTP into RNA in isolated nuclei; however, because the antibody did not affect the activity of purified RNA polymerase II, the factor is probably not a component of the polymerase.[359,360] The antibody has been used in immunofluorescent microscopic studies to localize the factor to the nucleoplasm of mouse 3T3 cells.[359] Other RNA polymerase II stimulatory factors have been isolated from lamb thymus,[361] rat liver,[362] and mouse myeloma cells.[300]

A protein cofactor which stimulates RNA polymerase I was found by Goldberg et al. to be crucial for transcription from native calf thymus DNA, but not from polydeoxycytidylic acid.[363] The stimulatory activity may be due to either or both of two proteins (11,000 and 12,000 daltons) which were resolved by SDS polyacrylamide gel electrophoresis of a rRNA Sepharose®-bound fraction. Kuehn et al. reported a phosphorylated NHC protein of *Physarum polycephalum* which stimulates the activity of RNA polymerase I.[364] This protein exists as a dimer with a monomer molecular weight of 70,000 daltons. A similar factor which stimulates rRNA synthesis twenty-fold was discovered in *X. laevis* embryos by Crampton and Woodland.[365]

3. HMG Proteins

Among the most extensively studied NHC proteins are the HMG proteins (see Reference 366 for a review of the properties of the HMG proteins). This group of low molecular weight, salt-extractable (0.35 *M* NaCl), and acid soluble proteins includes HMG 1, HMG 2, HMG 14, and HMG 17 all found in a number of organisms, but originally in calf thymus,[295,367] HMG 6 and HMG T in trout testis,[368] and HMG E in chick erythrocytes.[369] All HMG proteins are similar to one another in that they are quite lysine-rich (19 to 24%) and have a high content of acidic amino acids.[370] The molecular weights have been reported as 26,500 daltons for HMG 1 and 26,000 for HMG 2 as determined by equilibrium sedimentation.[371,372] HMG 1 and HMG 2 have two tryptophans each[373] and a least one cysteine each.[374] Despite the high lysine content, the acidic amino acids cause the pI of HMG 1 to be between 6 and 8, and that of HMG 2 to be between 7 and 9.[374] The unusual amino acid composition of these proteins is exemplified by the fact that HMG 1 contains a continuous sequence of 41 aspartic and glutamic acid residues.[375] HMG 2 contains a similar but not identical fragment.[376] Almost complete sequences of HMG 1 and 2 are now available.[377] HMG E (sometimes called HMG2B)[378] and HMG T[368] are analogous proteins.

HMG 14 and 17 from calf thymus[295] and HMG 6 (originally called histone 6) from trout testis[379] have molecular weights of 9,000 to 14,000 daltons and, while they are rich in lysine and acidic amino acids, they have no methionine, cysteine, isoleucine, or aromatic amino acids.[366] The amino acid sequences of calf HMG 14[380] and 17[381] and chick HMG 17[382] are now available. The amino acid sequence of HMG 6 has been shown to be very similar to that of HMG 14 and 17 of calf.[383]

A number of studies utilizing chromatin digestion and fractionation have suggested that HMG 1 and HMG 2 are associated with actively transcribing chromatin.[384-386] However, this notion is contradictory to reports of higher levels of these HMGs in less transcriptionally active cells.[387] Others have observed a correlation between the level of HMG 1 and 2 and the potential for differentiation (in mouse neuroblastoma and Friend cells).[388] HMG 1 and HMG 2 have been shown to have a binding preference for single-stranded DNA;[389] they do not bind to double-stranded DNA-agarose[390] or DNA-cellulose[391] columns in 0.2 *M* NaCl (physiological conditions). In addition, Javaherian and his colleagues have reported that HMG 1 and 2 reduced the linking number of supercoiled DNA, causing a net unwinding of 22° and 26° per molecule.[392,393] HMG 1 and 2 are able to bind specifically to histone H1, differing in their binding to the various H1 subfractions.[394]

HMG 14 and 17 bind to core nucleosomes and have a major effect on nucleosome structure. An examination of mononucleosomes prepared by digestion of chromatin DNA to either 145 and 160 bp of DNA showed that the 160 bp containing nucleosomes retained HMG 14 and 17, while the 145 bp containing nucleosomes did not.[387] Sandeen et al.[395] and Mardian et al.[396] have demonstrated two binding sites for HMG 14 or 17 per mononucleosome. The model proposed is of one molecule of HMG 14 and/or 17

associated with the DNA at or near each entry point of the DNA into a nucleosome core. It seems likely that the basic N-terminal region of the HMG binds to the DNA while the acidic C-terminal region binds to the nucleosome core. Such studies have not yet considered the interaction between HMGs and oligonucleosomes and, therefore, may not reflect the in vivo situation. The interaction of HMG 14 and 17 with actively transcribing chromatin has been the subject of intensive study and is discussed in detail in Section V.

4. Protein A24

Protein A24 (also called uH2A) is a complex of 28,451 daltons, in which ubiquitin is linked via a glycyl-glycine bridge to residue 119 of histone H2A[296,397] (for a review of A24, See reference 398). As much as 10% of the total H2A found in the nucleosomes of cultured mouse cells is conjugated with ubiquitin in this fashion.[17] An analogous protein, uH2B, present in smaller amounts, consists of ubiquitin linked to the carboxy terminal portion of H2B.[399] Martinson et al. showed A24 to be an integral part of a subset of trimmed, salt-washed, nucleosome cores.[400] In nucleosomes reconstituted using A24 instead of H2A, the HMG binding, H1 binding, DNase I sensitivity, and micrococcal digestion patterns were not altered by the presence of A24 in the core.[401] Studies in Novikoff ascites cells showing that H2A and A24 undergo the same modifications during the cell cycle have provided further evidence that A24 behaves in a similar manner as H2A.[402]

The conjugation of ubiquitin and H2A to form A24 is rapidly reversible in vivo.[403] Protein A24 lyase is an isopeptidase, cleaving the link between ubiquitin and H2A so as to leave each component intact.[404] The equilibrium between these two forms of H2A has been suggested to be involved in gene activation. Chromatin fractionation experiments demonstrate that free ubiquitin is found in fractions containing the mRNA coding sequences after nuclease digestion[405] and that A24 is found in the undigested (and inactive) fractions.[406] Levinger et al. have used electrophoresis to separate mononucleosomes and have then analyzed the complexes resolved for their DNA or protein content by an appropriate second dimensional electrophoresis step. When the DNA second dimension was examined with probes for active and inactive sequences and compared to the protein second dimension, A24 was found to be associated with mononucleosomes containing the inactive sequences.[407] Metaphase chromosomes lack A24[191,403] (see Section II.D). The general problems associated with chromatin fractionations leave the role of ubiquitin and A24 in gene regulation unsettled. A role for ubiquitin in protein degradation in the cytoplasm has been proposed.[408,409]

5. H1°

H1°[297], IP-25[410], and a butyrate-enhanced protein[411,412] are a group of proteins similar to histone H1 in structure. Subfractions of H1° have been isolated from CHO cells.[413] Although H1° is rich in lysine, it differs from H1 in its amino acid composition, particularly by the presence of a methionine residue.[412] The amino acid sequence of several H1° molecules is known; all contain an invariant region which shows a degree of homology with H5 and far less homology with H1,[414,415] although antibodies to both H1 and H5 will cross react with H1°.[416] The H1° group has usually been found associated with nondividing cells in a prolonged G_1 ("G_o") state.[297,410,417–419] It has been suggested from digestion experiments that H1° may replace H1 in association with linker DNA.[420]

6. Nuclear Matrix Proteins

Since 1949, numerous reports have been made of structure in the nucleus in addition to the chromatin fibers.[421] The nomenclature of this structure(s) has varied with the method

of the study, but it has generally come to be known as the nuclear matrix. For detailed information on this subject, see References 422 and 423. The nonrandom arrangement of chromatin in interphase nuclei has been discussed earlier. Several models of organization have been proposed utilizing the idea of regular attachment of the chromatin fiber to the matrix, lamina, or nuclear pores (e.g., Reference 424). Such an association would presumably be reversed during metaphase condensation; indeed lamins A, B, and C have been shown by immunofluorescence to be dispersed during mitosis.[425] Although with the exception of any "attachment" proteins the matrix is not chromosomal and its proteins are not, strictly speaking, chromosomal proteins, it is nonetheless an important structure with functions that may have some bearing on gene activation in the broadest sense.

The matrix complex can be isolated by treating nuclei with 2.0 *M* NaCl, removing the nuclear membrane with 1% Triton® X-100, and digesting with DNase and RNase.[426] The three major acidic proteins of the nuclear matrix account for 10 to 20% of the total nuclear protein content[427] and are approximately 69,000, 66,000, and 62,000 daltons.[428] Using two-dimensional gels, the three major protein bands have been resolved into a large number of protein spots.[429] Also present were proteins known to be associated with hnRNA.

The function(s) of the nuclear matrix remain unknown; however there is evidence which suggests an involvement of the matrix in DNA replication. Preparations of the nuclear matrix, which contain very little DNA, contain a significant proportion of the newly synthesized DNA.[426,430,431] A role for the nuclear matrix in the initiation of transcription has also been suggested.[432] HnRNP proteins appear to be an integral part of the nuclear matrix,[429] and hnRNPs remain associated with the matrix after DNA depletion and removal of histones by salt.[433-435] In a recent study, cross-linking experiments demonstrated that the 41,500 and 43,000 dalton nuclear matrix proteins were bound to hnRNP.[436] These data are consistent with an involvement of the nuclear matrix in some aspect of transcription or RNA metabolism; the most likely alternative is that posttransciptional processing of RNA occurs in the matrix as suggested by Revel and Groner.[437]

7. Proteins Involved in Microtubule-Kinetochore Association

The binding of microtubules of the spindle apparatus to the kinetochore has been examined in a number of EM studies. Ris and Witt showed that the microtubules are joined directly to the chromatin fibers at the outer disc of the kinetochore.[438] Less is known about the actual molecular binding, although one presumes that specific proteins are involved. It has been reported that some microtubule associated proteins, but not tubulin, will bind preferentially to eukaryotic satellite DNA.[439-441] One may hope that the kinetochore-specific lupus antisera recently obtained will aid in identifying the specific proteins involved.

B. Distribution Studies

In several cases, a determination of a protein's distribution in chromosomes or otherwise in relation to DNA sequences has provided clues to its function. In distribution studies one is limited to either (1) cytological techniques which allow one to probe a relatively intact structure, but which have inherent limits imposed by problems of fixation, or (2) biochemical techniques which frequently disrupt the nucleus and chromatin structure, but can permit a more detailed characterization of strong interactions. Results obtained by immunological techniques, by affinity chromatography using chromosomal proteins, and by specific DNA binding techniques will be discussed.

1. Immunological Techniques

An immunofluorescent staining assay for observing the distribution of specific NHC proteins on the polytene chromosomes of the salivary glands of third instar and prepupal *Drosophila* has been developed (see Figure 4). This technique relies on the pattern of visible bands (chromomeres) in polytene chromosomes, which allows correlation with genetic and biochemical data. In particular, it is known that the puffs, or decondensed bands, are sites of intense RNA synthesis. The pattern of puffs (active loci) is developmentally determined; it has been characterized in detail by Ashburner.[442] The immunofluorescence staining procedure involves incubation of a polytene chromosome preparation ("squash") with an antiserum against the protein in question followed by incubation with a fluroescein-conjugated secondary antibody. This allows visualization of the bound antibodies under ultraviolet illumination. Although the technique is extremely useful, it is limited by the level of resolution of the chromosome bands. A detailed description of the method as commonly used can be found in References 443 and 444. Problems of protein extraction and fixation can be minimized by using hand dissected chromosomes,[445] although this has rarely been done. A detailed review of all results to date can be found in Reference;[446] those most pertinent will be summarized here.

Analyses of this type, using the salivary glands of *D. melanogaster,* have suggested that at least four classes of NHC proteins showing the following distribution patterns can be identified:

1. Staining of all chromosomal loci in proportion to DNA distribution, indicating a protein likely to be a component of the basic machinery needed to fold and organize the DNA.
2. Staining of large regions within the chromatin (e.g., euchromatin or heterochromatin).
3. Staining of a set of loci, each of which is active at some point during the developmental period for this cell type (defined as the "developmentally active" loci). This pattern might be indicative of structural components required for gene activity or of receptors and/or signals involved in the response of these loci to subsequent stimuli.
4. Staining only of those loci which are being transcribed at the time of testing (the set of "immediately active" loci). This pattern should be indicative of a protein involved in the transcription process.

It should be kept in mind that these classifications constitute a working model based on the data at hand and must, of course, be expanded and modified to meet the demands of new information. Specific and reproducible patterns of protein:DNA interaction which do not fit into this classification scheme have already been obtained by using a variety of techniques. Examples of these include DNA binding proteins such as DB-2, which have a sequence specific interaction with DNA.[447,448] Sufficient information to consider the function of proteins of this type is not yet available and they will not be discussed in detail.

a. Staining in Proportion to the DNA Distribution

One clear case of this type has been observed using antibodies to a protein isolated by sequential isoelectric focusing and SDS polyacrylamide gel electrophoresis. An antiserum prepared against this *Drosophila* NHC protein of pI 5.2 and 21,000 daltons produced a pattern of staining showing widespread distribution.[449] The chromocenter was prominently stained; puffs were also stained, but at a very low level. This very

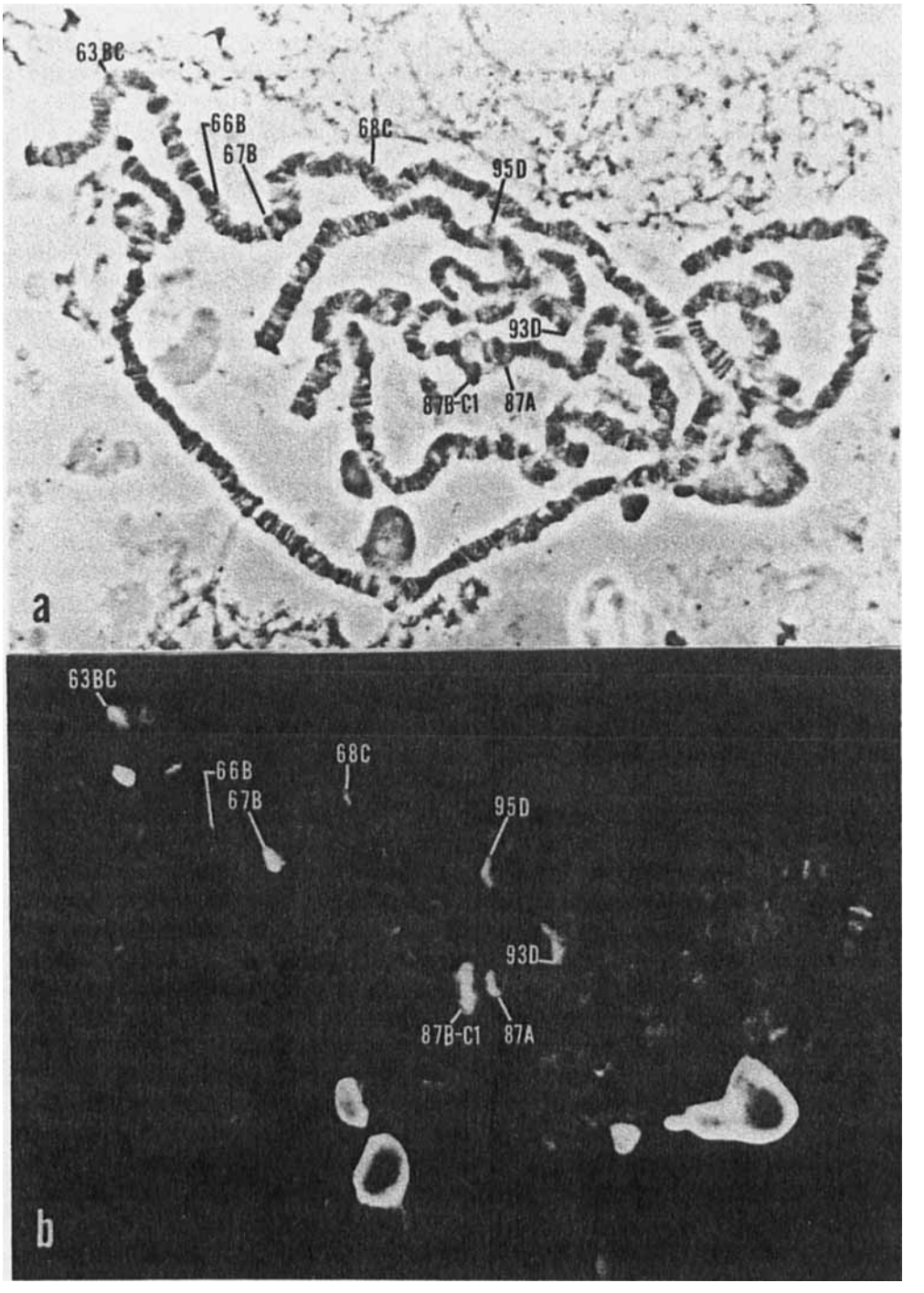

FIGURE 4. Light micrographs of polytene chromosomes from the salivary gland of a heat shocked larva stained with antibodies against RNA polymerase II. The antiserum was a gift from Arno Greenleaf and E. Bautz, prepared as described.[474] (a) Phase contrast, (b) fluorescence.[703]

general, apparently nonspecific interaction between the protein and the chromatin fiber suggests a protein with a general structural role. Since rough quantitative estimates of the protein yield indicated that only one molecule of this protein is present for every 10 to 100

nucleosomes, one might suggest a role in packaging or stabilizing higher order structures, such as the 300-Å fiber. The putative scaffold proteins (discussed previously in Section II.D), could have a distribution pattern and quantitative representation of this type. Monoclonal antibodies showing a distribution of this type have been obtained and characterized by Saumweber and his colleagues.[450]

A second set of proteins possibly of this type has been reported from studies using Ehrlich ascites tumor cells. Two NHC proteins of 54,000 and 68,000 daltons were isolated by virtue of their tight binding to DNA under alkaline conditions.[451] Antibodies to these proteins gave an intense nuclear staining in addition to a filamentous staining in the cytoplasm in Ptk-1 cells (giant rat kangaroo cells). No staining was observed using metaphase cells; this may be a result of the structural proteins being obscured. These results and others led the authors to suggest that these tight-binding proteins might stabilize the superhelical structure. Although the authors further suggest that this protein fraction may include a peptide linker which joins adjacent single-stranded DNA segments,[452] supporting evidence has not been forthcoming.

Antibodies prepared against HMG 1 and HMG 2 of calf thymus give very general staining of the polytene chromosomes of *C. thummi.* However, Kurth and Bustin reported that the antigenic determinants appeared to vary in accessibility between loci as a function of developmental period.[453] In some instances it has been observed that HMG1 antiserum will stain the cytoplasm at a rather high level. However, this appears to be an artifact due to the extraction properties of HMG 1. Microinjected ^{125}I-HMG 1 will enter the cell nucleus rapidly and concentrate there to better than 90%, but is capable of equilibrating into a second nucleus within a few hours after cell fusion.[454] Thus it has been inferred that at least a portion of HMG 1 is in rapid equilibrium with the cytoplasm. It has also been observed that a significant amount of HMG 1 and HMG 2 are readily lost during isolation of nuclei by conventional means, whereas essentially all the HMG 1 and 2 are found in nuclei isolated by cytochalasin-induced enucleation.[455] Methanol fixation, frequently used in cytological preparations, can also apparently induce loss of HMG 1 to the cytoplasm.[456] HMG 1 and HMG 2 are immunologically cross reactive, and are generally observed to show the same behavior in vivo and in vitro.

b. Staining of Large Regions within the Chromatin

Several types of proteins have been found which can be classified in this group of the NHC proteins. Will and Bautz have produced an antiserum against an NHC protein fraction obtained by hydroxylapatite chromatography which gives very clear staining of the centromeric heterochromatin of *Drosophila* polytene chromosomes in addition to staining a few bands known to contain satellite DNA.[457] A single peptide of 38,000 daltons was shown to be responsible for this pattern. Another DNA binding protein (BA), isolated from rat liver and human lymphocytes, is associated with nongrowing cells. This 31,000 dalton protein was shown to be present in heterochromatin by immunoelectron microscopy and peroxidase-antiperoxidase localization of the antigen, and to have a preference for AT-rich DNA.[458] The authors suggested that BA stabilizes heterochromatin and is involved in general repression. The NHC protein Dl of *Drosophila,* isolated by Rodriguez-Alfageme et al., appears to be concentrated at a limited number of locations in formaldehyde fixed polytene chromosomes.[459] These locations include the proximal heterochromatin of the X chromosome and pericentric heterochromatin of the three autosomes, a pattern which corresponds closely with quinacrine staining of AT-rich DNA. In unfixed chromosomes, Dl appears to be extracted, resulting in a general low level pattern. Again, a repressor function has been suggested. The molecular weight of Dl has not been accurately determined because of its unconventional electrophoretic properties in comparison with standard proteins.

Much interest is currently centered on the antisera from patients suffering from

autoimmune diseases. An antibody has been found in the serum of scleroderma patients which reacts with a protein of 70,000 daltons (Scl-70) and which interacts strongly with interphase chromatin and chromosomes in mitosis.[460] It is estimated that there are approximately 26,000 molecules of antigen per genome. Sera from different patients with autoimmune diseases exhibited several types of nuclear staining, one of which was a pattern of prominent association with the kinetochores of mitotic cells, leaving the chromosome arms completely unstained. This centromeric pattern seems to remain unchanged in interphase cells, with staining of discrete foci in the nucleus observed. Conserved in mammalian cells, the antigenic component has been found in human, hamster, and mouse cells.[461,462] In addition, antibodies from patients with other autoimmune diseases have been shown to stain polytene chromosomes of *Drosophila,* resulting in a number of distribution patterns, including the prominent staining of either condensed chromatin or puffs.[463]

While examples exist for preferential staining of the heterochromatin, none has been found having a clear and exclusive preference for euchromatin in the polytene chromosomes. There are several antisera against NHC proteins which do not stain the chromocenter, but it is difficult to evaluate this data because of the relative reduction in number of gene copies in this region and the consequent decrease in the sensitivity of the assay. Potential candidates for such proteins probably would have to be tested by immunofluorescent staining on fixed metaphase chromosomes.

In addition to general structural proteins, one might expect this subgroup to include certain enzymes. A 25,000 dalton serine protease has been found which can bind to DNA. Labeled protease inhibitors have been used to demonstrate indirectly its widespread distribution on polytene chromosomes. Analyses using inhibitors in conjunction with induction of heat shock indicated that this protease is not required for puffing.[464]

c. Staining of the Set of Developmentally Active Loci

In late larval and prepupal development, the salivary gland chromosomes of *Drosophila* exhibit a highly regulated pattern of puff formation and regression. This specific pattern of gene activity has been studied in detail and the loci mapped on the polytene chromosomes by Ashburner.[442] Indirect immunofluorescence has been used to correlate the puffing patterns with the localization of proteins or protein fractions. The first positive experiment of this type utilized a 80 to 110,000 dalton subfraction (referred to as ρ) of the total NHC proteins of *Drosophila* as immunogen. An antiserum against this protein subfraction produced a fluorescent pattern which included sites which were immediately active (puffs), had recently been active, or were to be active. Careful analysis of those sites prominently and consistently stained on chromosome 3 established a 90% correlation with those sites listed by Ashburner as puffing in the third instar and prepupal stages.[465] A similar correlation was found with an antiserum to a 60 to 65,000 dalton subfraction of the NHC proteins released by mild DNase I digestion of *Drosophila* nuclei, termed the "band 2" antiserum.[466] Recently, results with monoclonal antibodies prepared against the band 2 immunogen have confirmed that a distinct 62,000 dalton protein exists with this characteristic distribution pattern[467] (see Figure 5). The staining patterns in these three cases, while not completely identical, correlated well with the potential for and history of gene activity. A similar pattern has been observed by Saumweber et al. using monoclonal antibodies against a protein of 38,000 daltons.[450]

That some antigens of this type might be evolutionarily conserved has been demonstrated by staining of *Sciara coprophila* nuclei as well as *D. melanogaster* nuclei with the ρ antiserum.[468] The pattern of staining on *Sciara* polytene chromosomes was relatively specific and similar to that on *Drosophila* chromosomes with the exception of one telomeric region which had an unusually intense staining in *Sciara.* It should be

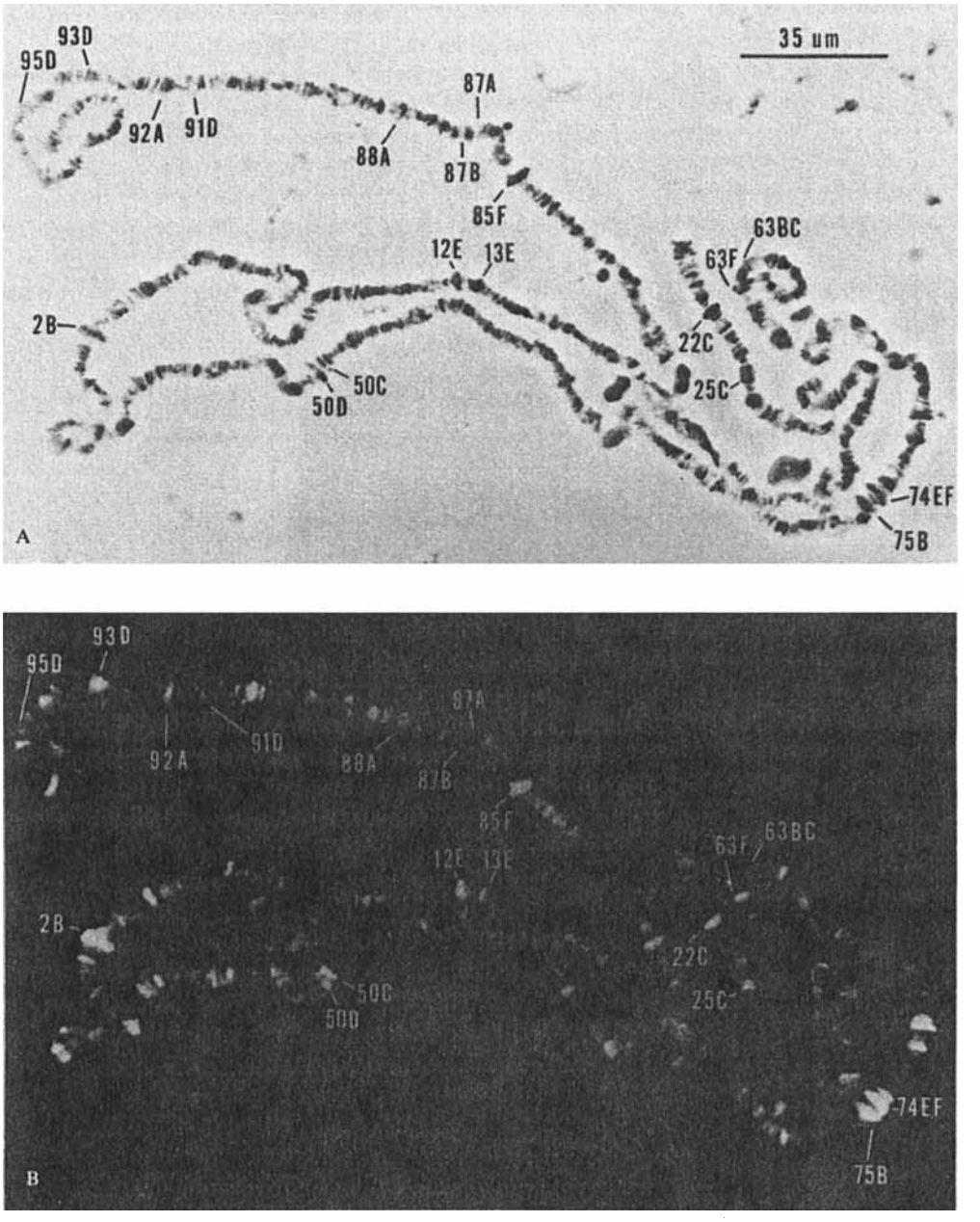

FIGURE 5. Light micrographs of polytene chromosomes from a normal larva stained with antibodies secreted by clone 28. (A) Phase contrast, (B) fluorescence. (From Howard, G. C., Abmayr, S. M., Shinefeld, L., Sato, V. L., and Elgin, S. C. R., *J. Cell Biol.*, 88, 219, 1981. With permission.)

noted that the ρ antiserium reacts with several proteins,[699] and consequently one cannot infer conservation of a particular antigen from this work. That the chromosomal proteins are in general conserved is of course argued by the success of cell hybridization.[469]

It is possible to alter the pattern of gene transcription in *Drosophila* by exposing the animal (or cells) to temperatures of 29 to 37° C, routinely 35° C. Such heat shock induces major puffing of a small set of loci concurrent with the rapid inactivation of most normally active loci. Some of the heat shock loci are, however, puffed at specific

developmental stages, and biochemical evidence shows 95D to be active in many cell types at room temperature.[470] The immunofluorescent staining pattern on chromosomes from heat shocked animals can be studied and an additional correlation made between the antigen location and puff sites. The staining pattern obtained using ρ antiserum, band 2 antiserum, and clone 28 antibodies under these conditions included the heat shock loci, which previously were not prominently stained, as well as sites that stained prior to heat shock (although in some cases staining had decreased at these sites).[465,466] Positive staining therefore may indicate an antigen which is necessary, but not sufficient for transcription. The possibility that the change in distribution is observed simply as a function of a change in accessibility for the antibody probe seems unlikely, since appropriate unpuffed sites do stain.

Another protein which exhibits localization related to developmental programs of transcription is the 20-hydroxyecdysone receptor. Known to be required for the induction of RNA synthesis at certain genes, 20-hydroxyecdysone induces immediate puffing at some loci ("early"), while causing activation of other loci with a time lag of nearly 3 hr ("late"). Gronemeyer and Pongs have taken advantage of the ability of 20-hydroxyecdysone to be photoactivated by irradiation and thereby cross linked to proteins in the chromatin complex *in situ*.[471] Following irradiation, *D. melanogaster* salivary glands were fixed with ethanol/acetic acid, squashed in formaldehyde/acetic acid, and stained with fluorescent antibodies against 20-hydroxyecdysone. There was bright staining at those puffs which respond to the hormone, both early and late, including those puffs which regress. A gel blotting technique was used to demonstrate that 20-hydroxyecdysone is bound to a single 150,000 dalton protein in *Drosophila*.[472] In *C. tentans*, 20-hydroxyecdysone is also located at several puff sites.[473] This pattern appeared to be stage-specific.

d. Staining of the Immediately Active Loci

In contrast to the previous subgroup, antibodies against this class of NHC proteins produce a pattern of staining correlated only with immediately active loci — those in the process of synthesizing RNA at the time of assay. These proteins are thus expected to exhibit a pattern of localization dependent on the developmental stage of the larva or on other means of induction or repression such as heat shock. This type of pattern was seen with antibodies against RNA polymerase II prepared by Greenleaf and colleagues.[474] With this antiserum, prominent staining of puffs was obtained with no staining of the centromeric heterochromatin or of inactive euchromatic bands.[304,475,476] Following heat shock, staining is lost at the developmental loci, but was prominent at the newly induced loci.[477,478] The pattern of staining was similar to the pattern of uridine incorporation, corresponding to RNA synthesis. This result has recently been repeated using monoclonal antibodies prepared against the high molecular weight subunits of RNA polymerase II.[479] These antibodies have also been used in staining the primary spermatocytes of *D. hydei*. The loop structures of the Y chromosome, known to be active by uridine incorporation, showed preferential staining indicating that these regions are transcribed by RNA polymerase II.[480]

In addition to enzymes involved in transcription, proteins involved in packaging and processing RNA might be expected to be associated with active sites. This pattern has been obtained using an antiserum prepared against a 34,000 dalton hnRNA binding protein from *Physarum*.[481] The antiserum reacts with a protein of similar molecular weight from *Drosophila* and has been shown to immunoprecipitate hnRNP particles from HeLa. Indirect immunofluorescence on polytene chromosomes showed the protein to be concentrated only at those loci which are immediately active. During heat shock, the newly active loci are prominently stained, while other staining decreases. Other

investigators studying RNA-binding proteins have found them localized in the nucleus of human tissue culture, amphibian, and chicken neural and glial cells.[482] In addition, several antisera to proteins (larger than 20,000 daltons) recovered from RNPs have been shown to bind to the transcriptionally active lateral loops of lampbrush chromosomes.[483] Based on these results, one might propose that proteins involved in transcription and immediate processing of RNA are found only in association with the active site and the nascent RNA located there.

Techniques for the localization (and subsequent isolation) of certain chromosomal proteins by means of their binding to small molecules have been developed as an extension of the approach of immunofluorescent staining of polytene chromosomes. Small molecules such as lectins, cAMP, and 20-hydroxyecdysone, as previously described, have been used as probes. These molecules are visualized either by direct labeling or by binding of fluorescent antibodies. Concanavalin A has been shown to bind to puffs of polytene chromosomes and to transcriptionally active chromatin in rat liver.[484,485] This is proposed to be a result of its affinity for sugar residues on modified proteins. One can suggest similar experiments to localize enzymes of interest by means of inhibitors, for example to localize RNA polymerase II by binding α-amantin to chromosomes. The distribution of cyclic nucleotides on formaldehyde-fixed polytene chromosomes has been studied.[486] Results indicate that cGMP, but not cAMP, correlate with sites of dispersed chromatin (interbands, diffuse bands, and puffs), but not highly condensed chromatin (dark bands). This suggests that cGMP plays a role in gene expression, possibly by inducing kinase activation and subsequent phosphorylation of chromosomal proteins. This technique can be further expanded by combining immunofluorescence with incorporation of radiolabeled precursors and autoradiography.[487] This provides the potential for the simultaneous location of an antigen and of an enzymatic activity, and is especially applicable to the enzymes with synthetic activity and RNA and DNA polymerases.

2. Chromatin Fractionation

Although a considerable body of work exists on chromatin fractionation, most will not be discussed in this review. In order to fractionate chromatin, it is necessary to cut or break it into smaller fragments and to separate these on the basis of some property related to transcription. So little is known about active chromatin that the selection of such property has been difficult. A method has been developed to separate active from inactive chromatin taking advantage of the differential nuclease sensitivity which is successful by the criteria of fractionation of DNA sequences.[488] Unfortunately it appears that the cleavage of the chromatin can result in rearrangement of some chromosomal proteins.[489-491] Evidence that a structure has retained its integrity during isolation is difficult to obtain. Some of these problems can be avoided by utilizing discrete structures such as nucleoli[492,493] or metaphase chromosomes (e.g., Reference 494), and these preparations may yield new information.

Weisbrod and Weintraub have used a type of affinity chromatography dependent upon the interaction of fixed HMG 14 and 17 with "stripped" nucleosomes.[495] In these experiments, purified HMG 14 and 17 were covalently linked to a column matrix. Chromatin, digested to oligonucleosomes and stripped of its endogeneous HMG 14 and 17 by extraction with 0.4 *M* NaCl, was reassociated with the column-bound HMG proteins. This procedure allowed the separation of the chromatin into two fractions: one which did not bind to the column in 0.01 *M* NaCl, and one which did bind and was removed from the column by 0.4 *M* NaCl. This strategy was successful in isolating a fraction of chromatin greatly enriched for genes known to be active in the particular tissue used. The data support the idea that nucleosome structure includes characteristics

allowing specific interaction with HMGs. These characteristics were able to survive both isolation of mononucleosomes and salt washing. Unfortunately, these characteristics remain obscure; patterns of NHC proteins, histones, and histone modifications were identical in the different fractions. In the chick system there is a correlation with a lack of methylation of the DNA of active regions which may be relevant. In order to determine the generality of these observations, it would be of interest to determine the distribution of equivalent HMG proteins in *Drosophila* polytene chromosomes by immunofluorescence techniques. Unfortunately, HMG proteins of *Drosophila* have not yet been identified. This strategy of reassociation binding might also be extended to other proteins which show a specific distribution in vivo.

3. Protein Distributions Assayed by DNA-Binding Techniques

A number of in vitro systems have been developed to determine the distribution of NHC proteins by virtue of their sequence-specific interactions with defined regions of DNA. In some cases, these systems also facilitate the purification of the protein(s) of interest. Foremost has been the filter-binding assay developed originally in studies of *lac* repressor-operator interactions.[496-498] In principle, specific protein-DNA interactions can be identified by the ability of nitrocellulose to retain such complexes on filtration, while allowing unbound DNA to flow through (and hence, with suitable radioactive labeling, allowing quantitation of the interaction). However, in searches for proteins binding to single copy sequences in an eukaryotic genome, the sensitivity of the technique is not sufficient to allow identification of specific interactions unless some purification of the proteins or DNA sequences (or both) is performed. With this in mind, Weideli et al.[499] used DNA-cellulose chromatography[500] to partially purify a number of DNA-binding proteins in *Drosophila*. One particular protein fraction (DB-1) was then used in a filter-binding assay to screen pools of radioactive recombinant plasmids from a library of the *Drosophila* genome. If some degree of binding was detected, the DNA was eluted from the filter and used to transform bacteria, which was then further screened until individual sequences were identified. By this rather tedious reiterative procedure, three recombinant clones were detected which showed highly specific binding to a major polypeptide in the protein fraction (assayed by competition assay with other, nonspecific, DNA probes). One of these clones was localized to the nucleolus by *in situ* hybridization experiments and hybridized specifically with 18S and 28S rRNA. In a second instance a clone which bound specifically to another protein fraction (DB-2) was found to hybridize to a single site in polytene chromosomes.[448] If sufficient amounts of these proteins can be purified to prepare antibodies, the proteins of interest could be investigated further by immunofluorescence studies (as previously outlined) and their distribution in vivo confirmed.

It is also possible to use a cloned recombinant DNA to search directly for proteins binding specifically to repetitious sequences using the filter-binding technology. Hsieh and Brutlag have thus identified a protein that binds specifically to a highly repetitive satellite element of *Drosophila*.[501] By employing suitable restriction fragments of the DNA it was possible to localize the interaction to a 117-bp DNA sequence. Competitive binding assays showed that the protein did not display a general affinity for A-T-rich regions, and that it was specific in its interaction with supercoiled but not linear DNA. Jack et al. have used similar techniques in their search for proteins in crude nuclear extracts specific for a *Drosophila* heat-shock gene region.[502] A defined region upstream from the presumed start of transcription of a 70,000-dalton heat-shock protein was identified as the effective protein binding site. This technique clearly has the potential to be scaled-up to preparative proportions.

Weideli and Gehring have performed DNA-cellulose chromatography using the

plasmids identified in the work on DB-2 and have purified the cognate binding proteins from crude nuclear lysates.[447] Using only the plasmid affinity column and a gel filtration step, purification of as much as 200,000-fold has been achieved. Use of sheared calf thymus DNA premixed with the crude protein preparation decreased the nonspecific binding which is often a problem with such DNA chromatography.

A new development of the filter hybridization technique that depends on the ability of radioactive DNA to interact specifically with proteins immobilized on nitrocellulose replicas of polyacrylamide gels has recently been described by Bowen et al.[503] The technique appears to offer the opportunity to rapidly screen protein preparations for binding affinity to defined sequences of DNA. Jack et al. used the technique to confirm the specificity of the binding interaction between a defined region of heat-shock associated DNA and a protein fraction purified by filter binding.[502] The autoradiogram of the protein blot slowed that the specific restriction fragment bound to each of three polypeptides of molecular weight 35,000, 34,000, and 29,000 daltons. The protein fractions were isolated from heat shocked cells; however no attempt was made to determine whether the proteins of interest were heat-shock specific (i.e., whether they were not also present in control cells).

One reservation about all of the approaches discussed is the concern that in vitro binding may not reflect any in vivo situation. Binding conditions cannot duplicate those within a cell, so results may be misleading. Only if there is a functional assay of the relevance of a given DNA:protein interaction can binding studies be given full credence. In vitro binding studies do, however, allow precise analysis of the interaction between a known protein and a specific binding site on DNA. Tjian localized the binding site for a T-antigen-related protein in an adenovirus-SV40 hybrid using DNase I digestion of the DNA-protein complex formed in vitro.[504] Three fragments of DNA were protected by protein binding. Subsequent restriction analysis of the isolated fragments identified a tandemly repeated sequence near the origin of replication as the specific binding region. The technique appears to be of general use and was used to identify specific locations of interaction of protein DB-2 on *Drosophila* DNA[448] and wheat germ RNA polymerase II on SV40 DNA.[505]

A very elegant version of this technique, known as "footprinting", was developed by Galas and Schmitz.[506] If a given ^{32}P end-labeled fragment of DNA is bound to its cognate binding protein, then limited DNase I digestion (an average of one cut per DNA molecule) will generate a series of fragments with one end defined by the ^{32}P label and the other by the DNase I-cleavage site. Display of this fragment collection on a polyacrylamide gel produces a pattern which looks much like a sequencing ladder. However, the region of DNA covered by the protein is now immediately obvious since most of the internucleotide cleavage sites are protected from the enzyme. The corresponding gap in the fragment array has been termed the protein "footprint", and the region of the binding interaction is thus easily mapped. The method was used in a most convincing way with the demonstration that a *Xenopus* 5S DNA transcription factor bound with high specificity to an internal region of the 5S DNA sequence.[352,353] The region protected from DNase I coincided almost exactly with an internal sequence previously identified as crucial for correct initiation of transcription of *Xenopus* 5S RNA in vitro.[239,240]

A somewhat analogous technique was developed by Johnsrud.[507] Instead of cleavage with DNase I however, the DNA-protein complex (in this case *lac* promoter:RNA polymerase) was treated in a limited reaction with dimethyl sulfate, which can alkylate both guanine and adenine, if they are in the appropriate positions in the major and minor grooves (guanine in the major groove and adenine in the minor). Subsequent selective depurination and alkaline cleavage at the exposed sugar residue generates a series of 5′

end-labeled fragments which can be separated and visualized on a gel. Sites of high or low susceptibility to methylation can be easily detected compared to controls using naked DNA. In this way, the precise location of the polymerase on *lac* promoter was determined and information regarding the relative degree of contact between protein and DNA in the major and minor grooves was also derived. These techniques should also prove useful in the analysis of chromatin structure. While the histone-DNA interactions do affect the pattern of nuclease digestion of DNA, they do not substantially block access to the major or minor groove by dimethyl sulfate. Hence even within the overall chromatin structure, this technique might be used to map the position of regulatory proteins.

V. CHANGES RELATED TO GENE EXPRESSION

A. Changes in Packaging

It is now certain that the conversion of a gene from a quiescent DNA sequence into a transcriptionally active locus involves a series of structural changes in the hierarchical complex known as chromatin. Much knowledge of the operational parameters defining the "active" state has accumulated within the last 5 years, thanks to a variety of experiments, but chiefly those utilizing endonucleases to dissect the chromosomal fiber. Although it is not simple to integrate the diverse results which have increased the understanding of the mechanisms involved, it would seem that an outline of the process of gene activation can now be suggested as follows.

The genetic information of a eukaryote consists of a multitude of coding sequences interspersed with an even greater number of spacer regions of unknown function. Relatively large, sometimes polycistronic blocks of DNA are somehow insulated from each other in a reproducible manner to form "domains" within each chromosome. These are formally analogous to the chromomeres long known in the Diptera. During the course of differentiation, each domain is designated as either competent or incompetent for expression in a tissue-specific manner. It seems likely that this involves a stable, heritable alteration in the chromatin; this has been detected as a difference in susceptibility to digestion by DNase I, with competent domains being more rapidly solubilized. One or more DNase I-hypersensitive sites are acquired near the 5′ ends of competent genes prior to their expression. In general, the relevant chromatin structure is assembled before transcription commences, persists after transcription has ended, and in at least some cases is transmitted vertically during replication, independent of transcription. An altered nucleosomal configuration directly associated with the transcriptional event has been revealed by studies using micrococcal nuclease.

It has become evident that these important structural changes are mediated by chemical modifications of the component macromolecules in chromatin and by changing patterns of association among those components. For example, in some animal systems transcribed DNA sequences are undermethylated in comparison with adjacent sequences or with the same sequence in nonexpressing cells. In chick chromatin, the nucleosomes of transcribed regions have a strong affinity for the high mobility group proteins HMG 14 and 17. Circumstantial evidence indicates that the histones associated with transcribed regions may be highly acetylated, but this has not yet been supported by direct tests. Changes in the distribution of NHC proteins on *Drosophila* polytene chromosomes in response to changing patterns of gene expression have been clearly demonstrated. Many of these characteristics are probably shared by genes transcribed at widely varying rates. (The actual rates of transcription could be controlled both by the relative strengths of promoters and by the facility of elongation, both of which will be readily affected by local chromatin structure.) Some of these characteristics appear to be structural modifications independent of the transcriptional event.

Many of the earliest observations of altered chromatin structures related to transcription were made directly by visualizing identifiable active genes with the electron microscope. Several early studies of ribosomal genes and of loops from lampbrush chromosomes in amphibian oocytes found that the central fiber of active genes (identified by RNP transcription complexes) exhibited a smooth, nonbeaded appearance, while nontranscribed regions displayed the beaded morphology indicative of a normal nucleosomal array.[508-511] Variations of the low-salt spreading method developed by Miller and Beatty[512] were used to visualize these transcription units. Under these conditions, no structures more complex than the 100-Å fiber survive, and variable DNA packaging ratios of about two are commonly found for "inactive" chromatin regions instead of the five- to sevenfold compaction expected for closely packed nucleosomal arrays. Packaging ratios of one to two are generally observed for active genes.

While it is therefore difficult to interpret directly the structure of the smooth fiber, several interesting observations on the temporal and physical course of gene activation and transcription have emerged. Foe, studying embryogenesis in *Oncopeltus fasciatus,* noted that in the late blastoderm stage only beaded chromatin could be seen, while in the early germ band stage (shortly before rRNA could be detected biochemically), nonbeaded stretches of rDNA could be seen, many as yet devoid of RNA transcription complexes.[508] Scheer and Franke, studying the oocytes of several amphibian species, also found the nucleolar chromatin fiber to be smooth whether it was densely or sparsely covered with RNP particles; intervening nontranscribed spacers were nonbeaded as well.[509,510] During inactivation as a result of either normal development or treatment with actinomycin D, a beaded appearance was progressively assumed in RNA-fibril-free regions. Similar results were noted for lampbrush loops, except that some stretches of beaded chromatin appeared in nontranscribed regions. These authors concluded that the conformational shift observed was not directly associated in space or time with the transcriptional event itself. Rather, the "unraveling" of nucleosomes preceded the transcription of rDNA, and the rearrangement into beaded nucleosomes followed the cessation of transcription after a short period. They also noted a similarity in appearance of the central fiber of genes transcribed at different rates. While the nature of the smooth fiber remains obscure (and is likely to have been affected by the preparative procedures), it seems likely that the active sequences are still associated with structural proteins. When rDNA was stained with phosphotungstic acid it exhibited a width of 73 ± 17 Å as opposed to 20Å for purified DNA.[508] In addition, Scheer and his colleagues observed that antibodies against histone H2B as well as those against RNA polymerase II specifically interfered with transcription of the lampbrush loops when injected into *Pleurodeles* oocytes.[513,514] The loops were visibly retracted, forming globular fibers as seen in the electron microscope. This may prove to be a sensitive assay for proteins in active chromatin. An extensive review of the early EM studies of active chromatin has been given by Mathis et al.[111]

More recently, altered conditions have been used in an attempt to preserve more of the native structure of the chromatin. Pruitt and Grainger report that by using various dilutions of a stock solution (70 m*M* NaCl/10 mm KCl/0.7 m*M* $MgCl_2$, pH 7.5) for dispersion, a continuum of chromatin unfolding could be seen.[515] On examining the ribosomal gene repeats (identified as uniform products of restriction digests) in stage two (pretranscription) and stage five (transcribing) nucleoli of *Xenopus* oocytes in low-salt conditions, they found that in both cases the coding sequences are in a relatively open conformation but display a normal nucleosomal morphology, while the nontranscribed spacer regions were highly (20×) compacted in both cases. It was clear that differential packaging of transcribed regions was established in the oocytes days or weeks prior to initiation of transcription. Greimers and Deltour also report a nucleosomal configura-

tion for transcribing nonribosomal genes in *Zea mays.*[516] In contrast, recent thin-section studies of algal and amphibian oocyte lampbrush chromosomes revealed nonbeaded loop axes measuring only 40 to 70 Å thick in the transcriptionally active chromatin.[517] In this procedure, either whole nuclei or isolated chromosomes were prepared and fixed under physiological salt conditions and supranucleosomal chromatin fibers were clearly preserved in inactive regions. The authors have cited evidence from immunofluorescence studies indicating the presence of histones on the loop axes, however, and maintain that an extended form of nucleosomal packaging persists even in these highly active genes. Andersson et al., also using thin-section techniques, have reported a similar "extended nucleofilament", 50 Å in. diameter, associated with the growing RNP particles of the Balbiani rings in *C. tentans.*[126]

Transcribing genes were inferred to be associated with histones from some of the earliest biochemical studies utilizing the nucleosome model. Axel and his colleagues showed by reassociation kinetics that DNA purified from micrococcal nuclease-generated 11S particles (mononucleosomes) contained essentially all sequences represented in the chicken and rat genomes.[252,518] The presence of actively transcribed sequences in such mononucleosomes was specifically demonstrated by hybridization with cDNA prepared from total polysomal poly A+ mRNA in rat liver, from globin mRNA in duck reticulocytes, and from ovalbumin mRNA in chicken oviduct. Virtually all investigators who have examined the question since have confirmed that DNA of active genes can be so recovered.

The first biochemical clue that the structural packaging of "competent" genes is altered was the finding of an increased sensitivity of the hemoglobin genes in chick red blood cells to DNase I.[109] While sequences encoding the globin genes were definitely present in 11S mononucleosome particles, it was equally clear that the majority of these sequences were rendered acid-soluble after an extent of digestion with DNase I which solubilized only 10% of the genome as a whole. This result was found only with nuclei obtained from red blood cells and was not observed with nuclei from fibroblasts, brain tissue, or even a population of red blood cell precursors. The authors did, however, find that the results were the same with both 5-day (actively transcribing) and 18-day (inactive) red blood cell nuclei. Concurrent work with the chick ovalbumin genes[518] also revealed such tissue-specific sensitivity. When oviduct nuclei were incubated with DNase I such that only 10% of the genomic DNA was rendered acid-soluble, roughly 70% of the ovalbumin-coding sequences were eliminated. When nuclei from chick liver were similarly treated, no preferential digestion of the ovalbumin gene occurred. In subsequent experiments the DNA from DNase I treated oviduct nuclei was hybridized with cDNA prepared from ovalbumin mRNA and from total cellular mRNA selected for low copy number, allowing an analysis of the structure of genes transcribed at widely varying rates.[519] Sequences encoding genes transcribed at a very low rate were digested with kinetics similar to ovalbumin, which is transcribed at a very high rate in these cells. These findings clearly suggested that the alteration of chromatin configuration detected by DNase I was not related to the rate of transcription of active genes, but rather to a property inherent to sequences which can be transcribed.

A novel application of the conventional "nick translation" reaction using *Esherichia coli* DNA polymerase I has also substantiated the generality of DNase I sensitivity.[520,521] When intact oviduct nuclei were incubated with very low concentrations of DNase I and the other reaction components, over 85% of the incorporated 3H-UTP was found in sequences which were transcribed in that tissue. Similar treatment of naked DNA resulted in uniform labeling of all sequences. When labeled nuclei were subsequently redigested with DNase I, the labeled sequences were liberated in acid-soluble form seven times faster than bulk DNA. When the nuclei were treated with micrococcal nuclease, it

was shown that the labeled sequences were recovered within nucleosomal structures.

Many investigators also looked for a preferential digestion of active genes by micrococcal nuclease and found none. In retrospect, this can be attributed to the mode of action of micrococcal nuclease which does not rapidly render DNA into acid-soluble form. More recently, a tendency for active genes to be preferentially cleaved into mononucleosome particles has been demonstrated. This effect is readily detected by Southern blot analysis, but not by reassociation kinetics. Bellard et al., having failed to find selective digestion of the ovalbumin gene in chick oviduct nuclei using cDNA hybridization, examined the DNA after separation on agarose gels.[522] Hybridization analysis of these "purified" fractions revealed that most of the ovalbumin sequences were located in the mononucleosomes and dinucleosomes, while the globin genes were underrepresented there. They also found, however, that in the nuclei of hen erythrocytes the two genes were cleaved at identical rates.

The latter results were clarified by Bloom and Anderson, who found that while the globin genes in both mature (inactive) and immature (active) chick red blood cells were more sensitive than bulk chromatin to DNase I, only in immature erythrocyte nuclei were the globin genes preferentially cleaved by micrococcal nuclease.[523] Using an approach similar to that of Bellard et al. (involving excision of four size classes of DNA fragments from agarose gels, followed by hybridization analysis), they showed that when oviduct nuclei from estrogen-treated or withdrawn chicks were mildly digested with micrococcal nuclease (to 1 to 3% acid-solubility of the DNA), the concentration of ovalbumin genes in mononucleosome length fragments was six times greater than in the $\geq$1300-bp size fraction. As the time after hormonal withdrawal increased, the selective cleavage of ovalbumin genes decreased; the change correlated temporally with the decrease in concentration of nuclear estrogen receptor and presumably with transcription. A similar correlation between accessibility of genes to the nuclease and rate of transcription had been found for the rRNA genes.[524] Hence it appeared that while DNase I could detect an altered chromatin state broadly associated with the potential for a gene to be expressed in a given tissue, the sensitivity of transcribed sequences to micrococcal nuclease was closely associated with their frequency of transcription.

The accessibility of the extra chromosomal ribosomal RNA genes of *Physarum polycephalum* to micrococcal nuclease was found to be similar to that described above.[525,526] The extrachromosomal palindrome containing the 19S and 26S ribosomal RNA genes was not preferentially degraded to acid-soluble products by micrococcal nuclease, but the coding sequences were more rapidly cut to monomer-sized fragments. In addition, an anomalous distribution of these sequences was found in agarose gels; large amounts of DNA had nonintegral nucleosomal repeat lengths.[526] Johnson et al. described a class of submononucleosomes named the "A" particles. The rDNA was preferentially digested to this form, which migrated more slowly in sucrose gradients and displayed an altered, more "extended" configuration in the electron microscope. The rDNA-containing A particles contained a different subset of nonhistone proteins from most mononucleosomes.[525,527,528] In the transcriptionally inactive micronucleus, however, no significant smearing of the nucleosomal ladder was evident, and no A particles were found.[526]

Direct examination of the nucleosomal pattern corresponding to a specific DNA sequence in both the active and inactive state has yielded additional information. The heat-shock genes of *Drosophila* may exhibit the most rapid and sizable induction of RNA synthesis known in the animal kingdom.[529] When *Drosophila* nuclei were mildly digested with micrococcal nuclease and the resulting DNA fragments displayed on agarose gels, no obvious disruption of the normal chromatin structure, visualized in terms of the nucleosomal ladder, could be detected as a consequence of heat shock. When

the DNA was transferred to nitrocellulose and probed with cloned heat-shock gene sequences, however, it was seen that in addition to becoming more sensitive to digestion upon activation, the genes also exhibited a highly smeared set of digestion products with little or no discrete monomer or oligomer length DNA. When cells were permitted to recover from heat shock prior to digestion so that heat-shock RNA was no longer being synthesized at the time of assay, a normal nucleosomal pattern was regained.[110] At this point, it was clear that micrococcal nuclease revealed an altered nucleosomal configuration associated exclusively and transiently with actively transcribing genes. It should be noted that to date most detailed studies on the chromatin of specific active genes have examined genes transcribed at a high rate which could well differ in some aspects from genes normally expressed at low levels. In similar study of an active thymidine kinase gene from herpes simplex virus in mouse cells, an oligonucleosome pattern was detected; however, this was less distinct than the oligonucleosome pattern for the genome as a whole.[530]

The foregoing results in sum have clearly shown active genes to possess an altered structure which allows greater accessibility of the DNA to at least some enzymes. In the past it seemed reasonable that exogenous bacterial RNA polymerase might serve as a probe of chromatin structure, and numerous examples of experiments using this approach appeared in the early literature. However, it has been demonstrated that hormonal administration does not affect the ability of *E. coli* RNA polymerase to bind or specifically transcribe the ovalbumin gene at a time when transcription by endogenous RNA polymerase has increased 30-fold.[531] After high levels of a specific mRNA have accumulated, artifactual transcription from this RNA template can give the appearance of specific transcription.[532,533] Due to findings such as these, *E. coli* polymerase is not much utilized at present in studies of chromatin template activity. Useful information has been derived from studies employing other probes, e.g., the "limited but exact" method of analysis afforded by restriction enzymes,[284] as discussed in Section III.B.

The nuclease probes of chromatin structure have perhaps been most intensively and successfully applied to the problem of hemoglobin induction, in both erythropoietic tissues and cultured cells. Weintraub and his collaborators have now suggested a timeline for the events involved in globin gene activation during erythropoiesis in the chicken embryo,[220,221,534,535] which is given here in brief. Erythroid differentiation is detectable at about 35 hr of development. While the primitive erythroblasts (2- to 5 days-old) make only embryonic β-globin, both the embryonic and adult genes are DNase I-sensitive, as shown using both cDNA and genomic clones of the β-globin genes. In fact, a very large region, perhaps 50 to 100 kb in extent, has actually become sensitive at this point, in keeping with the concept of a genome organized into extensive functional domains. It was found that both of the β-globin genes were digested at the same rate as assayed by blot hybridization, which measures the susceptibility of the first cutting site within a given fragment. However, the embryonic gene was clearly the more sensitive of the two as assayed by solution hybridization, which monitors the overall conversion of a sequence into acid-soluble fragments. Moreover, a DNase I-hypersensitive site was detected only at the 5′ end of the embryonic gene in these cells. In no case were the globin genes observed to be more sensitive than bulk chromatin in nonerythropoetic tissues such as brain during this time period. At 6 days of embryonic development, adult erythroid cell lines have formed and the embryonic gene was found to have a moderate level of sensitivity to DNase I (but above that of inactive genes) while the adult gene had become the more sensitive. In these cells a DNase I-hypersensitive site was observed for the adult, but not the embryonic gene. Bellard et al., who also have described several levels of DNase I sensitivity, also found that the most sensitive sequences were those currently being transcribed.[536]

Detailed analysis of the sensitive transcribing regions of the α-globin locus has

revealed a striking correlation, exact to within a few hundred base pairs of DNA, between the active, most sensitive subdomain (i.e., a 5 kb stretch containing the adult α-globin genes in adult erythrocytes) and both sequences which are undermethylated at CCGG and associated with RNA polymerase II. In definitive erythrocytes, the embryonic β-globin gene (which is not transcribed) becomes methylated, loses its DNase I-hypersensitive site, no longer binds polymerase, loses its high affinity HMG protein-binding sites, and becomes less sensitive to DNase I than the adult gene.[220,221,534,535]

While more is now being learned about the mechanics of globin gene induction, the identity of the factors responsible for initiating the necessary sequential changes in chromatin structure remain obscure. Studies of hormonally regulated genes may provide a tractable system for isolation of factors responsible for induction of gene activity since the agent for one level of control is already known and is often readily available. The synthesis of vitellogenin, the precursor of the major yolk proteins, is controlled by estrogen in the livers of oviparous vertebrates by means of regulation of the level of vitellogenin mRNA synthesis. Administration of estrogen will induce vitellogenin synthesis in male *Xenopus,* which normally produce no transcripts for this gene at all.[537] The vitellogenin gene exhibited the same baseline sensitivity to DNase I as the β-globin gene in male livers. After estrogen administration, the gene quickly became twofold more sensitive to DNase I digestion in this tissue. No change occurred in the vitellogenin gene in erythrocytes of stimulated animals, nor was there any change in the globin or ovalbumin genes in the liver of stimulated animals. The reversibility of structural changes involved in gene activation has been explored using chick oviduct by withdrawing estrogen; after 3 days 1000-fold less ovalbumin mRNA was found and the concentration of estrogen receptors had fallen 20-fold, but the gene was still DNase I-sensitive and capable of 98% of full response on reexposure to hormone.[538] The hormonal inhibitor tamoxifen also had no effect on the DNase I sensitivity of the gene.[539] It will be of interest to investigate the extent of the changes in chromatin structure caused by hormone binding, the localization of the hormone-receptor complex on the chromatin fiber, and the factors required for sequence-specific binding of the complex.

It should be observed that the domain organization apparent in large eukaryotic chromosomes may not be universal, particularly in simple eukaryotes. The finding that yeast chromatin is uniformly digested by DNase I as assayed by solution hybridization[540] may simply reflect the fact that at least 40% of the genome of *Saccharomyces cerevisiae* is transcribed. Neither heterochromatization nor mitotic condensation have been observed in yeast. During the transition from growing to stationary phase, when transcription falls by 30-fold, the entire genome becomes less DNase I-sensitive. This implies that this developmentally simple organism does not utilize the genomic compartmentalization evident in higher eukaryotes as differential DNase I sensitivity, or that all compartments are maintained in the same state at a given time in the cell cycle. Another case of this type is the SV40 minichromosome, which maintains its minichromosomal structure (as determined by sedimentation, density, and EM studies) during transcription, when the entire E strand is transcribed and the whole virus becomes DNase I-sensitive.[541,542]

Several studies have been carried out on the conformation of viral sequences in vivo, but many are complicated by the presence of multiple copies and/or incomplete copies of the viral genome.[543-548] This can sometimes be dealt with; it seemed clear, for example, that one of the two integrated RAV-O retrovirus genomes of chick embryo fibroblasts was DNase I-sensitive.[545] Upon superinfection, one copy was integrated and it was completely sensitive, while the configuration of the two original copies was maintained. It would be of interest to determine whether the viral integration process selects for "active" domains or whether the integrating virus is capable of "opening" a chromosomal region and if so, how extensive this region might be.

The fact that some viruses may have evolved by incorporating bits of genes from

eukaryotic hosts does not require that the derivative viruses will share regulatory signals in common with their hosts. When thymidine kinase (TK) genes from a variety of sources (including whole human metaphase chromosomes) were used to transform cells, the viral gene was constitutively expressed while all others were regulated as in normal TK^+ cells. Furthermore these findings persisted whether the transgene was stably integrated or persisted in an unstable episomal state.[549] It was recently found that when mouse L-cells were TK-cotransformed with plasmids containing a cloned *Drosophila* heat-shock gene, cell lines arose which either repressed the integrated gene entirely or permitted transcription only under conditions which induced the native heat-shock genes of mouse.[550] In this experiment the input DNA included 1.1 kb of 5′ flanking sequence, extending significantly beyond the normal position of the DNase I-hypersensitive site associated with this gene. Investigation of the nuclease sensitivities of the integrated and adjacent genomic sequences in these cell lines may provide valuable insights into the causality of gene activation.

The possibility of detecting various levels of compaction within inactive regions of chromatin is raised by a mathematical modeling study of the rates of disappearance of genomic restriction fragments after DNase I digestion.[551] The globin, albumin, and vitellogenin genes of *Xenopus* were found to exhibit slight differences in sensitivity as naked DNA and nonparallel differences as packaged, inactive genes; the globin gene, while most sensitive as naked DNA, was threefold less sensitive in hepatocyte chromatin than the others were in erythrocyte chromatin (where both were inactive). It is quite possible that multiple levels of tertiary compaction may subdivide the nonexpressed sequences of higher eukaryotes, even as the expressed genes exhibit multiple levels of organization, each of which offers another possibility for control.

It is evident that the chromatin structures responsible for maintaining the active state of an expressed gene can be faithfully perpetuated during DNA replication. Weintraub has obtained evidence for the very rapid assembly of an active chromatin structure during replication.[552] Using ^{3}H-thymidine pulse-labeling of transformed chicken leukemia cells followed by incubation of their nuclei with micrococcal nuclease or DNase I, he found that active genes were assembled into nucleosomes within 2 kb (that is, within 1 min) of the replication fork. These nucleosomes had already assumed a DNase I-sensitive configuration within 4 kb of the fork. Experiments with inhibitors of protein synthesis showed that the appropriate configuration could be assembled subsequent to replication, if necessary. McKnight and Miller, in an EM study of the ultrastructure of newly replicated chromatin in *Drosophila* embryos also found a beaded morphology indistinguishable from normal nucleosomal chromatin very near the replication forks.[553] Cell division is a logical point for changes in gene expression, and there is now evidence from several systems, including chick erythropoietic tissue, implicating chromatin replication in a mechanism for gene activation.[535] It should be borne in mind, however, that in the cases of the vitellogenin and heat-shock genes mentioned, gene induction was quickly achieved without prior replication. The existence of multiple pathways serving different purposes for gene induction in eukaryotic cells is quite probable, although it seems likely that many structural features are common to all active genes.

B. Changes in Macromolecules

It is clear from the preceding discussion that profound changes occur in chromatin structure in relation to gene activation and transcription. Some of these apparently precede transcription and therefore are possible regulatory events. However, in order to identify and characterize these events in a convincing way, it will first be necessary to locate them in time and space. An understanding of the mechanisms involved in establishing and maintaining an active gene in general will be very useful. One would like

to know what alterations occur in the DNA, histones, and NHC proteins of a locus in the process of activation, including rearrangements of material and modification of the previously present macromolecules.

1. DNA

The constancy of the genome at the level of DNA sequence is a basic tenet of molecular genetics. Nonetheless, an increasing number of exceptions are being recognized involving amplification and rearrangement of sequences. Amplification of a gene expressed specifically in a terminally differentiated cell is attractive as a means of achieving high levels of specific RNA synthesis. In most cases tested to date, such specific differential amplification does not occur (e.g., globin genes in the mouse reticulocyte;[554] ovalbumin gene in the chick oviduct;[555] silk fibroin gene in *B. mori;*[556] vitellogenin gene in avian liver[557]). One exception observed is the amplification of the genes for two (or more) closely linked chorion proteins in the follicular cells of the egg chamber of *D. melanogaster.*[558,559] These genes are amplified about 16-fold; DNA on both sides of the locus is amplified to a lesser degree. In all, the region affected is 80 to 100 kb, a region that covers several bands of the polytene chromosomes.[560]

Rearrangement does not appear to be a common event during development. Even in the case of a single gene which is regulated differently in different tissues, constancy of the DNA sequence has been reported. For example, the chicken transferin gene is present in the same copy number (one per haploid genome) and in an identical sequence conformation (to the level of restriction analysis) in liver, where it is inducible by decreased iron level, oviduct, where it is under hormonal control, and erythrocyte, where it is always inactive.[561] Exceptions include the rearrangement of sequences observed for selection of mating type in yeast[562] and for selection of flagellin in *Salmonella;*[563–565] one suspects that other cases of this type will be found in the unicellular eukaryotes. The evidence for transposable elements in yeast[566] and *Drosophila*[567] has suggested that cases may be identified in higher eukaryotes where changes in DNA sequence arrangement alter gene expression, as observed for corn (reviewed in Reference 568). Generally, these examples involve a change in position of a promoter or other regulatory sequence with respect to the mRNA-encoding sequence. However, a recent study of the immunoglobulin genes suggests that rearrangement can critically affect the chromatin structure of a gene as well. Transposition of a variable region gene to a position 5′ to the constant region gene is necessary for expression of both immunoglobin light and heavy chains. The constant region gene for kappa light chains is observed to be DNase I-sensitive both in nonimmune (B-cell lymphoma) cells and in immunocompetent cells. However, the variable region gene (which includes the promoter) is DNase I-sensitive only after the rearrangement event.[569] It is tempting to interpret these findings as indicating a gene activation process involving movement of a promoter and coding region from a closed to an open domain. In this context it will be interesting to determine the chromatin structure state of expressed and nonexpressed genes inserted into the genome by transformation (see Section V.A).

The possibility that modification of the bases in DNA can be utilized in the regulation of gene expression in eukaryotes is currently a subject of intense interest. Recent findings will be summarized here; see References 570 and 571 for a detailed review. The DNA of plants and of some lower animals is very highly modified, which complicates analysis. In vertebrates, however, 5-methylcytosine (m^5Cyt) is the predominant modified base. The amount of m^5Cyt in animal genomes varies widely, from several per cent of the cytosine in many vertebrates to very low amounts (<1%) in arthropods.[161] This wide variation in amount of base modification makes it unlikely that there is a single use for m^5Cyt across the evolutionary spectrum.

One role of base modification is suggested by the tissue-specific methylation patterns in the DNA of vertebrates as shown by analysis using pairs of restriction enzymes that are or are not sensitive to the presence of m^5Cyt in the same restriction site.[572] In particular, a lack of m^5Cyt at some, but not necessarily all sites in or around a highly active gene in a terminally differentiated cell has been reported (chick β-globin gene;[573] human globin genes;[574] mouse J-chain immunoglobin genes;[575] chick ovalbumin gene;[576] chick lens δ-crystallin genes[577]). A general correlation between m^5Cyt and nuclease-resistant chromatin has been observed.[578] Working with several vertebrate tissues, Naveh-Many and Cedar reported that while 70% of the CpG in DNA is methylated, only 30 to 40% of the CpG in DNase I-sensitive chromatin sequences is methylated, and only 20 to 30% of the CpG in DNA complementary to mRNA is methylated.[579] Specific correlations of DNase I sensitivity and loss of m^5Cyt have been reported for the chick ovalbumin[580] and hemoglobin genes.[221]

There is, in addition, experimental evidence that undermethylation of DNA affects the transcription of certain genes. For example, ethionine, an effective inducer of globin gene expression in Friend cells, does cause undermethylation of the DNA.[581,582] More definitive results have been obtained by Taylor and Jones, who have shown that 5-azacytidine and other analogues which are incorporated into DNA but because of their structure block methylation, will cause the differentiation of a mouse embryo cell line into muscle cells, adipocytes, and chondrocytes.[583-585] A correlation between hypomethylation of the DNA and transcriptional activation of an endogenous retrovirus using 5 azacytidine has also been reported.[586] The metallothionein-1 gene in W7 mouse thymoma cells is not expressed; however, after growth in 5-azacytidine, the gene is inducible. The effect is blocked by inhibition of DNA synthesis. The DNA of the inducible derivative lines is not methylated at this locus.[587]

One might hypothesize that genes expressed as a part of terminal differentiation must not be methylated. However, the correlation is not a simple one, since some sites within active genes remain methylated and some sites outside the genes lose their methyl groups. Sites within particular genes have been observed to be unmethylated in some tissues in which the gene is not transcribed.[574] The correlation between a lack of m^5Cyt and transcription cannot be a direct one.

The generality of such a role for DNA methylation can also be questioned on other grounds. Some organisms do not have m^5Cyt in their DNA; for those that do, much of the pattern is invariant over development.[588-590] One might suggest that the hypomethylation of DNA of very active genes is a consequence of the chromatin structure of the active state (e.g., involving shielding of the DNA from methylating enzymes) rather than a cause of the chromatin structural transition as has been inferred. One can, nonetheless, speculate on mechanisms by which the presence of m^5Cyt might affect chromatin structure. Methylation of DNA does have a destabilizing effect, but this is generally not sufficient to favor open structures.[591] Recent work, described in Section II, does however show that m^5Cyt can favor the transition to an alternative DNA structure such as Z-form DNA.[158] Obviously either the presence of the methyl group as such or a switch to an alternative DNA helix could cause a change in the binding affinity for a particular protein. It will be of interest to learn whether or not the histone-DNA interaction is perturbed in regions where the DNA is wound in other than the B-form. It should of course be remembered that arthropods have the same sort of DNA and histones as do vertebrates; gene activation appears to involve similar mechanisms as detected by changes in DNase I sensitivity and micrococcal nuclease sensitivity of the chromatin. The lack of m^5Cyt in these organisms poses a puzzle.

2. Histones

The histones, while among the most highly conserved proteins, are also among the

most readily modified proteins. Among the unusual amino acids observed in histones are ϵ-*N*-methylysine in the mono-, di-, and trimethyl forms, ω-*N*-methylarginine, 3-methylhistidine, α-*N*-acetylserine, ϵ-*N*-acetyllysine, *O*-phosphoserine, *O*-phosphothreonine, *N*-phospholysine, and *N*-phosphohistidine. Poly-(ADP)-ribosylation can occur at glutamic acid residues. Here only those cases where there is reasonable circumstantial evidence that the modification is part of the mechanism of gene activation will be reviewed. A more detailed discussion covering the earlier literature has been presented by Allfrey.[114]

The core histones are modified by acetylation of the ϵ-amino groups of lysine residues in the *N*-terminal third of the protein. Up to four sites are available in histones H3 and H4. Histones are acetylated by a number of enzymes, some of which are general and others of which show different affinities for each histone or for histones free in solution as compared to those in association with DNA in a nucleosome (e.g., see Reference 592). Histone acetylation in vivo is rapid and reversible, independent of histone synthesis or degradation. All histones can be acetylated in vivo; however almost 20% of the H4 of a rat hepatoma cell line remains unacetylated, regardless of treatment, suggesting that their accessibility is not uniform.[593] Two populations of histones have been recognized by their kinetics of acetylation.[594,595]

There is considerable circumstantial evidence linking histone acetylation with gene activation. Increases in histone acetylation are observed at times of increased transcriptional activity, such as at gastrulation, or following inductive stimuli. The rate of histone acetylation for the highly active SV40 minichromosome is about four times higher than for the host cell chromatin.[596] Yeast chromatin also has particularly high levels of acetylated H3 and H4.[597] In contrast, there is little acetylation of H4 in the transcriptionally inactive chick erythrocyte chromatin.[598] Chromatin fractionation experiments, utilizing DNase II digestion or micrococcal nuclease digestion to preferentially release active chromatin, indicate that the histones in these fractions are highly acetylated.[599,600] A careful study of H4 acetylation as a function of the cell cycle of *Physarum* has suggested that it is the presence of the tetraacetylated form which is correlated with transcription.[601]

Riggs et al. discovered that treatment of cells with millimolar amounts of *n*-butyrate led to greatly enhanced levels of acetylation of histones H3 and H4.[602] This finding was greeted with tremendous enthusiasm, and it was soon convincingly demonstrated that the effect was due to an inhibition of histone deacetylase activity by *n*-butyrate.[603-606] Observations on several tissue culture lines suggest that the cells enter an extended G_1 state, with concomitant increases in HI^o.[411,604]

A correlation has been found between an increase in histone acetylation and an increase in nuclease sensitivity of the chromatin.[607,608] The pattern of nuclease sensitivity mimics that seen for active genes described in Section V.A. The associated sequences are five- to tenfold more sensitive to DNase I, and are more rapidly degraded to small oligonucleosomes (but not acid soluble fragments) by micrococcal nuclease.[609-613] Evidence for a direct role in transcription is more difficult to obtain, given the pleiotropic effects of butyrate on cells. Butyrate can both induce new transcription[614] and block induction of new transcription by hormones.[615] The rate of in vitro transcription from nuclei is the same for normal material and that containing histones hyperacetylated in vivo.[616,617] A direct analysis can be carried out using as a template SV40 DNA (form I) complexed with histone octamers from normal or butyrate treated cells. The latter complex is relatively DNase I-sensitive. Using this approach it is possible to show that highly acetylated core histones can inhibit transcription by *E. coli* RNA polymerase and calf thymus RNA polymerases I and II as efficiently as the normal core histones.[610]

It is obvious that the acetylation of the *N*-terminal portions of the histones will neutralize positive charges and weaken the electrostatic interactions between these histone tails and DNA. Note, however, that histone octamers with the *N*-terminal

portions removed are capable of folding DNA.[618] In studies of mononucleosomes, changes in the interhistone contacts are observed,[619] as well as a loosening of the histone-DNA contacts somewhat similar to those reported when the histone tails are removed by proteolysis.[620] It is attractive to consider the effects on higher order structure in the context of the model for the 300-Å fiber stabilized by the association of the histone tails with the DNA of the adjacent nucleosome (see Section II.B). This model implies that histone acetylation will lead to a loss of nucleosome-nucleosome interaction, destabilizing the coiled structure. Indeed, a correlation between histone acetylation and the susceptibility of the linker region to DNase I is observed.[621] An analysis of the binding interactions between the normal or acetylated histone core complex and SV40 DNA found no significant difference between the association constants, but a large reduction in the cooperativity of binding for the aceylated histone cores.[622] It is reasonable to conclude that histone acetylation is a necessary but not sufficient change for gene activation, most probably having an effect on nucleosome-nucleosome interaction and hence on the higher order structure of the chromatin fiber.

In addition to the preceding, phosphorylation of histone 1 has been implicated in gene activation. Histone 1 is generally present in two to five subtypes differing from one another in the *N*-terminal region of the molecule.[623] Site-, subtype-, and enzyme-specific phosphorylation of H1 can occur in response to hormone stimulation.[624-626] Sites involved in phosphorylation in response to hormone stimulation are different from those involved in cell cycle modulated modification.[111] Phosphorylation of H1 has been shown to decrease its affinity for DNA.[627] Unfortunately, the complexity of the system and of the response has not as yet permitted any direct analysis of the role of H1 phosphorylation in gene activation. It has been suggested several times that H1 has been removed from active loci (and this is attractive in terms of models of unfolding the chromatin fiber), but as yet the issue is unresolved.[111]

3. *The NHC Proteins*

It is clear that a number of NHC proteins will be involved in establishing an active gene. Observations to date suggest that these can be classified either according to their distribution relative to the transcriptional sites, according to their time of action in the transcriptional process, or according to whether their role is enzymatic or structural. The evidence for structural NHC proteins involved in altering and/or maintaining an active chromatin configuration will be examined here. Unfortunately, it has not been easy to develop useful assays for the identification and analysis of such proteins. A straightforward approach might be to develop an in vitro transcription system using defined components which operate with the same selective efficiency as the in vivo system. As a result of considerable effort, in vitro transcription systems have been established using eukaryotic DNA as a template and eukaryotic RNA polymerases (with additional factors) as enzymes.[628-630] To date these transcription systems fail to show evidence of regulation, most being completely promiscuous. For example, the embryonic, fetal, juvenile, and adult β-globin genes of goat, regulated in vivo, are all transcribed to the same extent in vitro.[631] If a promoter is present in the DNA, it will be used. This problem may be attributed in part to the template; naked DNA does not exist in the nucleus. So few of the important characteristics of the template are known as yet; the required size, winding number, degree of folding, associated macromolecules, etc. remain obscure.

Because of these difficulties, it has been necessary to use other, less direct assays to identify the proteins involved in gene activity. The technique of immunofluorescence has been used to identify proteins which are associated with the developmentally active class of genes on the polytene chromosomes. Preferential digestion of active genes has been

used in attempts to identify associated proteins. Finally, extractions of chromatin with salt washes have been used to remove specific proteins. DNase I sensitivity has frequently been used to identify the "active" template, although, as discussed in Section V.A, this condition is probably necessary, but not sufficient, for gene activity.

Immunofluorescence studies of the distribution patterns of NHC proteins on polytene chromosomes have indicated that there are several proteins ("class c") which are preferentially associated with active and potentially active loci, as discussed in Section IV.B. The distribution pattern of these proteins is analogous to what is believed to be the pattern of broad DNase I sensitivity, which involves large areas of the genome, perhaps chromomeres, and is characteristic of loci which have been, are, or can be active in the cell type under study. Only one group of genes, those inducible by heat shock, have been studied by both techniques. In this case, both DNase I sensitivity and association with class c proteins are correlated with activation, and are reversible (see References 110 and 700). The bulk of the data on DNase I sensitivity of specific genes involves genes expressed at high levels in terminally differentiated cells. More work is needed on other types of loci. It would be of interest to obtain an overall pattern of DNase I sensitivity in the polytene chromosomes of the *Drosophila* salivary gland. Use of monoclonal antibodies has established that the distribution pattern under discussion is the property of individual proteins, but little is known about these proteins because it has been difficult to isolate sufficient amounts for full characterization. However, it should be possible to clone the genes for such proteins, allowing more extensive study. The major objection to the immunofluorescent technique is that it is applicable only to organisms with large, meticulously characterized polytene chromosomes such as seen in *Drosophila.*

Chromatin fractionation has been used to differentiate between proteins associated with active and inactive genes. Unfortunately even the best fractionations give only enrichments, not purification, of active chromatin. The possibility of protein rearrangement and aggregation is also a major drawback.[489,491] One approach to fractionation is to digest chromatin with nucleases and to select nucleosomes either on the basis of the kinetics of release or on the basis of solubility in salt.[488,632] Such rapidly released and/or soluble nucleosomes are generally reported to be enriched in transcribed DNA sequences and in HMG 14 and 17 as well as in a variety of other uncharacterized NHC proteins;[384,633,634] occasionally HMG 1 and 2 are also reported.[385,387] In contrast, Mathew et al. have reported the presence of HMG 14 and 17 in chromatin fractions enriched for satellite DNA.[635] Nucleosomes have also been separated electrophoretically, and the distribution of various DNA sequences in nucleosomes and subnucleosomal particles have been identified by Southern blotting.[636,637,407] Using this approach, the HMG proteins are found in association with a wide range of DNA sequences including inactive, highly repeated DNA.[407] Of course, it is not certain that in a limited number of fractions, all nucleosomes that segregate together in fact share all of the same characteristics.

The best evidence for a role of HMG 14 and 17 in gene activation comes from salt extraction experiments. As first noted by Weisbrod and Weintraub[638] and confirmed by Gazit et al.,[639] 0.35 *M* salt will extract a subclass of nuclear proteins apparently responsible for maintaining the broad DNase I-sensitive configuration in chick erythrocyte chromatin. Reconstitution of the depleted chromatin with either the whole extract or with purified HMG 14 and 17 will restore its DNase I sensitivity. Furthermore, reconstitution using an extract from erythrocyte nuclei with extracted brain chromatin has no effect on the sensitivity of the globin gene, while reconstitution using an extract from brain nuclei with depleted erythrocyte chromatin fully restores the sensitivity of the globin genes. The lack of tissue specificity of the HMG proteins implicates them as

general modulators of gene activity. Work from several laboratories has established that there are two binding sites for HMG 14 and 17 per nucleosome;[395,396] the two proteins appear to be very similar and either alone can achieve the effect.[640] Sandeen and her colleagues have confirmed preferential binding of HMG 14 and 17 to active genes in stripped chromatin, while pointing out that with further addition of protein all nucleosomes will bind these HMGs.[395] This work has been extended to the use of immobilized HMG 14 and 17 to isolate active nucleosomes by a type of affinity chromatography.[495]

There remain many questions concerning what is necessary and sufficient to generate and maintain a DNase I-sensitive chromatin structure. Chambon and his colleagues were able to reconstitute a DNase I-sensitive chromatin with SV40 DNA and highly acetylated histone cores.[610,641] Weintraub and his colleagues, however, have manipulated this parameter using 0.35 *M* NaCl, which does not extract histones.[638] One hypothesis which reconciles these observations is the idea that HMG 14 and 17 are inhibitors of histone deacetylase, and that it is the maintenance of the acetylated histone core that is critical.[642,643] Senear and Palmiter working with the active chick ovalbumin gene report that although a shift in DNase I sensitivity occurs when chromatin is washed with 0.35 *M* NaCl, oligonucleosomes retain a differential sensitivity to DNase I even after washing with 0.6 *M* NaCl, which removes H1 and much of the NHC protein, including HMGs.[644] It is often difficult to reconcile different data because the DNase I sensitivity has been presented in relative terms with little quantitization. However, the most general hypothesis would be that both histone modification and the association of HMG 14 and 17 are required to generate the DNase I-sensitive state associated with enhanced transcription in vivo.

C. Boundaries of Change

One method of correlating particular structural changes in chromatin with steps in the process of gene activation is to map the boundaries of these changes on the DNA map of the locus. Recombinant DNA techniques have enabled the construction of a fairly detailed map of several regions of transcription and the surrounding few kilobases of DNA. Investigations of in vitro transcription have demonstrated that the eukaryotic RNA polymerases are capable of recognizing and utilizing precisely a promoter as defined solely by its DNA sequence.[628,630,646–648] Correct initiation by RNA polymerase in vitro generally appears to require a TATA or Goldberg-Hogness sequence 25 to 30 bp upstream from the initiation site.[649–651] In studies aimed at evaluating the significance of the TATA box in vivo it was observed that precise initiation required the presence of the sequence, but that its deletion merely served to produce RNA with heterogenous 5′ ends and did not abolish transcription.[652,653] Deletions ranging from one hundred to several hundred base pairs upstream of the initiation site (leaving the TATA box intact) virtually abolished transcription altogether.[650,653,654] An upstream control region is also required for successful transformation with the TK gene.[655] Sequences downstream from the initiation site (within the transcribed region) are a critical part of the promoter for RNA polymerase III,[239,240,656] and apparently can have an effect on polymerase I.[657] Sites of termination of transcription have been determined for the 5S RNA,[658] but there is some controversy concerning genes transcribed by RNA polymerase II. While in one case evidence has been obtained that the site of termination of transcription is the site of poly A addition seen in the final mRNA (e.g., for ovalbumin[659,660]), Hofer and Darnell have reported transcription in a nuclear system at the mouse β-globin gene extending at least 1 kb downstream from this site.[661] They suggest a model of continuing transcription with specific RNA cleavage at the poly A addition site. In another case, multiple termination sites have been reported.[662,663] However, the sum of the data indicates that RNA

polymerase interacts specifically with the DNA encoding the RNA transcript plus perhaps 1 to 2 kb of DNA on either side. This defines a primary domain of interest for chromatin structure mapping, apparently one with fairly well-defined boundaries.

One must also consider the question of a significantly larger domain in gene activation. Classical genetic techniques have estimated the size of a gene as tens of kilobases. The notion of distinct large chromosomal domains with fixed boundaries developed from the description by Bridges of a reproducible pattern of about five thousand distinctive bands in fixed polytene chromosomes of *Drosophila*.[664] The observation of distinctive puffing patterns temporally correlated with developmental gene activity[442] and biochemical evidence confirming the physical association between this phenomenon and transcriptional activity in *Diptera*[147] increased interest in the system. While the old concept of "one chromomere, one gene" now appears simplistic, the idea of a chromomere as a functional domain for gene activation still seems reasonable. The observations of specific protein distribution patterns which can be correlated with the band patterns reinforce this idea. Overall, a two-level system of transcription control has emerged from these studies: first, the immediately transcribed region with associated promoter sequences and second, a large domain, perceived both genetically and cytologically.

However, there is little information on how the boundary of a chromomere is established and what its genetic significance might be. Some pieces of information lead one to question the importance of chromomeres in this regard. Large puffs apparently disrupt the structure of adjacent chromomeres. Position effects, which are suspected to involve chromatin structure, occur over several chromomeres. While one can suggest that each chromomere will have boundaries, it seems likely that at least part of the boundary function will be general; deletion and rearrangement mutants with break points such that the normal boundary is eliminated can in certain cases still be properly activated (e.g., Reference 665). Correlation of results from genetics, cytology, and molecular biology should help solve this puzzle. For example, the combined work of McGinnis et al.[152] and Muskavitch and Hogness[151] has defined 16 to 19 kb of DNA necessary for the proper function of the glue protein 4 gene in vivo, and placed the boundaries on the DNA map. The unit of transcription is approximately 0.95 kb. Several regions of interest in the *Drosophila* genome have now been cloned and the DNA mapped over several chromomeres. Thus the DNA of chromomere boundaries is now available; perhaps such boundaries can be recognized by mapping of chromatin structure. If an appropriate region could be identified, one could search the DNA sequence for portions that might promote alternative DNA helix forms and thus alter the ability of the DNA to fold into nucleosomes. It is observed, for example, that poly(dA), poly(dT), with circa 10 bp per turn, does not form nucleosomes;[666,667] A + T-rich DNA sequences occur at 10 to 100 kb intervals in the DNA of several eukaryotes.[668]

A similar two-tiered pattern of structure has been suggested by nuclease sensitivity studies of chromatin. Analysis of chick globin system has indicated that a small domain, on the order of a few kilobases and closely associated with the gene in question, is characterized by increased sensitivity to micrococcal nuclease and a high degree of sensitivity to DNase 1.[220,221] Beyond this is a relatively large domain, distinguished by its moderate sensitivity to DNase I (still above the level of bulk chromatin), extends over a region covering tens of kilobases. Using the affinity chromatography method discussed in Section IV.B, Weintraub et al. have provided evidence that it is approximately the region of transcription itself that is also characterized by hypomethylation of the DNA and by association of HMG 14 and 17 with the nucleosomes in the chick red blood cell system.[221,535] For example, the region of structural difference at the α globin locus in adult cells is presented as including both the α^D and α^A transcripts, the region in between these genes and up to 1.5 kb to the 3′ side. The structural alterations detected are thought

to be analogous to those detected as a smearing of the nucleosome pattern. The finding that the region of disruption for the 70,000 dalton heat-shock gene at 87A extends for several kilobases beyond the 3′ end of the stable mRNA map position[701] raises the question of where the boundries of perturbation occur in relation to transcription. Such boundaries appear to be fairly sharp, perhaps within the transition from one nucleosome to the next.[221,231]

That the domain of general DNase I sensitivity is quite large (tens of kilobases) is also supported by work on the ovalbumin gene. Lawson et al.[669] and Bellard et al.[536] have reported that in this case, the DNase I-sensitive domain contains at least three genes (ovalbumin, X, and Y) and extends over at least 54 kb of DNA. Bellard et al. also reported that a minimum of 25 kb could be distinguished by its sensitivity to micrococcal nuclease. No mapping of this type has as yet been completed in *Drosophila*, where it may be possible to correlate the genetic unit with the unit of perturbation. One would like to know to what extent these observations will prove to be general, in particular with regard to constitutive genes. It is possible that only some alterations are necessary to make a gene accessible to transcription, while others are specifically required for high-level transcription. How to distinguish when a gene is "off", when it is "on" only by virtue of read-through transcription (noise), and when it is "on" at a low level for biological purposes[670] remains a vexing problem. Some understanding may be gained by analysis of different regions of the genome thought to be in these categories using the appropriate cloned DNA. One would like to know how the results discussed previously for genes transcribed at high levels compare with results for genes for necessary enzymes expressed at the level of 5 to 40 mRNA copies per cell.

It is proving more difficult to try to establish the temporal sequence for chromatin structure alteration in relation to gene activation. There is evidence that at least two events occur prior to transcription. As discussed previously, a DNase I-hypersensitive site at or near the 5′ end of the gene appears to be necessary, but not sufficient for transcription. Such sites are present at the heat-shock genes of *Drosophila*[212,214] at times when these genes are inactive but inducible.[671,701] While these sites are not observed in very early precursor cells, Weintraub[702] has isolated by transformation a chick erythrocyte precursor cell in which globin is not transcribed, but a DNase I-hypersensitive site for the embryonic gene is observed.

The broad DNase I sensitivity of the active chromatin also appears to be independent of the transcriptional event per se. Both the chick globin genes[109] and ovalbumin gene[539] in the terminally differentiated cells are sensitive to DNase I at times when the gene is no longer actually being transcribed. Chambon and his colleagues have found RNA polymerase in association with such globin genes and, therefore, suggest its necessity.[672] However, Weintraub does observe DNase I sensitivity for newly replicated genes of the class transcribed at a low level, implying that passage by the polymerase cannot be required.[552] Further, Storb et al. found that the immunoglobin constant region kappa gene is DNase I-sensitive prior to the rearrangement that brings the promoter on the variable region kappa gene into place.[569] Thus the broad DNase I sensitivity must be a property of the chromatin which can precede transcription. The immunofluorescence pattern of the chromosomal proteins identified as ρ, band 2, or clone 28 suggests that these proteins might be involved in maintaining the DNase I-sensitive structure. However, direct evidence is not yet available. The pattern suggests that all genes inducible as part of the developmentally regulated program in the terminally differentiated salivary gland cell have already adopted this configuration. However, it should be noted that in male *Xenopus* liver the vitellogenin gene is not DNase I-sensitive prior to its rapid induction by estrogen;[537] *Drosophila* heat-shock genes are also insensitive prior to induction.

D. Transcription of Chromatin

In conclusion an attempt will be made to summarize the requirements for an active template in the eukaryotic nucleus. Observations on DNA sequences injected into *Xenopus* oocytes have offered two important insights. First, the template must be double-stranded DNA. If single-stranded DNA is injected, it is converted to the double-stranded form prior to transcription.[673] Closed or nicked circular DNA is a much better template than a linear fragment.[674,675] Second, the DNA is assembled into nucleoprotein (chromatin) prior to transcription.[674,676]

These requirements point to the possibility that the winding number of the DNA is of critical importance both in regulation and transcription. Control of winding implies that the end points of the domain are anchored, as is true for a circle or for a domain in native chromatin.[98] Winding could have critical effects on the histone: DNA interactions and, as such, could be used as a mechanism for regulation from a sequence at some distance from the region of transcription. Transcription itself requires a small amount of DNA unwinding, the Watson-Crick base pairing being perturbed for about 15 bp.[677] In this context it is interesting that HMG proteins have been found to unwind DNA[392,393] and to bind preferentially to the single-stranded form under physiological conditions.[390,678] It might be supposed also that a mechanism must exist to prevent nascent RNA from winding around the template DNA. It seems quite likely that nicking-closing enzymes will be found in association with the template DNA, allowing it to rotate as required. Interestingly, there is a report that novobiocin, which inhibits topoisomerase II, inhibits rDNA transcription;[679] it had earlier been found that a nicking-closing activity is isolated with polymerase activity in nucleolar chromatin from *Xenopus*.[680] Recent work indicates that the amounts of topoisomerases in nuclei may be roughly comparable to the amounts of RNA polymerase.[681] A clear picture of the winding problem and its solution awaits further work.

A simplistic approach would be to view the potentially active gene as a region of free DNA, analogous to the *E. coli* chromosome. This seems highly unlikely, as will be discussed. However, the DNase I-hypersensitive sites at the 5′ ends of active genes can at present be hypothesized to result from an absence of nucleosomes (as visualized for SV40). This presumably increases the accessibility of that DNA sequence for interaction with polymerase and its cofactors or with other regulatory molecules. Expression of the early genes of SV40 may be accomplished by binding of the RNA polymerase to DNA sequences mapped at the left hand side of the DNase I-hypersensitive region.[504,682] One could suggest that after, but not before, the DNase I-hypersensitive site of this type is established, regulation of gene expression may be accomplished by the same mechanisms as found for prokaryotic genes (direct protein-DNA interactions at this site, polymerase-cofactor interactions, etc.). However, it should be noted that in several cases the DNase I-hypersensitive site(s) appear to be significantly upstream of the TATA box. That the DNase I-hypersensitive site may represent a necessary activator function of some sort, perhaps related to the winding number of the domain, is reinforced by the intriguing observation that deletions of portions of DNA upstream from initiation sites (in regions presumed or known to contain such hypersensitive sites) results in abolition of transcription of that locus in vivo (see Sections III.A and V.C). Of course, it is not known what structure is indicated by "an absence of nucleosomes"; it seems unlikely that free DNA will be found in the eukaryotic nucleus. In contrast, the work by Chao et al. on the *lac* operator/repressor system had indicated that regulatory protein-DNA interactions need not be interfered with by the presence of nucleosomes.[57,58] However, no direct test of this sort on a eukaryotic system has yet been carried out, although it seems probable that this could be done using the SV40 origin region, RNA polymerase, and T antigen.

Let us now consider the histone: DNA interactions in the region to be traversed by the RNA polymerase. Three basic models have been proposed: (1) histones are not present along this DNA; (2) histones are present, but the normal octamer structure has been perturbed; (3) histone octamers are present, but switch out of the way by binding to the nontranscribed strand of DNA, to the nucleosome immediately adjacent or to the nascent RNP.

One of the first hypotheses concerning the role of histones was that they acted as repressors of template activity as well as general structural components.[683] Following establishment of the nucleosome model of chromatin structure, the idea of an absence of histones at active loci was supported by the observation that one obtained a smear, rather than a pattern of oligonucleosomes, on digestion of active regions of chromatin with micrococcal nuclease; however, other interpretations are possible. EM observation of spread chromatin fibers active in transcription has led to the report that both the transcribed and spacer rDNA gene regions are in smooth fibers of an appropriate length to be double-stranded DNA.[684] However, studies on genes transcribed at a low level have found beads of an appropriate size to be nucleosomes along the template fiber. The rate of transcription may be pertinent. Artificial or native complexes of viral DNA with histones in a nucleosomal arrangement can be transcribed, albeit at a low rate.[641,685] While it is known that histone cores can move along the DNA fiber,[45] there is no evidence for the dissociation or sliding of cores under physiological conditions at rates relevant to transcription.[641,686]

Soon after the nucleosome model of chromatin structure was established, the recovery of active DNA sequences in 11S mononucleosomes was presented as evidence for this structure in active genes. However, the recovery of a DNA-protein complex as an 11S particle in a sucrose gradient can hardly be considered a definitive identification of nucleosomes. The report that affinity chromatography with HMG 14 and 17 can retain DNA: histone complexes significantly enriched for active DNA sequences and showing a stoichiometric complement of histones provides much stronger evidence that the histones are present in some way at the active gene. The fact that mono- and oligonucleosomes can be generated by micrococcal nuclease such that the associated active gene sequences are still preferentially digested by DNase I further indicates that the DNA: histone complexes isolated retain at least some of the features of the complex in vivo.[109,687] Interestingly, antibodies against H2B will inhibit transcription and cause a collapse of the loops of lampbrush chromosomes.[513,514] One might even suggest that the histones play a role in maintaining the gene in the active (partially unwound?) state, being part of the activation mechanism as well as part of the repression mechanism.

If histone cores are present, what is their form? It seems likely, as previously discussed, that the histones will be modified by acetylation in the NH_2-terminal regions and that a specific complement of NHC proteins will be associated with them.[688] Alberts et al.[689] have suggested that the core may dissociate into two half-nucleosomes, each composed of a heterotypic tetramer of histones and each remaining in association with one strand of the DNA. The model is appealing in that it allows for strand separation and DNA uncoiling with perhaps a minimal disruption of structure, but there is at present little direct evidence to support it. Since the dissociation is thought to occur only transiently, it is compatible with the EM studies that show an absence of beads for genes transcribed at a high level, but the presence of beads on genes transcribed at a low level.

Alternatively, it has been proposed that the histone octamer may remain intact but move out of the way by binding to the nontranscribed DNA strand or to another nucleosome.[690] Histone octamers cross linked by chemical means are capable of folding DNA into nucleosomes, although treatment with protein denaturants appears to be required.[691]. Likewise, cross-linked octamers are no different from regular octamers in a reconstituted template transcribed by *E. coli* RNA polymerase.[690] This suggests that

disruption of the histone octamer is not a requirement for in vitro transcription. Histone octamers can bind efficiently to single-stranded DNA, forming a nucleosome-like complex, but do not do so with RNA.[618,692] The presence of RNA in a double helix with a DNA strand also inhibits the formation of nucleosomes.[693] A model of histone association with the nontranscribed strand could be tested by repeating certain nuclease digestion studies using single-strand probes. Nucleosomes do, however, have a capacity to bind additional histone octamers, at least one with high affinity, suggesting that a transient shift to such a conformation leaving some accessible DNA is possible.[694-696] One wonders, however, how this sort of system could be maintained for genes transcribed at a very high rate. The strategies employed may differ significantly depending on the rate of transcription required. Knowledge of the structure of the active gene has developed rapidly in just a few years. The processes involved in the establishment and commitment to such a state represent a challenge which will undoubtedly attract much future investigation.

ADDENDUM

There have been several recent reports concerning DNase I hypersensitive sites. In yeast, two loci coding for alcohol dehydrogenase isozymes have stable 5′-hypersensitive sites, even though one is a constitutive locus and the other is ethanol inducible; the inducible locus does, however, increase rapidly in overall DNase I sensitivity or activation.[704] Genetically inherited integrated murine proviral sequences and a cellular oncogene (c-mos) show no DNase I sensitivity in tumorous tissues of leukemic mice.[705] However, the somatically recombined proviral squences show both hypermethylation and DNase I hypersensitive sites, located some 400 to 500 bp upstream from transcriptional initiation, at the junction of cellular sequences and the 5′ long terminal repeat of the provirus.[705] In contrast, DNase I hypersensitive sites apparently not associated with transcription per se have also been found. In T lymphocytes, hypersensitive sites are apparently located at a DNA rearrangement (switch) site between J_H and C_μ genes; these are not seen in the liver.[706] However, it is interesting to note that in β-cell lineages, the C kappa region is transcriptionally promiscuous at a low level in the unrearranged form with initiation in the region near the J sequences.[707] The V kappa gene is inactive at this stage and only becomes competent when rearrangement has taken place,[707,708] moving it to an active domain. In a separate case, it was found that the extrachromosomal DNA of *Tetrahymena* contains hypersensitive sites at both the region of transcriptional initiation, a promoter for RNA polymerase I, and the origin of replication.[709]

The special features of these sites remain intriguing. One particular sequence from the DNase I sensitive region of SV40 (the 72 bp tandem repeat) has been found to act as a *cis* enhancer of transcription in in vivo transcription experiments.[710,711] It will work in either orientation and can be located proximal or distal to the gene itself over quite large distances. A sequence of similar function (although of unrelated DNA sequence) has been found in polyoma.[712] The 72 bp repeat of SV40 has been replaced by an unrelated 72 bp sequence repeat from the Moloney sarcoma virus, giving rise to a viable SV40 recombinant.[713] This enhancer sequence is unrelated to other upstream sequences close to the initiation site required for efficient in vivo transcription[714,715] (i.e., it is not a promoter); its effect on DNase I sensitivity and/or hypersensitivity will be interesting to investigate.

The actual limits of the broad DNase I sensitive domain for the ovalbumin gene cluster of the chick have been defined as spanning a region of 80 to 100 kb with a 10 kb transition region at one end. In nonexpressing tissue there is no DNase I sensitivity.[716] Another type

of structural boundary has been found for a tandemly repeated cluster of tRNA genes in *Xenopus* 718. A 250 bp gap in the nucleosomal array across these genes was found using micrococcal nuclease in tissue where the genes were not expressed. The precise positioning of the genes reported might well be dictated by proceeding from this boundary (where a protein might reside) across the cluster of genes to the next 250 bp gap. In liver and kidney cells the genes are active, and, while still present in nucleosomes, no longer show evidence of any specific positioning. The nature of structural boundaries will be receiving much attention in the future. The possibility that particular DNA sequences are involved seems strong, but it is not necessarily true that a sequence-specific protein-DNA interaction will create the boundary in every case. As previously mentioned, Z-DNA has been located in vivo in *Drosophila* polytene chromosomes.[154] Histone core-DNA interaction stabilizes B-DNA and, in fact, Z-form DNA may be incompatible with normal nucleosome structure.[718] Similarly, an A-T rich section of DNA might constitute a boundary, as even quite short sketches of poly(dA).poly(dT) (20-80 bp) discoverage nucleosome formation to a marked degree.[719]

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