

STRUCTURE, DYNAMICS, AND FUNCTION OF CHROMATIN IN VITRO

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ABSTRACT

The substrates for the essential biological processes of transcription, replication, recombination, DNA repair, and cell division are not naked DNA; rather, they are protein-DNA complexes known as chromatin, in one or another stage of a hierarchical series of compactations. These are exciting times for students of chromatin. New studies provide incontrovertible evidence linking chromatin structure to function. Exceptional progress has been made in studies of the structure of chromatin subunits. Surprising new dynamic properties have been discovered. And, much progress has been made in dissecting the functional roles of specific chromatin proteins and domains. This review focuses on in vitro studies of chromatin structure, dynamics, and function.

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INTRODUCTION, PERSPECTIVES, AND OVERVIEW

The substrates for the essential biological processes of transcription, replication, recombination, DNA repair, and cell division are not naked DNA; rather, they are protein-DNA complexes known as chromatin, in one or another stage of a hierarchical series of compactions. Cells regulate the folding state of their chromatin both temporally and spatially: There are progressive changes in bulk chromatin folding throughout the cell cycle, and, at any moment in time, the chromatin structure is altered at specific positions along each chromosomal DNA molecule. For these reasons, it is important to determine the structures of each level of chromosome folding, to determine the mechanisms by which cells regulate the folded state of their chromatin, and to elucidate the relationships between chromosome structure and chromosome function.

This is a period of tremendous and increasing excitement for students of chromatin structure and function, for two main reasons: First, new studies provide incontrovertible evidence linking chromatin structure to essential aspects of function. Most notably, they establish a clear relationship between chromosome structure and gene regulation. Connections are established to both gene activation and silencing; numerous mutants and suppressor mutations point to a host of regulatory and effector molecules providing bridges between chromosome structure and function; and suddenly, several of these molecules have been identified, isolated, and their genes cloned. Second, there has been great progress in studies of chromosome structure and dynamics. High resolution structures of key chromatin components and subassemblies have been determined. Much progress has been made toward understanding higher levels of chromosome structure, although here the studies are at low resolution and many conclusions are tentative and controversial. Surprising new dynamical properties have been discovered. And, much progress has been made dissecting the

functional roles of specific chromatin proteins and domains. Collectively, these data take us well on our way toward an understanding of the molecular basis of chromosome function.

At the same time, this progress raises a wealth of important new questions and highlights some significant puzzles. Many treasures await discovery. The coming years promise to be exceptionally interesting.

This review will focus on *in vitro* studies of chromatin structure, dynamics, and function, and especially on results obtained since the author's previous review in 1989 (112). Much of what had been learned at that time continues to be important in current thinking, so the interested reader should consult that review for more detailed discussion of certain points. For general background information in this field, three books are particularly helpful (102, 107, 122). Other helpful recent reviews of aspects of chromatin structure and function include (24, 49, 50, 81, 82, 105).

STRUCTURE OF THE NUCLEOSOME AND NUCLEOSOME CORE PARTICLE

DNA in chromatin is closely associated with a number of highly conserved proteins known as histones that fold the DNA in a hierarchical series of stages, ultimately yielding a $\approx 10,000$ -fold linear compaction preparatory to cell division. The initial or lowest level of chromatin organization consists of the local wrapping of a short stretch of DNA, 147 bp in length, in $\approx 1 \frac{3}{4}$ turns of a flat superhelix around an octameric histone protein core, which is composed of two molecules each of histones H2A, H2B, H3, and H4. This complex of the histone octamer and 147 bp of DNA is known as the nucleosome core particle. This local packing motif is repeated at intervals, millions of times along the entire DNA length, with short variable-length stretches of "linker" DNA between consecutive core particles.

In most cases *in vivo*, each nucleosome core particle is associated with one additional molecule of a "linker histone," H1 or H5 (a particular gene variant of H1; hereafter referred to simply as H1). Particles containing the complete core particle plus histone H1 and the linker DNA at each end are called nucleosomes. Nucleosomes are traditionally considered to be the fundamental units of chromatin structure.

Octamer and Core Particle Structure

There have been important new advances in our understanding of the structure of the nucleosome core particle. The structure of the histone octamer in the absence of DNA was solved by X-ray crystallography at 3.1 Å resolution (4); and, more recently, the crystal structure of the complete nucleosome core

particle was solved at 2.8 Å resolution [(61); for review, see (116)]. The new structure provides a detailed view of the protein and DNA organization; it provides tantalizing glimpses of the histone tail domains; it suggests a model for the structure of the 30 nm fiber; and it has many surprises. This very important contribution will serve as a focus for much future work.

The structures reveal a tripartite assembly of the octamer, reflecting its two H2A–H2B heterodimer and one H3₂–H4₂ tetramer subunits (82, 102, 112). Interestingly, the tetramer can be seen as a stable complex of two H3–H4 heterodimers, which have a “handshake” interlocking protein fold very similar in structure to that of the H2A–H2B heterodimers. This protein architectural motif is now referred to as the “histone fold.” Each histone dimer has a pseudo-twofold (dyad) symmetry, and the octamer has an overall pseudo-dyad symmetry. There is an evident positively charged superhelical ramp, important for the DNA organization. The histone fold domains organize the central 121 bp of DNA, with the additional 13 bp at each end organized by an N-terminal alpha-helical extension to the histone fold of H3 and preceding residues from the tail domain. Each histone dimer organizes ≈ 27 –28 bp, with 4 bp stretches between them.

DNA binding is primarily to the sugar phosphate backbone over the short stretches where the minor groove (and hence the DNA backbone) faces in towards the octamer surface. Each histone dimer contributes three main DNA binding motifs, in two types, referred to as L1L2 and “1”1, with overall pseudo-twofold symmetry for the three sites. Contacts between the histones and DNA include extensive salt bridges and hydrogen bonds to the phosphate groups contributed by both main-chain and side-chain groups; extensive nonpolar contacts with the DNA sugar; electrostatic interactions of the positively charged N-termini of alpha helices with DNA phosphates; and a smaller number of base-specific contacts, including nonpolar contact of the 5-methyl group of thymidine in the major groove.

The overall DNA trajectory approximates 1.65 turns of a superhelix, but the diameter and bending are not uniform. In general, short stretches of DNA centered on each L1L2 or “1”1 binding site appear only slightly bent, while the DNA is bent relatively sharply over a few bp between these sites (which are spaced ≈ 10 bp center-to-center) so as to create the overall superhelical path. The helical twist of the DNA averages $10.2 \text{ bp turn}^{-1}$, but varies in detail along the DNA.

Two additional modes of histone-DNA interaction are particularly striking. First, an arginine sidechain is inserted into the minor groove every time it faces inward to the histone surface. In most cases the arginine is held by additional bonds to protein functional groups so as to prevent it from penetrating deeply into the groove and making base-specific hydrogen bonding contacts.

The second striking additional mode of histone-DNA interaction arises from the histone tails. Each of the core histones has a $\approx 10\text{--}40$ aa-long highly positively charged N-terminal region; and histones H2A and H3 have shorter but analogous domains at their C-termini, as well. These domains are referred to as “tails” because they are known to be highly extended and mobile. They are relatively highly conserved throughout evolution, and are of great interest because they are the sites of numerous posttranslational modifications known to be essential in chromatin function. They are seen only for a fraction of their length in the crystal structure. Several of the tails act together to bracket turns of DNA, passing over and between the DNA gyres. Other aspects of the structure and function of the tail domains are discussed in more detail in the section on 30 nm fiber structure, below.

Histone H1

H1 STRUCTURE Progress on the structure and function of histone H1 has led to large new puzzles and surprises. H1 proteins have a conserved central ~ 80 aa globular domain, and two long, highly positively charged tails. The structure of the globular domain of histone H5 (GH5) has now been determined to high resolution by X-ray crystallography (83); and that of histone H1 (GH1) has been determined by NMR (14). Interestingly, the structures resemble those of several other prokaryotic and eukaryotic transcription factors. The long C-terminal extensions of the proteins have a propensity towards alpha helix formation (16).

LOCATION AND ROLE OF H1 IN THE NUCLEOSOME Earlier studies (112) established that H1 was located on the surface of the nucleosome and suggested that the globular domain was located on the nucleosomal dyad axis over the region where DNA enters and exits the core particle. Both H1 and GH1 (and H5, GH5) bind cooperatively to two molecules of double stranded DNA at once (21, 97), suggesting that the globular domain must have at least two DNA-binding surfaces, and that these might both be required for function in chromatin.

Analysis of the structure of GH5 identified a potential second DNA binding surface in addition to the one identified by homology to the other structurally similar known DNA binding domains. Mutation of this second site shows it to be required for formation of the cooperative complexes with pairs of DNA molecules and for proper binding to H1-depleted nucleosomes (33). New electron and atomic force microscopy studies confirm and extend earlier findings that H1 influences the entry/exit angle of linker DNA, i.e. the trajectory taken by DNA as it enters and exits the nucleosome (7, 36, 54). When H1 is present, the points at which DNA enters and exits the nucleosome are close, whereas when H1 is removed the points of DNA entry and exit are further apart, appearing to be on approximately opposite sides of the nucleosome from each

other. All of these data are consistent with the earlier model in which GH1 is located over the nucleosomal dyad, binding simultaneously to the pair of DNA segments entering and leaving the nucleosome.

It came as a great surprise, therefore, when a pair of papers appeared that used chemical crosslinking methods to map the location of GH5 in the nucleosome, and yielded a very different location (40, 80). These results suggest instead that GH5 packs underneath the DNA where the DNA leaves the core particle, i.e. that it effectively continues the superhelical ramp on one side of the histone octamer. At present, this matter is still open (82).

Adding further to the puzzles surrounding histone H1, a series of new discoveries leaves us knowing even less about its function than before. Many lines of evidence had suggested that H1 played an essential role in the 30 nm fiber, through its effects on the organization of nucleosomal linker DNA. Several new studies reopen this question. The genomic DNA sequence of the yeast *Saccharomyces cerevisiae* has been determined and reveals one and only one protein having significant homology to the conserved globular domain of higher cell H1s; this protein has now been eliminated by gene knockout, and cells remain viable (101), although there are detectable alterations in gene regulation. This is likely not to be a peculiarity unique to yeast. Physical studies of isolated chromatin in vitro show that at least some yeast chromatin is capable of higher order folding (58), as is H1-depleted chromatin from other sources (see section on 30 nm fiber structure, below). Consistent with the view that H1 may in general not be essential, no candidates for an H1-like protein have been identified in the embryogenic stages of *Drosophila* development, and the one identified candidate for an H1-like protein present during *Xenopus* early development can be eliminated, with little evident consequence for nuclear assembly or, indeed, for the development of the organism (10, 18, 72).

Fundamental Repeating Unit

These data force one to reevaluate the view that an H1-containing nucleosome is the fundamental or minimal repeating unit of chromosome structure. At least for *S. cerevisiae*, and quite likely for *Drosophila* and *Xenopus* during early development, this is simply not the case. The fundamental unit of chromosome organization is a nucleosome without H1.

Another surprise concerns the amount of DNA associated with each nucleosome. A long-held view, supported by analyses of the time-course of micrococcal nuclease digestion of chromatin, had been that H1 bound to and stabilized an additional 10 bp at each end of the core particle DNA, so that a minimal nucleosome contained at least 167 bp. Indeed, such particles (called chromatosomes) can be isolated by controlled micrococcal nuclease digestion of chromatin obtained from suitable cell types (112). However, recent measurements of the nucleosome repeat length, which yield the length of DNA per

nucleosome averaged over an entire genome or over distinct genomic regions (using hybridization methods), reveal numerous instances of a substantially shorter repeat length, $\approx 156\text{--}157$ bp (8, 31, 114). At this point, the fundamental repeating unit of chromosome structure appears to be a nucleosome containing only the core histone octamer and $\approx 156\text{--}157$ bp of DNA.

It is appropriate to define as linker DNA the total length of DNA associated with a nucleosome minus the 147 bp of the core particle. An analysis of measurements of nucleosome repeat lengths reveals that bulk average linker DNA lengths are not uniformly distributed; rather, they occur at a preferentially quantized set of lengths, differing by integral multiples of the DNA helical repeat, ≈ 10 bp (114). The preference for having a length equal to one of the quantized set is pronounced but not absolute.

Nucleosome Positioning

This area is the subject of much misunderstanding. The facts in outline are these: In vivo mapping methods reveal that in some cases nucleosomes are preferentially localized at specific genomic positions, often correlated with the underlying regulatory organization of genes (49). The preferred positions can be influenced by the presence of other proteins (92), but often are the same as those found when histones are reconstituted on the same naked DNA in vitro. And, when nucleosomes are reconstituted in vitro with a unique but arbitrary DNA sequence of length L that is longer than the 147 bp of the core particle (so that the octamer could in principle be located starting at any of the $L-146$ positions while still being fully occupied by DNA), it is often found that the octamer exhibits a pronounced preference for location at one or a few specific positions (i.e. for initiating nucleosomal wrapping at one or a few specific basepairs within the sequence).

We distinguish translational and rotational positioning. Translational positioning refers to the extent to which a histone octamer selects a particular contiguous stretch of 147 bp of DNA in preference to other stretches of the same length that are translated forwards or backwards along the DNA. Rotational positioning is a degenerate form of translational positioning in which a set of discrete translational positions, differing by integral multiples of the DNA helical repeat, are all occupied in preference to the set of other possible locations. DNA sequences that are intrinsically bent or are anisotropically bendable may lead to rotationally positioned nucleosomes (87, 93). As discussed below, other interactions too may contribute to translational or rotational positioning.

DNA SEQUENCE-DIRECTED POSITIONING The following discussion focuses on DNA sequence-directed nucleosome positioning, for which the determinants of positioning are contributed solely by the DNA sequence and the histone octamer.

Nucleosomes [lacking histone H1 (75)] are mobile in physiological conditions [(65, 98, 103); see section on nucleosome mobility, below]. Standard protocols for nucleosome reconstitution end in approximately physiological conditions or sweep slowly through such conditions prior to freezing-in the resulting particles at low ionic strength (59). It follows in either case that the resulting distribution of nucleosome positions is an equilibrium one.

This fact has several important consequences. It implies that nucleosome positioning is not “precise” as often stated, but rather is a statistical property, governed by the laws of chemical equilibrium. Thus, observations of apparent precise positioning actually reflect preferential occupancy of one position together with a general insensitivity of mapping methods to lower levels of occupancy at the set of all other positions. More careful recent studies of positioning *in vitro* (84) reveal occupancy of numerous translational positions that are not related by the DNA helical twist, although even these studies cannot quantify the nonzero occupancies that must exist at all possible positions.

Because nucleosome positioning is an equilibrium property, there exists a particular mathematical relationship between the free energy of histone–DNA interactions measured in competitive nucleosome reconstitution experiments, and the time- or ensemble-averaged probability of occupancy of the preferred site (59). Because free energies are finite, positioning *in vivo* will be statistical too, not precise, even though additional forces may contribute to establishing the positional biases. It is important to recognize this statistical property of positioning because it has substantial ramifications for mechanisms of gene regulation. When positioning is not precise, essential DNA sequences will sometimes be buried when they need to be accessible, or may be accessible when they need to be repressed (buried). Mechanisms proposed for gene regulation must be robust with respect to statistical fluctuations in nucleosome positioning, which are inevitable when free energies are finite.

Previous studies have identified particular protein and DNA determinants of positioning. Histone octamers lacking the trypsin sensitive tails, and the H3₂H4₂ tetramer on its own, adopt the same preferential position (41) as intact octamer. While these data suggest that the histone fold domains of the tetramer may be the dominant determinants of positioning on this sequence, there is no reason why this needs to be true in general. Indeed, two of the four sites of greatest DNA helical deformation in the nucleosome core structure are consequences of interactions with the H2A/H2B heterodimer. However, it could turn out in practice that, simply because so much of the DNA is organized and contacted by the tetramer, the tetramer usually dominates the positioning.

DNA determinants of nucleosome positioning have been discovered through analyses of DNA sequences present in isolated natural nucleosomes and of DNA sequences found by happenstance to be organized in preferentially positioned

nucleosomes [see (115) and references therein]. Many dinucleotide and longer sequence motifs have been discovered that recur with a ≈ 10.2 bp periodicity. This periodicity matches the average DNA helical repeat seen in the core particle structure (61), implying that genomic DNA has evolved to contribute to its own nucleosomal packaging (110). While such signals are readily detectable in genomic DNA (115), competition experiments show that $>95\%$ of bulk genomic DNA contribute 0 ± 0.2 kcal mol⁻¹ to the free energy of histone-DNA interactions in nucleosomes, relative to chemically synthetic random DNA (59). However, particular genomic sequences that have substantially greater affinities (i.e. substantially greater nucleosome positioning power) do exist and can be isolated using SELEX methods (109). It will be of great interest to correlate the locations of these high affinity sequences with the underlying genetic organization of the chromosome.

The relative phases of periodic signals from A/T- and G/C-rich motifs together with early notions about the structural and mechanical properties of various base-steps suggested that these motifs make the DNA statically or dynamically bent (anisotropically flexible), thereby reducing the free energy cost of bending and thus increasing the affinity. Artificial sequences according to these principles yield higher affinities in nucleosome reconstitution (93), and the resulting nucleosomes exhibit rotational positioning, as might be expected (20, 76, 93). However, recent compendia of the structural preferences of differing base-steps (23) simply do not uphold the assumptions on which these predictions are based.

It is useful to consider the problem more generally. Positioning achieves equilibrium, so the sites of preferential positioning will be those having minimum free energy. The net free energy for any particular position will reflect favorable contributions from the set of all the bonds that are formed (including van der Waals interactions, hydrophobic forces, etc. in addition to conventional bonds, and including all intermolecular, intramolecular, and solvent bonds) minus the free-energy cost of deforming the protein, the DNA, and the solvent away from their starting (uncomplexed) conformations into their core particle conformations. While the structures of the L1L2-DNA and "1"1-DNA interactions are relatively well conserved throughout the core particle (61), they differ in detail, probably from the necessity of accommodating differing local DNA sequences. We already know that the DNA changes structure upon nucleosome formation, and it is most likely that the histone octamer and the solvent do too. Thus the detailed equilibrium structures of the nucleosome as well as the net affinity will vary in detail with the DNA sequence.

The structure of the core particle shows myriad opportunities for particular DNA sequences to influence the number and strength of bonds that are made in the complex, as well as the energetic cost of changing the bondedness of the

separated partners. The structure also shows myriad locations where specific DNA bends or twists may be optimal. Thus, the DNA sequence can also contribute significantly to the energetics in at least four additional ways through the mechanical work involved in changing the position-dependent DNA bending and twist, which reflect contributions from (a) static bending, (b) the bendability (bending force constant), (c) static twist, and (d) the twistability (twisting force constant). Each of these quantities varies with the local DNA sequence (23), but at present the rules remain poorly understood.

A typical arbitrary DNA sequence will by chance combine some optimal local sequences and many suboptimal ones, which will most probably yield a near-average overall free energy in nucleosome reconstitution. The ability of protein side-chains and solvent to adapt to suboptimal sequences by adopting alternative conformations may act to constrain the standard deviation of the distribution of interaction free energies. Nevertheless, one can anticipate the existence of many DNA sequences in the tails of this distribution—i.e. having free energies that are much more favorable (or unfavorable) than average, simply because of the vast number of different sequences, $1/2 \times 4^{147} \approx 1.6 \times 10^{88}$, that exist for sequences of length 147 bp.

Based on these ideas, a SELEX experiment carried out on a large pool of chemically synthetic random DNA sequences has yielded a collection of sequences having higher affinity than previously known natural or nonnatural sequences (59a). Examination of these sequences reveals a large new set of sequence motifs (rules) having much greater statistical significance—and thus correspondingly greater positioning power—than the previously known ones. An important challenge for the future is to understand the functioning of these new rules in the context of the new crystal structure.

OTHER INFLUENCES ON NUCLEOSOME POSITIONING Biases in nucleosome positioning will arise in part from the population of DNA-binding proteins present in the cell at any moment, since protein binding to a target site will almost certainly restrict the translational or rotational positioning of that site in a nucleosome. These may be considered to be direct effects of proteins on nucleosome positioning. Assuming that positioning is fully equilibrated in vivo (65, 98, 103), these processes can only be understood as a large set of coupled chemical equilibria. Nucleosome positioning biases regulatory protein binding, and regulatory protein binding in turn biases nucleosome positioning.

Protein-dependent positioning can also be indirect. Such effects may arise when other DNA-binding proteins attract or exclude nucleosomes and so delineate a region to be filled-in statistically by other nucleosomes (51).

The requirements of chromatin higher order structure create biases for the mutual positioning of arrays of nucleosomes [(114, 126); and see the section on 30 nm fiber structure].

STRUCTURE OF THE NUCLEOSOME FILAMENT

The lowest level of chromosome organization is a repeating chain of nucleosomes, millions of nucleosomes in length, with nucleosomes separated by variable but preferentially quantized lengths of linker DNA. The actual 3-D structure is highly sensitive to the solution conditions. In solutions containing low concentrations of monovalent cations [M^+] and no multivalent cations [M^{n+}] (111), the chromatin fiber adopts an extended structure; individual nucleosomes separated by extended stretches of linker DNA are readily seen by electron microscopy. This state of chromatin is known as the nucleosome filament.

Previous physical studies reviewed in (112) establish that the nucleosome filament has an extended 3-dimensional zig-zag structure in solution. There are strong positional correlations between nucleosomes even when linker DNA is sufficiently long that the average distance between consecutive nucleosomes significantly exceeds the nucleosomal diameter. Removal of histone H1 leads to an increase in distance between nucleosomes and a loss or weakening of the positional correlations. These effects are likely consequences of an increase in the entry/exit angle of DNA from the nucleosome as expected from studies on individual nucleosomes summarized above.

New studies using atomic force microscopy (AFM) (54) and cryoelectron microscopy (7) are consistent with these prior conclusions and afford direct 3-dimensional views of the structures in the presence and absence of H1. However, while providing a direct determination of 3-D structure is an important benefit, these new methods also introduce their own problems, discussed below.

STRUCTURE OF THE 30 nm FIBER

In solution, titration of chromatin with increasing concentrations of monovalent or multivalent cations up toward physiological ionic conditions leads to a progressive folding of the nucleosome filament into a compact ≈ 30 nm wide filament, or 30 nm fiber (112). In vivo, most chromatin is maintained throughout most of the cell cycle in this 30 nm fiber state or in even more highly folded states reached by further compaction of the 30 nm fiber.

At the time of the previous review, there were several competing models for the structure of the 30 nm fiber. A preponderance of the evidence favored a "solenoid" model (25, 96, 119), although isolated results appear contrary to it. In the solenoid model, the chain of nucleosomes is organized in a one-start contact helix having roughly six nucleosomes per turn; nucleosomes are oriented with their dyad axes perpendicular to the solenoid axis, with the linker DNA entry/exit side facing inward toward the center of the solenoid.

Much progress has subsequently been made toward distinguishing between the competing models and toward an elucidation of the folding mechanism. However, a direct structural determination remains lacking and efforts toward that goal face serious obstacles.

Key Problems for Direct Structural Studies

There are two chief underlying obstacles to a direct determination of the structure of the 30 nm fiber. First, alternative models that have been proposed differ from the solenoid model only in subtle, high-resolution features; moreover, chromatin fibers are ordered but lack crystalline regularity, owing to variability in the length of linker DNA from nucleosome to nucleosome along the fiber. Thus, one obstacle is a general problem of obtaining high resolution structural information from objects that are both large and irregular. AFM or cryoelectron microscopy (cryo EM) both have significant problems. AFM studies are carried out in “tapping mode,” but this is something of a misnomer since very large amounts of energy can be deposited into the specimen with each “tap.” Moreover, samples are adsorbed to surfaces and examined in air at ambient humidity; there may be distortions upon adsorption, and the solution conditions are not well defined. Problems with cryo EM are discussed below.

The second significant obstacle is that the ionic conditions that stabilize the folded 30 nm fiber state of chromatin also cause its aggregation. Aggregation sets in before structure-sensitive solution probes such as the sedimentation coefficient or the sharpness of X-ray diffraction bands reach titration endpoints (111, 112). 30 nm fibers in the aggregates pack very closely together, so that electron density contrast between individual fibers is diminished or lost; such samples are unsuitable for EM tomographic analysis. On the other hand, when lower cation concentrations are used, the 30 nm fiber state is unstable or marginally stable, and thus subject to artifactual distortions.

Constraints from Short Linker Lengths

Crossed-linker models of chromatin (120) can be tested by examining the folding of chromatin from cell types having very short linker DNA lengths (i.e. very short nucleosome repeat lengths). Experiments on diverse sources of chromatin having short linker lengths revealed fibers having ordinary ≈ 30 nm diameters and a low pitch angle, in contrast to the requirements of crossed-linker models (58, 112). Recently characterized chromatin from sources having very short nucleosome repeat lengths of ≈ 156 – 157 bp (8, 31, 114) allow a stronger test of the model. Geometric calculations suggest that crossed-linker models cannot be constructed with such short linker lengths because there is not enough linker DNA to reach from one nucleosome across the fiber to the next, even if the two opposite nucleosomes are allowed to touch and some DNA is allowed to

be partially unwrapped off the surface of each nucleosome (31). Nevertheless, such chromatin undergoes cation-dependent higher order folding in vitro comparable to that of bulk chromatin (8).

These very short (≈ 156 – 157 bp) repeat lengths also place substantial constraints on the solenoid model. Geometric calculations suggest that solenoid models can be constructed with such short linker lengths only if the solenoid is right-handed (31).

DEPENDENCE OF FIBER DIAMETER ON LINKER LENGTH The solenoid model makes no specific prediction regarding the effects of large changes in linker DNA lengths (i.e. corresponding to an increase in the nucleosome repeat length, as opposed to a small change in length leading to twist errors discussed below). Varied linker lengths could be accommodated within the center of a solenoid having constant width, or in an additional loop nested between nucleosomes, or by small changes in the angle between consecutive nucleosomes leading to a changed diameter. Conflicting results have been obtained. One study suggests that the diameter is invariant for the chromatin in situ, but that it can change on isolation of the chromatin in vitro (123). Evidently fiber diameter depends on some uncontrolled experimental variable.

Linker DNA Topology

LINKER DNA BENDING The solenoid model has the striking requirement that linker DNA must bend to allow consecutive nucleosomes to pack together in space. In contrast, crossed-linker and extended chain models allow the linker DNA to remain straight. Can linker DNA bend as required by the solenoid model? And, does it do so in chromatin? These questions have been addressed in several recent studies, which have led to contradictory results.

Dinucleosomes—oligomers of chromatin containing just two nucleosomes separated by one linker—allow a detection of linker DNA bending through measurement of the nucleosome–nucleosome distance (124). Electron microscopy of fixed, unstained samples, together with quasielastic light scattering of dinucleosomes in solution, showed that, as the concentration of mono- or divalent cations is increased from 2 mM M^+ to 18 mM NaCl or 2 mM $MgCl_2$, the average edge-to-edge separation of the two nucleosomes decreases from ≈ 15 nm (corresponding to fully extended linker DNA) to near-contact. These results are confirmed and extended in subsequent studies on the roles of histone H1 and the core histone tail domains, described below (27, 124). Another study detected a smaller increase in D_t (the translational diffusion coefficient) over the range ≈ 3.2 – 20 mM M^+ in the same buffer system, and came to a different structural conclusion (7). However, it is not clear that these results are really in conflict. First, much of the overall increase in D_t occurs in the 1–3 mM (M^+) range, which

was not investigated in (7) or in frequently cited earlier studies such as (11). The relevant variable is the total monovalent cation concentration (111), not just (NaCl). Second, the data analysis method used in (7) is not the most appropriate for sensitive detection of modest changes in D_i in monodisperse systems.

Cryoelectron microscopy has been used to probe (M^+)-dependent changes in the structure of dinucleosomes and trinucleosomes (7), and led to the conclusion that (M^+)-dependent folding consists only of small changes in the entry/exit angle of linker DNA, with no bending of linker DNA—in clear conflict with the observations using conventional EM on fixed, unstained preparations (124). Similarly, a tomographic reconstruction of 30 nm fibers in situ using low temperature embedding methods (44) revealed a structure in which linker DNA is extended, not bent, conferring an extended zig-zag structure on the chromatin fiber.

While the point of the cryoelectron microscopy and low temperature embedding methods is to better preserve native structure, the evidence suggests that, for the case of chromatin fibers, they have not. The extended zig-zag chain structures seen in the tomographic reconstructions have values of mass per unit length (number of nucleosomes per unit translation along the fiber axis) that are significantly lower than those measured by neutron (30) or X-ray (121) low-angle scattering, or scanning transmission EM (30), and fail to explain the observed protection of linker DNA against nuclease attack that accompanies chromatin folding (130). Rather, the tomographic structures are consistent with the nucleosome filament state of unfolded 30 nm fibers studied in low (M^+) in vitro, suggesting that the chromatin has unfolded during preparation.

Independent evidence that there may be a problem with cryoelectron microscopy methods for DNA-containing specimens comes from studies of the topology of plasmid DNA. Cryoelectron microscopy revealed an unexpected cation-dependent change in plasmid topology leading to tight interwinding of DNA segments (6). Subsequent studies, however, using several different solution physical methods (29, 85) or an in-vitro recombination assay (86) conclude that this unexpected topology does not in fact occur.

One plausible explanation for the discrepancy concerns the temperature dependence to the helical twist of DNA (29). Classic studies establish that there is a substantial temperature dependence to the helical twist of DNA, and provide data on the coefficient of this dependence down to $\approx 0^\circ\text{C}$. In cryo EM, samples are plunged into liquid ethane near its freezing point (-172°C). It is estimated that the vitrification process in cryo EM occurs in $\approx 10^{-4}$ – 10^{-5} s (6). Thus, for a period of 10^{-4} – 10^{-5} s, the solution will be rapidly cooling from ambient ($\approx 20^\circ\text{C}$) down toward -172°C . During this period the helical twist of DNA will be changing significantly, although one can only estimate its low-temperature value by lengthy extrapolation. In response to the changing helical twist, the overall topology changes. The question is, are such changes rapid or slow

compared to the cooling time? Experimental data on the viscosity of supercoiled water show that at temperatures as low as $\approx -35^\circ\text{C}$, the viscosity is only $\approx 15\times$ greater than at 20°C (3). Thus, rotational and translational dynamics that occur on an ns timescale at 20°C (e.g. translations and rotations of nucleosomes) will be slowed to a 10–1000 ns timescale—which is still 10^2 – $10^4\times$ faster than the cooling rate. Thus, while the cooling rate in cryo EM seems fast by human standards, it is still extremely slow on the timescale of molecular motions. The structures seen by cryo EM reflect structures formed after the DNA twist adopts values appropriate to the very low temperatures; and, as described in the following section, regular structures for the 30 nm fiber have strict requirements for particular values of the integrated twist of linker DNA.

Studies of pyrimidine dimer formation have been used as an indirect assay for the bending state of linker DNA in chromatin. These studies assume that quenching from neighboring proteins can be ignored. But the fact that the pattern near the middle of nucleosome core DNA is sensitive to the presence of H1 (74) suggests that proximity of proteins does contribute significantly.

In summary, present results are in conflict. Studies suggesting that linker DNA is straight have substantial caveats. Relatively fewer studies support the view that linker DNA may be bent; in any case those studies pertain only to dinucleosomes, not to long chromatin oligomers. New approaches to this central question are badly needed.

TWIST CONSTRAINTS IN LINKER DNA The solenoid model (indeed, any regular structure) has an interesting constraint regarding the twist of linker DNA. Definite protein–protein or protein–DNA contacts between neighboring nucleosomes require particular values for the integrated (total) twist of each linker DNA segment. This requirement may be satisfied not just by a single particular linker length, but by any of a quantized set of lengths that differ one from another by integral multiples of the DNA helical repeat. Of course, any arbitrary DNA length can in principle be under- or overwound to give a needed particular total twist; however, when the twist error approaches 0.5 turns, the free energy penalties become very large and might easily exceed the free energy of any nucleosome–nucleosome contact that could be made. Thus, one might expect that linker DNA lengths would be preferentially quantized. An analysis of nucleosome repeat lengths reveals that linker DNA lengths do indeed occur in such preferentially quantized lengths (114). The quantization is not perfect, and need not be, since there is only a small free energy penalty for small twist errors. Moreover, the DNA twist itself varies with local sequence and on interaction with proteins.

That linker DNA lengths come in preferentially quantized lengths is an experimental truth; however, this particular explanation for that observation is not

unique. Evidence in support of this structural explanation is provided by a study of the free energy coupling between (M^+)-dependent folding of dinucleosomes and intercalation of ethidium bromide into linker DNA (126). Consistent with a constraint on the twist of linker DNA, ethidium intercalation causes decondensation of dinucleosomes, and chromatin folding competes with ethidium binding. Results from other laboratories suggest that these effects of ethidium are due to ethidium-induced changes in the twist of linker DNA, and not to a variety of other effects.

Location and Roles of Histone H1 and the Core Histone Tails

LOCATION AND ROLE OF HISTONE H1 The location of histone H1 (more precisely, GH1) within the 30 nm fiber is a subject of considerable interest for two reasons. This information can shed light on the function of H1 itself; and, since the location of H1 within the nucleosome is known at least approximately, knowing the location of H1 within the 30 nm fiber places constraints on possible orientations for the nucleosomes within the 30 nm fiber. Previous studies of 30 nm fiber structure define aspects of the orientation consistent with the solenoid model (112, 118, 119), but do not suffice to specify the orientation with respect to rotations about the axis of the nucleosomal disk (112).

The location of H1 within the 30 nm fiber has been directly determined using neutron scattering (34). H1 is found to be internally located, at roughly the same radius as the innermost surface of nucleosomes in the solenoid model. This implies that nucleosomes have a constant rotational setting about their disk-axis, allowing consecutive nucleosomes to make equivalent nucleosome–nucleosome interactions. An alternative solenoid-like model that allows a variable rotational setting about the nucleosome disk-axis and lacks equivalent interactions between consecutive nucleosomes (112) is inconsistent with this result.

Early data suggested an essential role for H1 in 30 nm fiber structure (112); but H1-depleted chromatin exhibits a (M^+)-dependent compaction too [see (125) and references therein]. More recent studies on defined nucleosome oligomers (91, 125) show that H1-depleted oligonucleosomes can fold to approximately the same compactness as those containing H1. Moreover, this compaction involves a bending of the linker DNA (27), as found for dinucleosomes (124, 125). For folding induced by monovalent cations, the folding transitions are shifted to slightly higher (M^+) (124, 125). This means that H1 contributes to the free energy of stabilization of the folded state, but that it is not solely responsible, hence folding can proceed in its absence.

Electron microscopic and X-ray scattering studies of long H1-depleted chromatin show that the compact states that can be achieved lack the order

characteristic of folded native chromatin (125), and led to the conclusion that H1 may be essential for selecting a single ordered conformation from a set of disordered compact conformations that are produced in its absence. However, new data suggesting that H1 may not be essential for viability force one to reexamine these questions.

While H1 may not be an essential protein, its presence in most cell types as a stoichiometric component of chromatin, its evolutionary conservation, and the fact that it does contribute to chromatin folding all point to an important role for H1 in 30 nm fiber structure and function. Variants of H1 are segregated in blocks in chromatin (48, 66). This segregation is inherently a cooperative phenomenon, and indeed H1 does bind cooperatively to DNA (see above); a tendency to self-association has been detected for GH5 but not for GH1 (62). Variants of H1 (e.g. H5) have varied affinities for DNA (17), and exhibit preferences for particular DNA base compositions or methylated states (64). These properties allow H1, in principle, to contribute to the free energy of chromatin folding to a variable and location-specific extent.

ROLES OF THE CORE HISTONE TAILS The newly completed high resolution structure of the core particle will completely change the nature of future studies of the roles of the core histone tails. Prior to this structure determination, the majority of studies of tail-domain function *in vitro* focused primarily (although not exclusively) on the collective roles of sets of the trypsin-sensitive tail domains. An underappreciated point concerning many of these studies is that while the texts often refer to the roles of the trypsin-sensitive N-terminal domains, it is well-established that trypsin attacks sites in the N- and C-terminal regions of the histones H2A and H3 as well (102). Thus, effects reportedly due to the N-terminal tails may in some cases prove due to the C-terminal domains instead.

With that caveat, several important conclusions emerge. Oligonucleosomes reconstituted without H1 and lacking the trypsin-sensitive tail domains of all four core histones fail to exhibit the cation-dependent folding and linker DNA bending seen when the core histones are intact (12, 26, 27). Evidently, one or more of the tails has an essential role in chromatin folding. Oligonucleosomes reconstituted using hyperacetylated core histones behave similarly to those produced with trypsinized octamer (28). Moreover, hyperacetylation leads to a decrease in the (time-averaged) amount of DNA wrapped on the nucleosome (5, 68). These results mean that cells can regulate the stability of chromatin folding through changes in the pattern of core histone acetylation, and further suggests that other posttranslational modifications of the tail domains, as well as the use of other histone gene variants, may act *in vivo* to regulate the stability of chromatin folding.

Subsequent studies examined separately the roles of the tail domains from H2A/H2B heterodimers or H₃H₄₂ tetramers by reconstitution with histones in which one or the other subunits, but not both, have been trypsinized. Reconstituted oligonucleosomes lacking the tails of the H₃H₄₂ tetramers show little ability to undergo cation-dependent folding or self-aggregation, similar to oligonucleosomes lacking the tails of all four core histones (28, 90). This implies a major role for one or more of these domains in chromatin folding *in vitro*. In contrast, the tails of H2A/H2B contribute detectably but only slightly to chromatin folding (28).

Many other studies establish roles for these tail domains in protein-protein interactions with nonhistone chromosomal or chromosome-regulatory proteins. For example, the N-terminal tails of both H3 and H4 are recognized and bound by the yeast gene-silencing proteins Sir3 and Sir4, and are essential for the association of Sir3 with telomeric chromatin and for the perinuclear positioning of telomeres (42). The N-terminal tails of H3 and H4 are similarly implicated in direct interactions with the yeast global repressor protein Tup1, and moreover this interaction is negatively regulated by acetylation of the N-terminal domains (22).

There is a degree of functional overlap between the N-terminal domains of H3 and H4 and also between those of H2A and H2B, as assayed by the crude but important measure of viability (56, 89). Importantly, such functional overlap does not imply mechanistic overlap (106). Since their mechanisms of action may differ, it need not seem surprising that these pairs of N-terminal tails could show partial functional overlap despite their different locations in the core particle structure.

NEW INSIGHTS FROM THE CORE PARTICLE CRYSTAL STRUCTURE The high resolution structure of the nucleosome core particle (61) provides several important new insights into the structure of the 30 nm fiber. Model building studies (61) reportedly show that this structure for the nucleosome core particle is compatible with the solenoid model for the structure of the 30 nm fiber (25, 96, 119).

An especially intriguing observation in the core particle structure is that an N-terminal tail of histone H4 on one nucleosome reaches over and makes an extensive contact with an H2A/H2B heterodimer on an adjacent nucleosome. The contact involves numerous hydrogen bonds and salt links between four basic residues on the H4 tail and a cluster of seven acidic residues on H2A and H2B. This interaction seems likely to have a strongly attractive energy, and is found experimentally to be essential for crystallization in the conditions used for the structure determination (61). The packing of nucleosomes within the crystal differs from that proposed for the packing in the solenoid model, and consequently the H4–H2A/H2B contact in the crystal has a direction opposite to the

orientation that it would have in the solenoid model. However, because of the likely flexibility of the N-terminal tail of H4 and the nature of the binding interface, one presumes that similar contacts could be made with the orientation of the neighboring nucleosome reversed to resemble that in the solenoid. Evidence consistent with the possibility that this interaction contributes to chromatin folding *in vitro* comes from the observation that cation-dependent folding of reconstituted oligonucleosomes (lacking H1) requires that H2A/H2B heterodimers and the tail domains of the H3₂H4₂ tetramer both be present (37, 67, 90), while the tail domains of H2A/H2B contribute only slightly (67).

Because the N-terminal domain of H4 is not essential for viability, attention is drawn to other core histone domains, too, for their possible involvement in chromatin folding. The N-terminal tails of histone H3 (which include many conserved acetylation sites) pass between two gyres of DNA, anchoring the 13 bp at each end of the core particle DNA, and extend outward from the particle. Similarly the most C-terminal visible residue of the histone H2A tail is poised for interaction with linker DNA and moreover is the site of ubiquitin attachment (113). Either of these domains could also have direct roles in nucleosome-nucleosome interactions as well as influencing chromatin folding through effects on linker DNA.

Very importantly, studies carried out *in vitro* show that the N-terminal tail of H3 and the C-terminal tail of H2A do in fact interact with linker DNA. Earlier studies established that the histone octamer on its own provides measurable protection of 167 bp of DNA against attack by micrococcal nuclease [e.g. (55)]—notwithstanding the fact that the relative absence of such protection is often used as an assay for the reconstitution of histone H1. One or more core histone domain(s) must be responsible for this protection. More recently, a study of accessibility of residues to chemical modification provides direct evidence of an interaction of the N-terminal tail of H3 with linker DNA in chromatin (43). Another study analyzed the digestion by micrococcal nuclease of long chromatin reconstituted with chicken erythrocyte H2B, H3, and H4, and wheat H2A, which has an additional 19-amino acid C-terminal extension relative to chicken. The additional length of the H2A C-terminal tail led to the protection of an additional 16 bp of linker DNA against attack by the nuclease (55). This observation implicates the extended C-terminal tail of the wheat protein in an interaction with linker DNA, and it suggests that the shorter C-terminal tail of chicken erythrocyte H2A likely contributes to the protection of the 167 bp fragment and therefore interacts with linker DNA.

The structure suggests that several other tail domains could also be involved in nucleosome-nucleosome interactions, either laterally or vertically within a solenoid. The N-terminal tail of H4 could be involved in contacts distinct from the particular interaction seen in the crystal packing.

HIGHER LEVELS OF STRUCTURE

Low-angle X-ray scattering studies of isolated metaphase chromosomes (52) show a series of diffraction features characteristic of 30 nm chromatin fibers (111, 119), suggesting that metaphase chromosomes are produced by further folding of the basic 30 nm fiber. Scanning electron micrographs show stubby 50–70 nm diameter projections emanating from the body of metaphase chromosomes (2), and thin sectioning studies (2) suggest that these projections are composed of a loop of 30 nm fiber that folds back and twists around itself. Similar structures are formed spontaneously by long 30 nm fibers in vitro (111, 118, 119). Moreover, there is a good correlation between the phase diagram for 30 nm formation in vitro (111) and the appearance of metaphase chromosomes (2). If the concentration of Mg^{2+} or other higher valence cations is too high, the 50–70 nm diameter projections appear to merge together and can no longer be resolved. Taken together, these data provide strong evidence that the 30 nm fiber serves as a basic architectural unit that is further folded into mitotic and probably meiotic chromosomes.

Two studies of higher order chromatin structure have yielded particularly important results: One study uses the natural processes of mitotic chromosome decondensation during the progression from M phase to S phase, and of recondensation during the transition from G2 to M phase, to examine chromosomes when they are less densely packed so that elements of their internal organization may be exposed. This approach in combination with serial thin sectioning electron microscopy and image reconstruction provides evidence for two levels of folding above the 30 nm fiber: fibers with diameters of ≈ 60 –80 nm and ≈ 100 –130 nm (9). The data suggest that the 30 nm fibers are organized into 60–80 nm fibers by local folding rather than helical coiling; the 60–80 nm fibers may be helically coiled into the 100–130 nm fibers; and these in turn may be locally folded into chromatids. However, an alternative view suggesting that mitotic chromosomes may be organized with a final helical coiling cannot be excluded at present. An important corollary of these results is that a majority of chromatin in the interphase nucleus is organized in levels of folding above that of the 30 nm fiber.

A second important discovery concerns long-range correlations in spatial positions between defined genetic elements along a single chromosome (128) in the interphase nucleus. Experiments using fluorescence in situ hybridization (FISH) methods on fixed nuclei reveal evidence for random walk behavior of interphase chromatin on two lengthscales. On one lengthscale, chromatin appears to be organized in large loops, several Mbp in size, with the chromatin randomly folded within each loop. On an even longer lengthscale, the chromosome behaves as a long chain of such loops, again showing random walk behavior. Individual chromosomes occupy distinct domains in the interphase nucleus.

NUCLEOSOME DYNAMICS AND FUNCTION

Nucleosomes and chromatin fibers are dynamic, not inert. Their dynamic behavior is essential for chromatin function.

Regulatory Protein Binding

DNA sequences that are organized in nucleosomes are largely inaccessible to other proteins because of steric hindrance from the octamer surface and other nearby segments of DNA—both within a nucleosome and from other nucleosomes neighboring in space in the chromatin fiber. Yet most DNA *in vivo* is packaged in nucleosomes, and many DNA sequences are critical for biological regulation and so must be accessible to regulatory proteins at appropriate times. What, then, are the principles that guarantee that regulatory proteins will have access to their DNA target sequences when necessary? The answer to this important question is not known. For references and for discussion of current ideas and their limitations, see (59, 77, 78, 117).

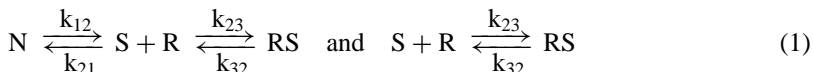
To investigate these questions, many research groups set up experiments *in vitro* that assess the ability of eukaryotic transcription factors to bind to their DNA target sites when those are incorporated into nucleosomes [see (77, 78, 117)].

The results were varied and confusing. In some cases, factors were able to bind to nucleosomal target sites, producing complexes that contained the factor, the DNA, and at least one molecule each of all four core histones. The affinities of the factors for their target sites were generally suppressed by one or more orders of magnitude. These studies left open the questions of why the factors had those measured affinities, and, strikingly, how the factors were able to bind to their target sites in the first place, when those sites were sterically occluded on the nucleosome! Even more puzzling, other factors were found not to be able to bind to nucleosomal target sites, including cases in which opposite outcomes were obtained with two closely related factors—one apparently “able” to bind, the other “not able.”

Site Exposure Model for Dynamics and Function of Nucleosomal DNA

A new “site exposure model” (77, 78) allows these and other results to be understood and, indeed, quantitatively predicted with no adjustable parameters. This model is illustrated in Figure 1*a*.

We make the simplifying assumption that sufficient nucleosomal DNA is exposed such that the rates and equilibria for binding to an exposed nucleosomal target sequence or to a naked DNA target sequence are identical. Thus,



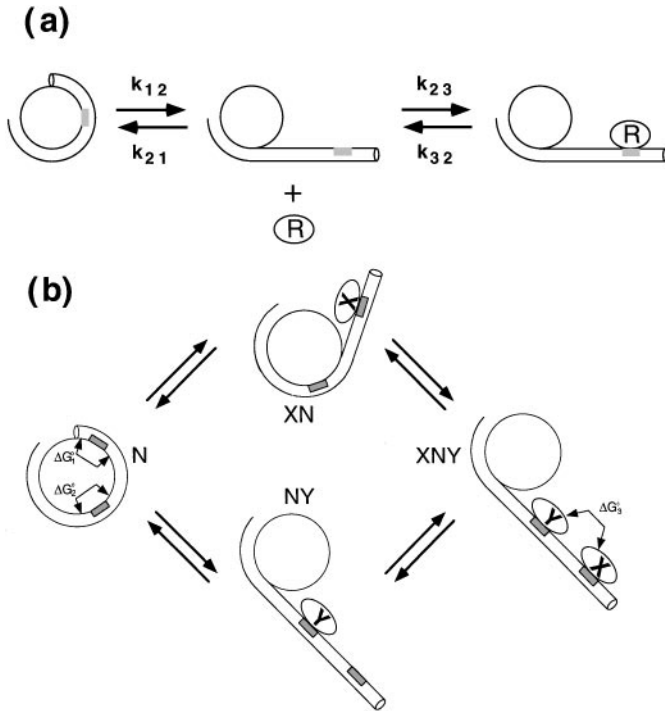


Figure 1 (a) The site exposure model illustrated for a single nucleosome. The histone octamer is shown from above as a disk with the DNA coiling around it. A particular DNA target sequence (*stippled*) is inaccessible to the regulatory protein (*R*) that acts on it. k_{12} and k_{21} are position-dependent apparent-rate constants for site exposure and recapture, respectively. Exposure of sites nearer the middle of the nucleosomal DNA may occur by several successive steps of exposure of shorter segments from an end as illustrated; each smaller step would have its own microscopic rate constant. k_{23} and k_{32} are microscopic-rate constants for binding and dissociation of *R* from its target site, and pertain to naked DNA as well as to the exposed state of nucleosomes. Real nucleosomes exist in long chains, but this need not prevent uncoiling such as illustrated. With just modest deformation of the linker DNA, a combined uncoiling coupled to a motion of the uncoiled DNA in a direction parallel to the axis of the nucleosomal disk allows uncoiling beyond the dyad (which is as far as necessary to allow binding anywhere) with no required crossings and with little motion of other nucleosomes. Higher levels of chromosome structure may need to be disassembled prior to the site-exposure process illustrated here, but are also believed to possess only marginal stability. (b) Cooperativity in the binding of multiple proteins to target sites in a single nucleosome. A nucleosome is shown containing binding sites (*stippled*) for two proteins (*X*) and (*Y*). *X* and *Y* may be two unrelated proteins, or two molecules of the same protein. *X* is defined as the protein binding to the outer site, and *Y* as binding to the inner site. ΔG_1 is the free energy cost for uncoiling enough DNA so as to expose the site for *X*. ΔG_2 is the additional free energy cost for uncoiling sufficient additional DNA so as to expose the site for *Y*. In some cases,

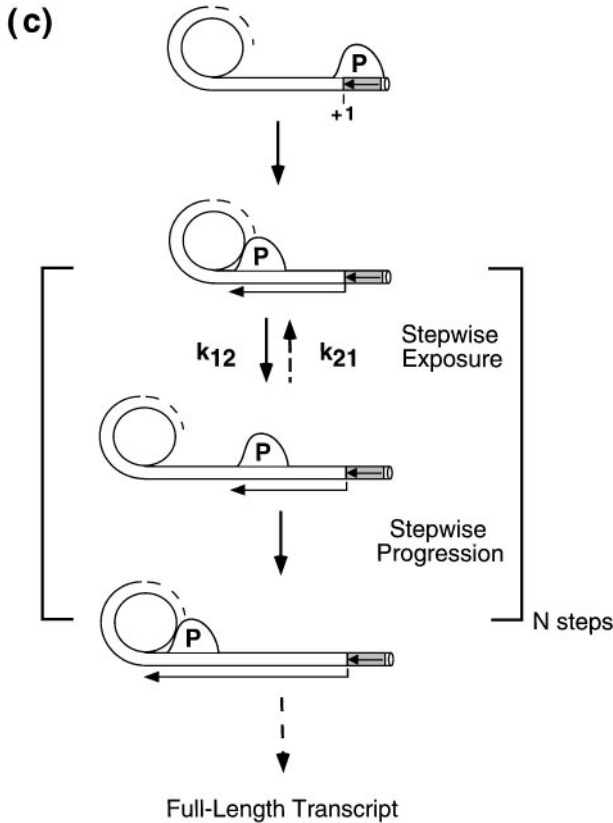


Figure 1 (Continued) X and Y may have “conventional” cooperative interactions, also detectable in their binding to naked DNA (e.g. from favorable protein-protein contacts between X and Y); these are collectively represented as ΔG_3 . (c) Site-exposure model for the mechanism of polymerase elongation through a nucleosomal template. Successive repetition of two steps—partial uncoiling, followed by elongation up to the next point of steric hindrance—allows full-length elongation. (The octamer could be transferred backward during this process, as has been suggested; see text). The number of such steps required for elongation through a complete nucleosome in this model depends on the length of DNA released in each step. Site exposure occurs nondissociatively, so the entire DNA chain is not released in just one step; thus, the number of site-exposure steps required for full-length nucleosome transcription is two or greater. The structure of the nucleosome suggests that contact points of the DNA and histones (likely sites of histone-DNA “bonds”) occur once each DNA helical turn, in which case DNA may be exposed in successive ≈ 10 bp-long steps. Similar suggestions for the mechanism of polymerase progression have been made previously (50).

for nucleosomes and naked DNA, respectively (N is the starting nucleosome, R a DNA-binding protein, and S the site-exposed state of the nucleosome, or naked DNA).

With this assumption, binding of a regulatory protein to a nucleosomal target sequence will occur with a net free energy change ΔG_{net}^0 , and an apparent dissociation constant $K_{\text{d}}^{\text{apparent}}$, given by:

$$\Delta G_{\text{net}}^0 = \Delta G_{\text{conf}}^0 + \Delta G_{\text{naked DNA}}^0 \quad \text{and} \quad K_{\text{d}}^{\text{apparent}} = K_{\text{d}}^{\text{naked DNA}} / K_{\text{eq}}^{\text{conf}}, \quad (2)$$

where $K_{\text{eq}}^{\text{conf}}$ is the equilibrium constant for site exposure in the nucleosome (k_{12}/k_{21}), $K_{\text{d}}^{\text{naked DNA}}$ is the dissociation constant for binding to naked DNA (k_{32}/k_{23}), and ΔG_{conf}^0 and $\Delta G_{\text{naked DNA}}^0$ are the corresponding free energies.

In this model, $K_{\text{eq}}^{\text{conf}}$ depends primarily on the translational position of the target sequence within the nucleosome (ΔG_{conf} depends on the length of DNA being uncoiled—i.e. on the length of protein-DNA interface that is disrupted). However, the effective $K_{\text{eq}}^{\text{conf}}$ will also depend on other factors such as the size and shape of the regulatory protein, the rotational setting of the target site around the periphery of the DNA helix, and on DNA bending induced by the protein, because these affect the amount of DNA that must be uncoiled to allow the protein to bind.

Studies of the accessibility of nucleosomal sites to restriction enzymes provide a test of this model and allow measurement of position-dependent equilibrium constants for site exposure (77, 78). The most important finding is that site exposure does in fact occur, even over the nucleosomal dyad (the middle and presumably least-accessible region of the nucleosomal DNA), with substantial values for $K_{\text{eq}}^{\text{conf}}$. This dynamic property intrinsic to nucleosomes provides a general mechanism guaranteeing that regulatory proteins may have access to their DNA target sequences.

The measured equilibrium constants for site exposure decrease more-or-less progressively as one moves inward from an end into the middle of the nucleosome, from $\approx 1-4 \times 10^{-2}$ just inside the core particle, to $\approx 10^{-4}-10^{-5}$ over the dyad axis. Such behavior is consistent with the simple uncoiling picture as illustrated. The structure shows the DNA to be wrapped on the histone surface as making contacts (“bonds”) in a small patch, every ~ 10 bp, each time the phosphodiester backbone (minor groove) faces inward toward the octamer (61). Thus, uncoiling would naturally proceed stepwise, with an incremental increase in energetic cost ΔG_{conf} (i.e. decreased equilibrium constant $K_{\text{eq}}^{\text{conf}}$) associated with each additional 10 bp-long segment uncoiled. Theoretical studies of a related model reveal additional stable states (63).

These results explain and clarify the confusing results of the direct binding studies. For those cases where proteins could bind, the site exposure model

provides a physically plausible mechanism for how they gain access to their target sites. Moreover, it allows us to predict the outcomes of the equilibrium binding studies: given the (readily measured) affinity of a protein for its target site on naked DNA, and the location of that target site in a nucleosome (for which our results provide the corresponding K_{eq}^{conf}), the predicted affinity for the nucleosomal target site is given by Equations 1 and 2.

The site exposure model also explains why in seemingly arbitrary cases it was found that certain proteins could not bind to their target sites within nucleosomes. In many (possibly all) such cases, the observed failure to bind can be attributed to a simple consequence of ending the titrations too soon, prior to reaching the $K_d^{apparent}$ that we predict for that system.

Studies of the kinetics of restriction enzyme digestion of nucleosomal DNA, and of equilibrium affinities for transcription factor binding to nucleosomal DNA, are unrelated except through the site-exposure model. Nevertheless, they yield equivalent results: That is, using the equations of this model, the results of either experiment allow the prediction of the other. Evidently this model and Equations 1 and 2 provide a framework for analysis and interpretation of the binding studies.

COOPERATIVITY The site exposure model has within it the potential for important novel cooperative (synergistic) interactions between multiple proteins binding simultaneously to sites within a single nucleosome (78). This cooperativity is distinct from any “conventional” (e.g. direct or other) cooperative interactions that may also exist between the proteins. The origin of this potential cooperativity (synergy) is illustrated in Figure 1*b*. It arises from the possibility that, once protein Y has bound, the binding of protein X may take place without having to pay the energetic penalty for site exposure (here defined as ΔG_{conf}^0), which otherwise would be required. Similarly, the ability of X to bind facilitates the subsequent binding of Y, since at least some of the final free energy penalty for the required conformational change is already paid. No special properties are required of X or Y: They need only bind DNA for this cooperativity to be manifested. X and Y may be two different proteins, or they may be two molecules of the same protein. The amount of cooperativity between X and Y (the coupling free energy $^*G_{XY}$ —the free energy by which the prior binding of X facilitates the binding of Y, or vice versa), equals $-\Delta G_{conf}^0$ —i.e. minus one times the energetic cost for exposing the outer site.

This model accounts quantitatively for a diverse set of experimental results on cooperative binding of various proteins to nucleosomal target sites obtained by another laboratory (1). Moreover, there is good agreement between the predictions of this model and the experimental data even using ΔG_{conf} obtained from the restriction enzyme digestion kinetic measurements instead of

the $*G_{XY}$ obtained directly from the primary cooperative binding data. These experiments are completely unrelated except through the site exposure model, so the agreement between the two provides strong evidence for the applicability of the site exposure model to the behavior of real nucleosomes.

These results have three important ramifications. (a) Real nucleosomes *in vitro* do behave in the manner described by Figure 1*b*, with the potential cooperativity of that model fully realized. Such behavior requires mechanical linkage between events at the two binding sites (78), consistent with uncoiling from an end as illustrated. (b) This cooperativity, which is intrinsic to nucleosomes, means that cells can control the occupancy at, for example, X's binding site, either by varying the concentration of X itself, or by varying the concentration of Y, with no requirement for conventional direct cooperative interactions between X and Y. This idea provides a natural mechanism for the construction of cooperative (synergistic) multi-protein control modules from the combinatorial action of independent and arbitrarily chosen parts. (c) The free energy of this cooperativity ($*G_{XY}$), obtained as ΔG_{conf} in our earlier studies, ranges from (minus) 2.5–6 kcal mol⁻¹. These large coupling free energies greatly increase the occupancies achieved by binding proteins that are present at realistic concentrations, compared to their occupancies if they act independently.

In real systems, X and Y may have direct “conventional” cooperative interactions, represented by ΔG_3^0 in Figure 1*b*, in addition to the cooperativity that arises from competing against a common competitor. In that case, the net cooperativity will be given by the sum of $*G_{XY} + \Delta G_3^0$. As one measure of the significance of the inherent cooperativity one can compare $*G_{XY}$ with typical values for ΔG_3^0 measured in real systems. A survey of several well-known conventional cooperative interactions having clearly established significance in gene regulation reveals typical values for ΔG_3^0 of ≈ 1 –2 kcal mol⁻¹ (78). The novel cooperativity free energies from the site exposure model are substantially greater than these free energies of previously recognized “conventional” cooperative interactions.

KINETICS In order for the site exposure model to be relevant in gene regulation *in vivo*, it is important that the rate of site exposure (measured by k_{12} in Figure 1 and Equation 1) be fast compared to the timescales of biological regulatory decisions (perhaps minutes or faster) or polymerase elongation (6–10 s per nucleosome for RNA pol II). Direct measurement of the rate constants for site exposure and recapture have not yet been made. Simple theoretical estimates, assuming either an activated or a diffusive process for recapture, lead to the expectation that site exposure will occur on the millisecond to microsecond

timescale (J Widom, unpublished information). Studies using coupled enzymatic reactions to detect site-exposure events show that site exposure occurs on a timescale of seconds or faster (79a).

NUCLEOSOME STABILITY Spontaneous site exposure processes are nondissociative: Exposed DNA segments are rapidly recaptured, and the time-averaged most prevalent state is that of an ordinary (fully wrapped) nucleosome. The possibility that simple dissociation of DNA from the histone octamer could explain the observed site exposure is explicitly ruled out by several independent experiments (77).

Site exposure occurring simultaneously on each side of the nucleosome could lead to dissociation of the DNA, but evidently does not do so. How can it be that nucleosomes are stable? The answer is not known. One plausible explanation is that site exposure events at the two ends may not be independent; release at one end may suppress release at the other. Mutual repulsion between nucleosomal DNA segments, which must serve to destabilize the wrapping of DNA, provides one such mechanism; additionally, conformational changes could be transmitted through the histone octamer, allowing binding events on one side to influence affinities and dynamics at the other.

Remarkably, nucleosomes may actually be less stable against DNA dissociation than has previously been imagined. One study reports that simple dilution of nucleosomes—in the presence of a poly dGdC competitor—leads to displacement of labeled DNA tracer from the histone octamer, as monitored both by solution footprinting and by native gel electrophoresis. This instability was apparently not a consequence of the use of a particular pathological DNA sequence (32).

Nucleosome-Disrupting Machines?

One idea that is popular in “active invasion” models of gene regulation [see (77, 78)] suggests that certain proteins might act as power-driven levers, harnessing the energy of ATP hydrolysis to pry DNA off a nucleosome. (Such an action is to be distinguished from a simple trapping of spontaneous nucleosome structural fluctuations such as the uncoiling fluctuations of site exposure.) Many protein candidates for such a role have been identified [for references, see (50, 77, 78)]. These proteins have ATPase activities; they are implicated in facilitating transcription factor access to nucleosomal DNA *in vitro*; and they are known to interact with nucleosomes and to be involved in transcriptional regulation *in vivo*.

Whatever the real activity of these proteins proves to be, they are unlikely to act by prying DNA off nucleosomes. Such an activity is not in accord with

real properties of molecular motors. Even if molecular motor proteins can act as power-driven levers, a simple calculation shows that RNA polymerase from *E. coli*—currently the strongest molecular motor known (127)—lacks the force by a factor of ten to break even individual, unnaturally weak, models of salt-bridges [bonds that help hold DNA on the nucleosome; see (61)], and can contribute, at most, negligibly or only a few-fold (in the weak-bonding and strong-bonding limits, respectively) to the rate at which DNA would be released in thermally driven (i.e. spontaneous) site-exposure processes (79a).

More plausible mechanisms through which such proteins might act to facilitate the binding of regulatory proteins include the following. (a) Simply by binding specifically or nonspecifically to DNA, such proteins would facilitate the ability of other site-specific proteins to bind, in accord with the cooperativity mechanism of Figure 1*b*. (b) As will be described in more detail below for the case of RNA polymerase, processive DNA-binding proteins can trap spontaneous uncoiling fluctuations of site exposure processes just after they occur, allowing other proteins to bind to the displaced naked DNA that accumulates behind them. In this case the role of ATP hydrolysis is simply to allow a processive protein to translocate forward when other obstacles do not prevent this. (c) The proteins might act by modifying the histones themselves, e.g. by phosphorylating one or more of them, thereby changing rates or equilibria for site exposure; these mechanisms are discussed below.

A particularly telling result is that, when the “swi/snf” class of such ATP-dependent proteins do play a role in expression of a particular gene *in vivo*, mutations in these proteins can in some cases be suppressed by second site mutations that map to the histones themselves [called “sin” mutants (38)]. The new structure of the nucleosome shows that many of the sin mutations could be expected to destabilize the wrapping of the nucleosomal DNA. Destabilizing the wrapping of DNA would increase the frequency and/or the lifetime of spontaneous site exposure processes, shifting the equilibrium constant for site exposure toward the “exposed,” or regulateable, state. *In vivo*, Sin⁻ mutants show increased susceptibility to attack from a variety of DNA nucleases and modifying enzymes (108).

The Nucleosome as a Binary Regulatory Switch

A property intrinsic to histone octamers potentially allows them to confer a binary “on/off” character to the binding of regulatory proteins to nucleosomal target sites, as illustrated in Figure 2.

Either simple equilibrium binding of multiple proteins to nucleosomal target sites, or the action of a processive DNA-binding protein (one model for active invasion), can lead to displacement of the histone octamer from its DNA. It is plausible that throughout most of the cell cycle (except, perhaps, S-phase?), the

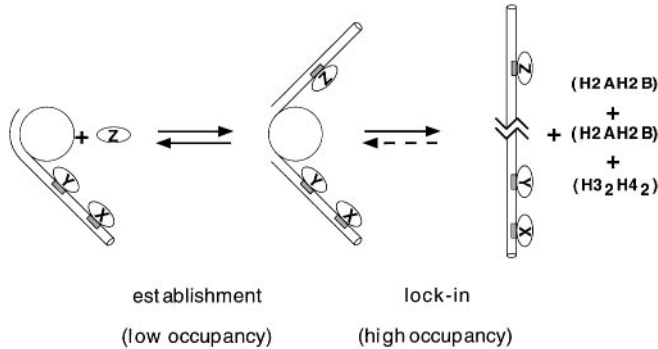


Figure 2 Locking in gene activation. Simple equilibrium binding of sufficient numbers of proteins to nucleosomal target sites, or the action of a processive DNA-binding protein (one model for active invasion) may lead to displacement of the histone octamer from its DNA. Histone octamers that lack their DNA are unstable in physiological conditions: they rapidly dissociate into the H3/H4 tetramer and 2 H2A/H2B heterodimers. Displacement of the histone octamer would then serve as a binary switch. In the initial “establishment” state, a nucleosome is present, and occupancy by bound regulatory proteins is low because of competition from site recapture (the process with rate constant k_{21} in Figure 1); nevertheless, site exposure does allow occupancy greater than zero. Displacement and dissociation of the histone octamer then “locks in” a new state, in which affinities and the corresponding occupancies are much greater, simply because the octamer is no longer an effective competitor.

Alternatively, simple equilibrium binding of proteins to nucleosomal target sites could lead to recruitment of a factor that posttranslationally modifies H1 or the core histones. Preliminary low-occupancy binding, followed by a posttranslational modification that increases site exposure, would provide another way in which a nucleosome could act as a binary regulatory switch.

concentration of free histone subunits may be sufficiently low that nucleosome reassembly—which may be a quaternary process—effectively does not occur. Displacement and dissociation of the histone octamer could switch a gene from an initial “establishment” state, having relatively low occupancy of target sites by regulatory proteins, to a new state, which, because the octamer is no longer an effective competitor, allows the same concentrations of regulatory proteins to achieve and “lock-in” much greater occupancy levels in this new quasi-equilibrium.

Alternatively, simple equilibrium binding of proteins to nucleosomal target sites could lead to recruitment of a factor that posttranslationally modifies H1 or the core histones. As will be discussed in a following section, site exposure processes can be greatly repressed or facilitated by such modifications. Preliminary low-occupancy binding, followed by a posttranslational modification that increases site exposure, would provide another way in which a nucleosome could act as a binary regulatory switch.

Nucleosome Mobility

Another dynamic behavior of nucleosomes that has been discovered is their ability to move along DNA in a process also referred to as nucleosome sliding (65, 76). Nucleosome mobility is readily detected by a 2-dimensional native gel assay, which takes advantage of the dependence of the electrophoretic mobility on the position of the octamer between the ends and the middle of a DNA fragment. The rate of nucleosome mobility is enhanced at elevated temperatures and ionic strengths, but mobility can also be detected in lower temperatures and approximately physiological ionic strengths. Mobility is strongly suppressed by histone H1 (75).

Can nucleosome mobility be the underlying mechanism of site exposure? If mobility were the underlying mechanism of site exposure, the analysis summarized above would still hold true. The measured equilibrium constants would describe the fraction of time that mobile nucleosomes spend in various locations so as to leave particular sites extending off their surface and accessible to other proteins.

The actual mechanism of site exposure has not yet been determined. However, the relative rates argue against a role for mobility in site exposure. Indeed, the evidence suggests that the converse is more likely to be the case: Site exposure may be the initial step in nucleosome mobility. Site exposure sufficient to expose all of the DNA occurs on a seconds timescale, whereas in comparable conditions nucleosome mobility occurs on the hours timescale.

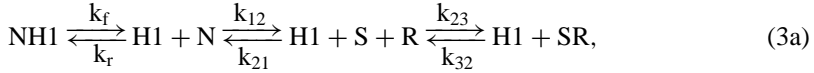
While mobility is often described as a “sliding” process, the nature of the histone:DNA interface would not allow a literal sliding motion; rather, a coupled rotation/translation of the DNA would be required in order to maintain the critical minor groove backbone contacts at multiple points on the histone surface. Such a process would be improbable (although not impossible). However, the site exposure mechanism provides a much simpler alternative, in which a segment of DNA released from the histone surface can loop back and be recaptured at a new position (most likely displaced by a multiple of the DNA helical repeat), creating a nucleosome with a “bulged” loop of DNA. Analogous structures have been postulated as intermediates during nucleosome transcription (50, 95). The bulge could propagate around the nucleosome in a relatively low-energy process; when it runs off the other end, the nucleosome would be found to have moved. Further studies are required to address the molecular mechanisms of both site exposure and nucleosome mobility.

Effects of Histone H1 and Histone Modifications

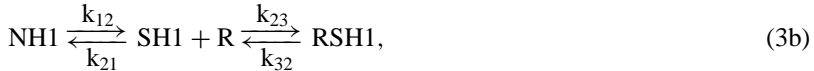
The presence of histone H1 on nucleosomes strongly suppresses the ability of other proteins to bind to nucleosomal DNA target sites (47), the ability of RNA polymerases to elongate through the nucleosomal DNA (71, 100), and the

mobility of nucleosomes on DNA (75). Studies of the direct effects of histone H1 on equilibrium constants for site exposure are not yet available.

From a formal perspective, H1 could act in either of at least two ways: In one mechanism, release of H1 must precede site exposure. Thus,



where NH1 is an H1-containing nucleosome. In this model, H1 acts effectively as a competitive inhibitor for site exposure. Cells may repress the genetic activity of regions of chromosomes either by increasing the concentration of H1, or by producing H1 variants or posttranslationally modified states of H1 that bind more tightly. Alternatively, histone H1 may always remain bound, but its presence may alter the equilibrium constants for site exposure, $K_{\text{eq}}^{\text{conf}}$, through effects on k_{12} , k_{21} , or both.



where SH1 is an H1-containing nucleosome in a conformational state able to bind R, and RSH1 is the ternary complex of nucleosome, R, and H1. In this model, cells may repress genetic activity by producing H1 variants or posttranslationally modified states of H1 that decrease $K_{\text{eq}}^{\text{conf}}$. These two models are readily tested and distinguished by experiments in vitro.

Acetylation of the core histones or complete removal of the trypsin-sensitive tail domains facilitates the ability of proteins to bind to nucleosomal target sites (53, 99, 104). Strikingly, several groups have recently identified transcriptional coactivators as having histone acetylase activity [for review, see (105)]. It remains to be seen whether the primary effects of core histone acetylation are direct, through chromatin structure and dynamics, or indirect, through interactions with other proteins as described above.

The site exposure model (Equation 1) provides one simple way in which the effects of histone acetylation may be understood. It is plausible that one effect of histone acetylation is to increase $K_{\text{eq}}^{\text{conf}}$, shifting the equilibrium toward the accessible (regulateable) state. Such a change in equilibrium constant is consistent with measured changes in the topology of nucleosomal DNA that accompany histone acetylation (5, 68). While these studies are normally interpreted in terms of a reduced amount of DNA wrapped around the acetylated histone core, this statement may be equivalent (averaged over time) to an increased equilibrium constant for site exposure.

New Roles for DNA Sequences in Gene Regulation

Genomic DNA sequences can contribute in two novel ways to gene regulation through their effects on nucleosome structure and dynamics.

NUCLEOSOME POSITIONING Nucleosomes having strongly biased positions definitely do exist *in vivo*, and may play important roles in both positive (60, 88) and negative (49, 50) gene regulation. These roles cannot depend on “precise” positioning, since, as discussed above, this does not exist. Instead, statistical biases in nucleosome positioning can contribute to gene regulation by biasing the time-averaged “accessibility” of regulatory sites. In the context of the site exposure model, one would say that biases in nucleosome positioning produce a particular weighted-average of equilibrium constants for site exposure. Depending on the distribution of occupancies, time-averaged biases in positioning can yield a very wide range of applicable K_{eq}^{conf} . If the predominant time-averaged placement of a regulatory site is in linker DNA regions, this will yield a relatively large K_{eq}^{conf} , possibly even one or greater. Conversely, if the predominant time-averaged placement of a regulatory site is near the nucleosomal dyad, this yields a small K_{eq}^{conf} , approaching 10^{-4} – 10^{-5} for an isolated nucleosome.

Importantly, as discussed above, DNA sequence preferences intrinsic to the histone octamer contribute strongly to nucleosome positioning. Many natural sequences that bias positioning at individual sites by factors of $100\times$ or greater are known. This means that the choice of particular genomic DNA sequences can bias, by factors of 100 or greater, whether the time-averaged K_{eq}^{conf} is a number closer to 1 or to 10^{-4} – 10^{-5} . Thus, genomic DNA sequence can play a dominant role in governing the occupancy of regulatory sites in chromatin, much greater than the effects attributable to many individual gene regulatory proteins.

POLY (DA:DT) ELEMENTS A second way that genomic DNA sequence can contribute to gene regulation is through effects on the structure and dynamics of nucleosomes that are (statistically) positioned by other forces. An especially interesting case is that of polypurine [chiefly poly(dA:dT)] tracts of length 15–30 bp that are found in many promoters. The general situation is illustrated in Figure 3.

A nucleosome is positioned (on average) through some combination of forces so as to cover the binding site for an essential gene activating protein and to position that site near the middle of the nucleosome. The presence of this nucleosome suppresses the accessibility of the binding site to the gene activating protein (and to other DNA binding proteins and enzymes that probe accessibility at that site). Nearby, between this binding site and the nearest end of the nucleosomal DNA, is a polypurine tract. Studies *in vivo* establish that these polypurine tracts are essential elements of the promoters, contributing roughly as much to gene activation as do the gene-activating proteins themselves (45). The function of these elements *in vivo* evidently depends on their intrinsic

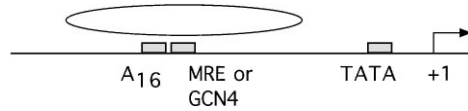


Figure 3 Polypurine elements in eukaryotic promoters. The figure illustrates cases studied in two yeasts (45, 129). A nucleosome (indicated by the *ellipse*, roughly to scale) is (on average) positioned so as to cover a binding site (indicated by *MRE* or *GCN4*) for a gene-activating protein. The positions of the TATA box and the start-site of transcription (+1) are indicated. Nearby the MRE or GCN4 sites, also present in that nucleosome, is a poly(dA:dT) element. The presence of this element is essential for the proper functioning of the promoter, contributing roughly as much to gene activation as does the gene-activating protein itself. These elements are thought to function by altering the structure or stability of the nucleosome in which they are packaged. The site-exposure model provides one way in which such effects may be understood.

structure or properties and not on their interaction with sequence-specific DNA binding proteins (45, 129). These elements increase the accessibility of the adjacent DNA regulatory sequences to the gene activating protein and to other proteins. These results suggest that the poly(dA:dT) elements lead to nucleosomes having altered structure or stability, thereby facilitating access by regulatory proteins at those genomic locations.

The site exposure model predicts such behavior and may account for the action of these promoter elements. Stretches of poly (dA:dT) of length 16 bp in a similar location decrease by $\approx 1.2 \text{ kcal mol}^{-1}$ the favorable free energy of histone-DNA interactions in nucleosome reconstitution (39). This decreases, by that same amount, the free energy penalty that must be paid in order to uncoil the nucleosomal DNA beyond the poly (dA:dT) stretch so that a protein can have access to a site further inside the nucleosome. Decreasing the free-energy penalty for exposing the site makes the overall process of protein binding more favorable. More generally, for any sequence element that decreases the favorable free energy of histone-DNA interactions by an amount $\Delta \Delta G_{\text{seq}}$, the site exposure model predicts that $K_{\text{eq}}^{\text{conf}}$ and the affinity for binding at a site further inside that same nucleosome will be increased by the factor $\exp(\Delta \Delta G_{\text{seq}}/RT)$.

Nucleosome Transcription and Replication

The nucleosomal organization of DNA presents obstacles for the elongation of RNA and DNA polymerase. Early studies from many groups on transcription of isolated nucleosomal templates led to the conclusion that RNA polymerase could not initiate if its promoter was inside a nucleosome; however, if the promoter is located off the nucleosome, the polymerase is able to elongate through the nucleosome to produce full-length transcripts [for reviews, see (24, 50)]. These studies were limited by the presence of multiple nucleosome positioning

isomers and the fact that the reactions were run asynchronously over numerous rounds of transcription, until the reactants were exhausted. In many cases, the possibility could not be excluded that most of the products arose from naked DNA, and not from nucleosomes at all.

Subsequent studies eliminated these uncertainties. Problems from multiple nucleosome positioning isomers are reduced or eliminated by purification of single isomers (using native gel electrophoresis) (69, 95), or through the use of nucleosome-positioning sequences that, together with a short overall fragment length, give predominant positioning at a single predominant location (79). Appropriately designed template sequences and experimental protocols allow study of synchronous transcription complexes, in real time, as they progress along the templates in a single passage (79, 95). Using bacteriophage T7 RNA polymerase (79), the timescale can be varied from faster to much slower than the natural rate of RNA pol II elongation, which is $\approx 6\text{--}10$ s per nucleosome.

Key conclusions include the following: Transcriptional elongation through a nucleosomal template can occur with high efficiency, approaching 100%. When the elongation rate is slow compared to the natural timescale, the velocities of polymerase progression (elongation) on the nucleosomal templates are slightly but reproducibly slower than on naked DNA. Evidently, polymerases can traverse through a nucleosome with little difficulty. The slight difference in rates is due to a slight increase in pausing on the nucleosomal templates. However, this increased pausing reflects properties of the DNA sequence itself, and not of its nucleosomal organization: The sites of this increased pausing are identical on nucleosomes and naked DNA. Studies on nucleosomes produced with crosslinked or disulfide-linked histone octamers show that complete disassembly or “lexosome” structural transitions of the histone octamer are not essential for polymerase elongation (70, 79).

When the elongation rate is close to or faster than the natural rate, the enhanced pausing is reduced on the second half of the nucleosomal template (79a, 95). This observation, together with a finding that nucleosome transcription is accompanied by backwards translocation of nucleosome on the DNA, led to a model in which the DNA ahead of the octamer uncoils from the histone surface while, at the same time, the DNA behind the polymerase coils back around it (94, 95a, 119a). Another study suggests that, in the presence of competitor DNA, nucleosome transcription leads to complete displacement of the template DNA from the histone octamer and its replacement by the competitor (69, 119a).

Polymerase elongation can lead to accumulation of positive superhelical tension ahead of the polymerase, and negative superhelical tension behind it (57). Positive supercoiling destabilizes nucleosomes while negative supercoiling stabilizes them (15). Thus, transfer of a nucleosome from ahead of to behind the polymerase is an energetically favorable process. Studies on the separate

histone subunits suggest that positive superhelical stress leads to displacement of H2A/H2B heterodimers, whereas H3₂H4₂ tetramers were found to have a preference for the positively supercoiled DNA (46), possibly accompanied by a major structural rearrangement (35).

How can the polymerase progress through a nucleosomal template? The polymerase cannot pass ghostlike through the atoms of the histone octamer and the non-template DNA strand. Rather, release of the DNA from the surface of the histone octamer must precede or accompany forward motion of the polymerase. (Histone subunits may, but need not be, released from the complex.) We argue above that a motor-like action of the polymerase cannot itself pry DNA off the histone surface. Rather, the polymerase must trap spontaneous uncoiling events originating from an end. Others have also reached this conclusion (50). We now know that spontaneous uncoiling events do in fact occur, and the available data are consistent with their extending inward from an end (see above). One example of this mechanism is illustrated in Figure 1c.

DYNAMICS AND FUNCTION OF HIGHER ORDER STRUCTURE

The lack of concrete information on the structure of the 30 nm chromatin fiber and on higher levels of structure precludes much discussion of their dynamics and function. Nevertheless, the following two points warrant consideration.

Dynamic Equilibrium

Is the 30 nm chromatin fiber an “inert,” static structure, so that internal regions of the fiber are inaccessible to proteins and other ligands, or is it “dynamic,” such that internal regions are frequently accessible? The question is an important one since most of the chromatin in a cell is maintained in a 30 nm fiber state (or an even higher level of structure), yet the chromatin must be able to unfold to some extent to allow binding by gene-regulatory proteins, elongation by RNA and DNA polymerases, and other essential processes having chromatin as the substrate.

Early studies show that the nucleosome filament folds gradually into the 30 nm fiber with increasing (M^{n+}) (112). For example, studies of the sedimentation coefficient of chromatin fragments show that *S* increases gradually with increasing (Na^+) (11) or (Mg^{2+}) (111). In general the chromatin precipitates before a clear titration endpoint is reached. Nevertheless, electron microscopic analysis reveals 30 nm fibers at appropriately high (M^{n+}) (111), and measurements of mass per length using STEM and neutron scattering show these to reach plateau values (30). Evidently, in physiological conditions, the 30 nm fiber is only marginally stable.

The simplest interpretation of these data is that the 30 nm fiber is in rapid dynamic equilibrium with the nucleosome filament state; titration with increasing (M^{n+}) gradually shifts the equilibrium constant from a value favoring the nucleosome filament to one favoring the 30 nm fiber. Consistent with this view, histone H1 is found to readily exchange between chromatin fragments in conditions in which the chromatin is folded (on average) into 30 nm fibers (13). Since we now know H1 to have an internal location in 30 nm fibers (34), free exchange of H1 requires dynamic local unfolding of the 30 nm fiber. In this view, post-translational modifications of the histones, or differing histone variants, could modulate the chromatin folding equilibrium, favoring one side or the other.

Other studies using electron microscopy to investigate the (M^{n+})-dependent folding of chromatin fibers on an individual molecule basis led to the conclusion that the folding of each fiber was a gradual process; at intermediate (M^{n+}), chromatin fibers had intermediate widths (96) and intermediate values of mass per length (30). Continuous folding of the chromatin filament could account for gradual changes in physical properties without a requirement for a dynamic folding equilibrium. This view of the folding process is less able to account for the exchange of free H1 between chromatin fibers.

Both of these views may be true at once if a dynamic folding equilibrium takes place over short lengthscales—e.g. the bending of individual linker segments between consecutive nucleosomes, or the wrapping of an individual turn of ≈ 6 – 7 nucleosomes in a solenoid. This would satisfy the data behind the dynamic equilibrium view, but could also account for the appearance of the images and the mass per length data since even these single-molecule studies represent averages over stretches of the chromatin fiber.

Site Exposure and Higher Order Structure

Can spontaneous site exposure processes occur in chains of nucleosomes, where the DNA ends are not free, and there may be folding into higher levels of structure? Recent studies suggest that site exposure does occur readily in chains of nucleosomes in ionic conditions where these chains are folded at least to some degree into higher order structures. These studies investigate the ability of GAL4 protein to bind to target sites in a central “test” nucleosome flanked on either side by five nucleosome-long tandem repeats of a nucleosome positioning sequence (73). The affinity of GAL4 protein for sites in the central test nucleosome is similar to that for sites in an isolated nucleosome measured earlier (77). In the context of the site-exposure model, measurements of affinity are equivalent to the restriction enzyme measurements of equilibrium constants for site exposure. Thus, these results imply that burying the test nucleosome in the middle of an 11-nucleosome-long chain has only little effect on the equilibrium constant for site exposure.

How can such binding events be understood structurally? In the nucleosome filament level of folding, a combined uncoiling of DNA on the test nucleosome coupled to a motion of the uncoiled DNA in a direction parallel to the axis of the nucleosomal disk, allows uncoiling beyond the dyad (which is as far as necessary to allow binding anywhere) with no required crossings, only minor deformation of linker DNA, and with little motion of other nucleosomes. Because the 30 nm filament is a marginally stable structure, evidently in dynamic equilibrium with the nucleosome filament state, binding should also be able to occur starting at this level of structure. The binding and chromatin folding equilibria are coupled. Posttranslational modifications of histones or differing histone variants that can influence the stability of the 30 nm fiber will also affect the binding equilibria.

CONCLUSIONS AND PROSPECTS

The tremendous progress in structural analysis of the lowest level of chromatin organization is not yet mirrored by progress in our understanding of higher levels of organization. There are formidable problems. New approaches are plainly required, yet it is not clear at this time what avenues may prove most productive.

Great progress has recently been made in identifying a set of structural proteins and enzymes that are somehow involved in modulating chromatin structure and activity. These represent only a small fraction of the full picture. A host of new proteins and enzymes remain to be discovered; and for all of these, both those discovered already and those yet to be discovered, there remains the problem of elucidating the structures and mechanisms through which they contribute to chromatin function.

It may be appropriate at this time to set ourselves the goal of achieving a quantitative, predictive understanding of gene regulation *in vivo*. In this case, two problems loom immediately. First, we need to learn how gene-regulatory proteins gain access to their target sites in chromatin, what happens to the chromatin when they do so, and how these processes are coupled energetically. Second, we need to learn what fraction of the total nuclear DNA is accessible to proteins at any moment and thus able to compete for regulatory protein binding.

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