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Histones H3 and H4 interact with the ends of nucleosome DNA

(chromatin subunit/methylation/arginine-rich histones/protein–DNA crosslinking)

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ABSTRACT **Isolated HeLa cell nucleosomes (core particles) were labeled at the 5′-termini of their DNA with ^{32}P using [γ-^{32}P]ATP and polynucleotide kinase. The label was crosslinked to a lysyl residue of a neighboring histone by sequential methylation, depurination, Schiff base formation, and reduction with sodium borohydride. After digestion of the noncrosslinked DNA by DNase I and venom phosphodiesterase, histones were separated by gel electrophoresis and those crosslinked to the 5′-termini were identified by ^{32}P-autoradiography. Histones H3 and H4 occur with equal frequency as the nearest protein neighbors to the end of the DNA in nucleosomes. Histone arrangements within the core particle compatible with these results are discussed.**

Morphological and biochemical studies have established the existence of a repetitive subunit structure for eukaryotic chromatin (1–5). Protein octamers, two each of histones H2A, H2B, H3, and H4, apparently are encircled by a 140–160 base pair length of DNA to form ν bodies or nucleosomes (6–13). A 40–60 base pair length of DNA, which may be associated with one molecule of H1, is interspersed between adjacent nucleosomes along the length of a chromatin fiber (11–16).

Emerging evidence has recently suggested features of the internal architecture of both histones and DNA within a nucleosome. Kornberg and Thomas (17) have suggested that the histone core is composed of a tetramer $(H3,H4)_2$, and two dimers, (H2A,H2B); on the other hand, Weintraub *et al.* (18) have observed a "heterotypic tetramer" (H2A,H2B,H3,H4) in histone solutions at high NaCl concentrations, and nearly all the possible pair-wise interactions between these four smaller histones occur in solution (19, 20).

A regular kinking of DNA folded around the protein core of a nucleosome was suggested by Crick and Klug (21); this postulate derives some support from Noll's and our demonstrations that DNase I and rodent liver endogenous endonuclease produce single-stranded DNA fragments which are multiples of ten nucleotides (12, 22, 23). Our studies of DNase I digestion of isolated nucleosomes labeled with a 5′-terminal-^{32}P using [γ-^{32}P]ATP and polynucleotide kinase demonstrated that, indeed, a single-strand scission site for this nuclease exists at 10-nucleotide intervals along the DNA within the nucleosome, although the relative susceptibilities of these potential sites to actual cleavage vary widely (23).

Little is known, however, of the contact interactions between histones and DNA in nucleosomes. The primary sequences of the histones suggested that the amino-terminal regions of these proteins might bind to DNA (24) and studies of Weintraub and his coworkers have documented the validity of this supposition (25). In this communication I describe a new approach to study protein–DNA interactions within the nucleosome. Isolated HeLa cell nucleosomes are labeled at their 5′-termini with ^{32}P and the method developed by Levina and Mirzabekov (26) is then used to crosslink the 5′-terminal-^{32}P label to its nearest histone neighbor. Guanylyl (and possibly adenylyl) residues at the 5′-termini are methylated with dimethylsulfate and depurinated under mild conditions; the aldehyde thus created reacts with a nearby histone lysyl residue to form a Schiff base, which is then reduced by sodium borohydride to form a covalent crosslink between a histone and the 5′-terminal-^{32}P label. Noncrosslinked DNA is digested with DNase I and venom phosphodiesterase, and the histones are separated by gel electrophoresis. The histones that interact with the 5′ end of the DNA in nucleosomes are identified by ^{32}P autoradiography.

EXPERIMENTAL PROCEDURES

Materials. Micrococcal nuclease (29,000 units/mg), DNase I (2940 units/mg), and venom phosphodiesterase (20 units/mg) were products of the Worthington Biochemical Corp. (units are as defined by the supplier). Polynucleotide kinase (30,000 units/mg) was purchased from Miles Corporation. [*methyl*-^{3}H]Thymidine (55 Ci/mmol) was a product of New England Nuclear Corp. and [γ-^{32}P]ATP (25 Ci/mmol) was from ICN Pharmaceuticals Co.

Cell Culture. HeLa cells, clone S3, were maintained in exponential growth in Eagle's spinner medium containing 5% horse serum, 50 μg of streptomycin per ml and 50 units of penicillin per ml. DNA was labeled by addition of 0.2–0.4 μCi of [^{3}H]thymidine per ml for 22 hr prior to harvesting the cells.

Preparation of Nucleosomes. Trimmed nucleosomes were prepared from isolated HeLa cell nuclei by micrococcal nuclease digestion and isokinetic sucrose gradient centrifugation as previously described (13). These particles have a histone/DNA ratio of 1.2 g/g, two each of the four smaller histones, no histone H1, and a DNA fragment 140 ± 5 base pairs in length without internal nicking (8, 13). Prior to modification with polynucleotide kinase and ATP, nucleosomes were concentrated by centrifugation for 18–24 hr at 55,000 rpm and 4° in the Beckman SW 60 rotor. The pellets were resuspended in 0.25 mM EDTA, pH 7.0, at a DNA concentration of 1 mg/ml and stored at −20° until used.

Labeling 5′-Termini and Crosslinking. Isolated nucleosomes were labeled at their 5′-termini exactly as described previously, using [γ-^{32}P]ATP and polynucleotide kinase (23). The concentration of ATP in the labeling reaction was 4 μM; the incorporated ^{32}P corresponded to substitution of 0.25 groups per 140 base pairs of DNA. After reisolation of nucleosomes, either by sucrose gradient centrifugation or by extensive dialysis, further modifications were carried out as follows: (*i*) [^{3}H]thymidine, [5′-^{32}P]nucleosomes were methylated with dimethylsulfate (30 mM) at a DNA concentration of 0.5–1.0 mg/ml in 0.01 M sodium cacodylate, 0.05 M EDTA, pH 7.0, for 16 hr at 4° with shaking; (*ii*) after dialysis into 0.01 M sodium phosphate, 0.05 M EDTA, 0.1 M NaCl, pH 6.0 ± 6 M urea, samples were depurinated for 8 or 20 hr at 37° or 22°, respectively; (*iii*) after depurination, $\frac{1}{10}$ volume of a freshly prepared 1.5 M $NaBH_4$ solution was added for 30 min at 0°, and the samples were dialyzed exhaustively versus 0.25 mM EDTA, pH 7.0, at 4°. The

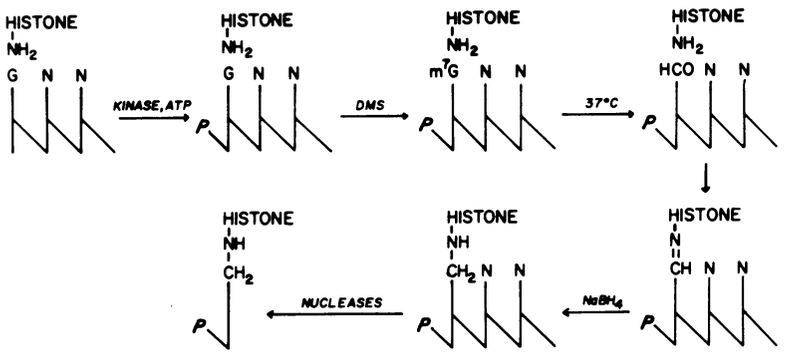

FIG. 1. Scheme for labeling the 5′-termini of nucleosome DNA with ^{32}P and crosslinking the labeled phosphate to a neighboring histone lysyl residue, combining previously described methodology (23, 26). DMS is dimethylsulfate.

expected reactions occurring at the 5′ end of DNA in an isolated nucleosome during this series of treatments are diagrammed in Fig. 1. Depurination and reduction of the Schiff base were carried out in both aqueous and 6 M urea-containing buffers in order to detect protein–nucleic acid interactions occurring both in the folded, compact nucleosome and in nucleosomes extended in urea, where only "primary sequence" histone–DNA interactions would be expected to occur (13, 27–29).

Digestion of Noncrosslinked DNA. Nucleosome samples crosslinked in the presence or absence of urea were adjusted to contain 5 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, warmed to 37°, and digested with 200 units of DNase I per ml. After 60 min of digestion with DNase I, samples were cooled to room temperature, adjusted to contain 0.02 M Tris·HCl, pH 8.9, 0.5 mM $CaCl_2$, and further digested with 2.5 units of venom per ml for 30 min. At the indicated times, aliquots of the digestion mixture were cooled to 0°, added to 250 μg of carrier calf thymus DNA, and precipitated with 10% trichloroacetic acid for at least 30 min at 0°. The precipitates were collected on glass-fiber filters, washed with 5% trichloroacetic acid and with 95% ethanol, and dried. NCS solubilizer (0.2 ml) (New England Nuclear Corp.) containing 10% H_2O was added and the samples were incubated for at least 2 hr at room temperature. After addition of 10 ml of scintillation fluid [0.6% (wt/vol) 2,5-diphenyloxazole, 0.1% (vol/vol) acetic acid in toluene], samples were counted in a Beckman LS 250 liquid scintillation counter. Appropriate corrections were made for spillover from the ^{32}P to the ^{3}H channel.

Gel Electrophoresis and Radioautography. After modification and combined DNase I and venom phosphodiesterase digestion, nucleosome proteins were dialyzed to water, lyophilized, and dissolved in sample buffer. Electrophoresis was carried out on sodium dodecyl sulfate discontinuous polyacrylamide slab gels using the buffer system of LeSturgeon and Rusch (30). The separating gel contained 18% acrylamide, 0.16% bis-acrylamide, while the stacking gel contained 3% acrylamide and 0.08% bis-acrylamide. The distance of migration for bromphenol blue was about 10 cm for most of the data presented; identical results were obtained using gels with a migration distance of 32 cm. Gels were stained for 1 hr in 0.2% Coomassie blue in 50% methanol, 7% acetic acid, and destained by diffusion in 20% methanol, 7% acetic acid containing Dowex 1X2 resin. Stained gels were photographed with Polaroid Type 55 P/N film using a Kodak no. 21 Wratten filter and scanned with an E-C Instruments Co. densitometer. For autoradiography, gels were wrapped in a single thickness of plastic wrap and placed on Kodak RP Royalfilm. Exposures of 3–12 hr were required. Developed autoradiograms were also scanned with the E-C Instruments Co. densitometer.

RESULTS

Fig. 1 shows the sequence of reactions beginning with the attachment of a ^{32}P label to the 5′ end of nucleosome DNA and ending with the covalent binding of that label to a neighboring histone. All modification reactions are performed under relatively mild conditions to minimize the possibility of disruption of nucleosome structure during labeling and crosslinking. The efficiency of the crosslinking reaction is high, as demonstrated by the results of nuclease digestion of particles labeled internally with [^{3}H]thymidine and at the 5′-termini with ^{32}P (see below).

In a previous study (23), digestion of similarly labeled, but not crosslinked, nucleosomes by DNase I led to a parallel loss of the two labels to acid-solubility. In contrast, when nucleosomes subjected to the Levina and Mirzabekov (26) crosslinking procedure are digested with DNase I, the two labels behave in disparate fashions (Fig. 2). At a concentration of 200 units/ml, DNase I degrades about 80% of the [^{3}H]thymidine label to acid-solubility in 60 min, while about 40% of the ^{32}P-5′-terminal label remains precipitable by trichloroacetic acid. Similar results are obtained for samples depurinated and fixed in the absence or presence of 6 M urea. Further digestion with venom phosphodiesterase reduces the acid-insoluble fraction of the [^{3}H]

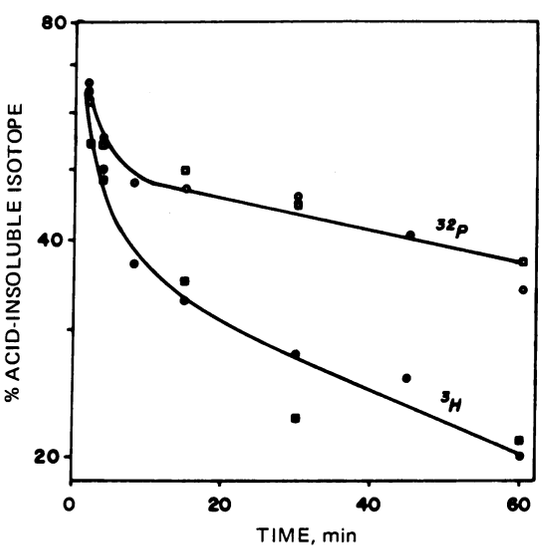

FIG. 2. Digestion of 5′-terminal-^{32}P (○, □) and [^{3}H]thymidine (●, ■) labeled nucleosomes with DNase I. Nucleosomes depurinated and fixed in the absence (○, ●) and presence (□, ■) of 6 M urea were digested for the indicated times with 200 units of DNase I per ml at 37° and the percent of the initial trichloroacetic-acid-insoluble radioactivity was determined.

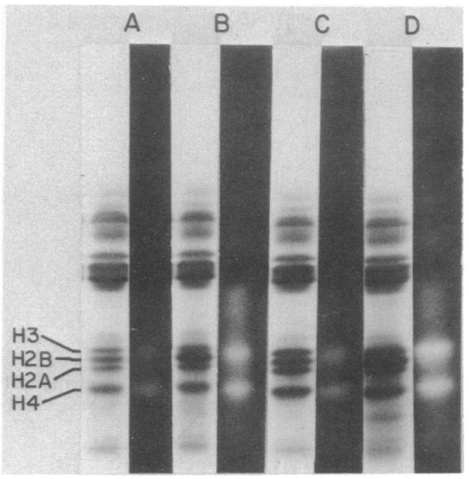

FIG. 3. Gel electrophoresis of histones crosslinked to the 5′-terminal-^{32}P of isolated nucleosomes. The left-hand sample of each pair is the Coomassie-blue-stained gel, while the right-hand sample is the autoradiogram. Conditions for depurination were as follows: (A) 8 hr, 37°; (B) 8 hr, 37°, 6 M urea; (C) 20 hr, 22°, (D) 20 hr, 22°, 6 M urea. All samples were reduced in the same buffer for 30 min at 0° with 0.15 M $NaBH_4$.

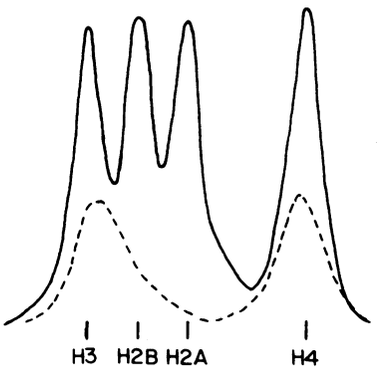

FIG. 4. Densitometric scans of the region of migration of the four smaller histones for the stained gel (—) and autoradiogram (- - -) for sample C of Fig. 3, but electrophoresed on a 32 cm long gel. Migration was from left to right. The positions of migration of the four smaller histones are shown.

thymidine and 5′-terminal-^{32}P labels to less than 5% and about 20%, respectively.

Since thymidine does not undergo methylation, depurination and subsequent crosslinking, the behavior of the ^{3}H label during nuclease digestion reflects the properties of bulk nucleosome DNA. In contrast, acid-precipitable ^{32}P label reflects, primarily, 5′-terminal [^{32}P]guanylyl residues that have been methylated and eliminated and the remaining deoxyribosephosphate crosslinked to histones, although there may be some contribution from formation of 3-methyladenine (31). The difference of about 15% in acid precipitability of the two labels after extensive nuclease digestion demonstrates an efficient level of the crosslinking reaction, since a 20% difference would be expected if this reaction had gone to completion with HeLa cell DNA of known 20% guanine content and a random distribution for the four bases at the 5-termini of isolated nucleosomes.

Quantitation of the residual acid-precipitable DNA phosphate, which might affect histone electrophoretic mobilities, leads to a *maximum* estimate of about one phosphate per histone deriving from internal phosphates, as judged by the ^{3}H label. The internal phosphates will be distributed randomly on all histones and hence should have little, if any, effect on the electrophoretic properties of the proteins. The estimate also includes DNA which may be of sufficient size to be precipitable by trichloroacetic acid and yet is not covalently crosslinked to histone; this DNA would dissociate in sodium dodecyl sulfate electrophoresis buffers.

Gel electrophoresis was carried out on the doubly digested histone samples (Fig. 3). In addition to the four smaller histones, a number of protein bands present in the nuclease preparations are visualized in the stained gel. The histones migrate with mobilities equal to those of purified calf thymus histones; any residual DNA phosphate bound to the proteins does not markedly affect their mobilities in this electrophoretic system. No differences in relative protein distribution are detected among the four samples crosslinked and fixed under the various conditions.

Autoradiography of this electrophoretic gel allows detection of the protein bands that have been crosslinked to the 5′-terminal-^{32}P label of nucleosome DNA. Clearly, only two bands contain the bulk of the ^{32}P label (Fig. 3). The mobilities of these bands correspond exactly to that of H4, on the one hand, and closely to that of H3, on the other, both visually on inspection of the gel and autoradiogram (Fig. 3) and in densitometric scans of the two samples (Fig. 4), even though the autoradiogram bands are much broader than those of the stained gel, due to the long range of the ^{32}P beta particle. The ^{32}P band near H3 migrates slightly faster than the stained protein, as would be expected for a protein modification that reduced the positive charge of the histone and, hence, made its electrophoretic behavior on sodium dodecyl sulfate gel electrophoresis less anomalous. No widening of the stained protein band corresponding to H3 is noted, since only trace amounts of the histone are actually crosslinked to the phosphate label. The band positions for ^{32}P and stained protein correspond exactly for histone H4. Again, only H3 and H4 are crosslinked to the ends of DNA in samples treated in the presence of 6 M urea, suggesting that the same histone–DNA interactions exist in folded and extended nucleosomes. H2A and H2B are not crosslinked to the ends of nucleosome DNA, demonstrating clearly that there is not a random association of histone proteins with DNA within the chromatin subunit.

DISCUSSION

Polynucleotide-kinase-catalyzed labeling of the 5′-termini of nucleic acids has been utilized in studies of nucleic acid sequences and repair. We previously applied this technique to labeling the ends of DNA in an isolated nucleoprotein, the eukaryotic nucleosome, and were able to define the positions of cleavage of nucleosome DNA by DNase I and judge the relative protection of potential scission sites by binding to histones (23). Here, I have used this method, in conjunction with the mild procedure for crosslinking DNA and protein developed by Levina and Mirzabekov (26), to begin investigations of protein–nucleic acid interactions within the chromatin subunit. Specifically, one can determine which histone(s) is (are) near the ends of the DNA in an intact nucleosome and thereby gain information as to the symmetry of histone arrangement along the DNA of the chromatin subunit. The results are clear—histones H3 and H4 are crosslinked with equal probability to the ends of nucleosome DNA and must therefore occupy positions within the particle at the ends of the DNA. In contrast, histones H2A and H2B are not crosslinked to the ends of the DNA and consequently must be located along internal stretches of DNA within the chromatin subunit.

Crosslinking experiments of this type potentially can reflect

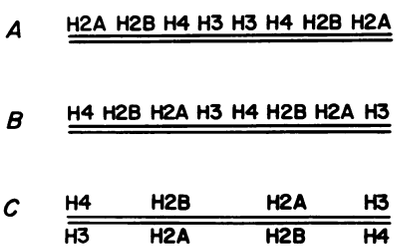

FIG. 5. Diagrammatic representation of three possible primary sequences of histones along the DNA within a nucleosome. The sequences represent the order of histones when histone–histone interactions have been disrupted (as by urea) sufficiently to allow the particle to unfold with extension of the DNA. (*A*) A symmetrical arrangement incorporating an $(H3,H4)_2$ tetramer core with (H2A,H2B) on the wings; (*B*) an arrangement with a polar sequence of histones along the DNA; and (*C*) a symmetric arrangement which requires that the H3,H4 dimer (minimally) be not dissociated in urea, even though interactions between H3,H4 dimers are disrupted.

two types of histone–DNA interactions. First, the label can be crosslinked to the histone that actually binds the 5′ end of nucleosome DNA. Second, if the end of the DNA possesses some mobility, the possibility exists that it might be crosslinked to histones that are near it only in the folded particle—not actually bound to the DNA but brought into the neighborhood by the histone–histone interactions that create the compact protein core of the chromatin subunit. To distinguish between these two types of crosslinking, depurination and fixation were carried out in both aqueous and urea-containing buffers. Urea disrupts some histone–histone interactions, generating an "extended" nucleosome, and thereby allows detection of primarily the first category of interactions. Interestingly, the crosslinks generated between DNA ends and histones are the same in the absence and presence of urea—that is, only histones H3 and H4 are crosslinked to the 5′-terminal-^{32}P label.

The most interesting result of the present study is the equal probability that the 5′ end of nucleosome DNA is crosslinked to either histone H3 or histone H4. Symmetry in the arrangement of histones within the nucleosome has been a feature of some models for the structure of the protein core of the chromatin particle (Fig. 5A; refs. 6, 18, and 32). The current study suggests that the arrangement of histones along DNA might be polar (Fig. 5B), with a molecule of H4 at one end of the nucleosome DNA and an H3 at the other. Alternatively, if partial, but not complete, disruption of histone–histone interactions occurs in 6 M urea (that is, H3–H4 stays as a dimer) and there is sufficient mobility in the 5′-label so that it couples to neighboring H3 or H4 with equal frequency, one can maintain a symmetric histone arrangement along DNA, such as is diagrammed in Fig. 5C. Although this latter possibility seemed unlikely in view of the demonstration that the isolated $(H3,H4)_2$ tetramer is dissociated by urea (33, 34) and on consideration of the limited length of the crosslinker, <8 Å from the phosphate backbone of the DNA to the modified lysyl residue, it derives credence from my unpublished observations which show that the H3–H4 dimer detected by carbodiimide crosslinking (35) is maintained in nucleosomes in 6 M urea. Thus, either histone arrangement 5B or 5C is consistent with the current data.

The ordering of the histones depicted in Fig. 5B and 5C includes the strong interactions between H3 and H4, on the one hand, and H2A and H2B, on the other (17). The orderings are also consistent with experiments using "zero-length" crosslinkers, which demonstrate the proximity of H4 to H2B, of H2A to H2B, and of H3 to H4 (35–37). If one folds the sequence depicted in Fig. 5B into a 2-turn helix or that in Fig. 5C into a 1-turn helix, it is apparent that an $(H3,H4)_2$ tetramer would be located on one side of the structure thus created. This tetramer would be located at the entry and exit points for DNA on the nucleosome, where the nucleic acid makes a transition in conformation from the bridge structure, likely extended B-form, to the conformation characteristic of DNA in the particle. The histones bound to DNA at these transition points are H3 and H4—an appropriate pair in terms of their very strong interactions with DNA, the rigid conservation of their primary sequences over long evolutionary periods, and their tendency to form a stable tetrameric molecule (17, 38). Further aspects of their role in folding DNA into a nucleosome are included in a model for structure of the chromatin subunit which will be presented.

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