begun a series of experiments designed to distinguish between these alternatives.

We currently are engaged in determining the precise locations of the various cross-links which have been described in this paper. We are also enlarging the collection of cross-linked species using these and other contact-site cross-linkers. We anticipate that it should be possible eventually to map fairly precisely the binding surfaces of the histones by means of saturation cross-linking of the type we have described.

Acknowledgments

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Specific Histone–Histone Contacts Are Ruptured When Nucleosomes Unfold at Low Ionic Strength†

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ABSTRACT: The ordered unfolding of the nucleosome core within chromatin at low ionic strengths has been studied. The results show that, when nuclei are lysed gently in solutions of very low ionic strength, their constituent nucleosomes rupture at a major H2B–H4 binding site but remain unperturbed at the site of the H2A–H2B interaction. These conclusions are based on data which show that at least four separate but closely spaced H2B–H4 contacts, identifiable by contact-site cross-linking in intact nuclei, are broken when nuclei are suspended in very dilute buffers. Appropriate controls on purified nucleosome monomers demonstrate that the H2B–H4 contacts being broken are indeed intranucleosomal. Sedimentation of nucleosomes in the ultracentrifuge at various salt concentrations reveals that a significant conformational transition occurs in the range of ionic strength over which the H2B–H4 binding site ruptures.

One of the paradoxes of chromatin function is that DNA, though tightly complexed with histones, nevertheless participates in the metabolic functions of replication and transcription. The fact that chromatin is structurally heterogeneous suggests that changes in chromatin structure may be prerequisite, or at least indicative, of changes in its functional state (Elgin & Weintraub, 1975). Now that the fundamental outlines of chromatin structure are known (Kornberg, 1977; Felsenfeld, 1978), it is possible to ask well-defined questions concerning the nature of the conformational transitions which accompany alterations in function (Weintraub et al., 1976).

Chromatin is now known to consist of subunits called nucleosomes [for reviews, see Kornberg (1977) and Felsenfeld (1978)], each composed of a core of eight tightly complexed histones about which 144 or more base pairs of DNA are wrapped. The core consists of two each of histones 2A, 2B, 3, and 4. Histone 1 is not considered part of the core, although most or all nucleosomes may bear H1 as a peripheral component.

Controlled digestion of nuclei with staphylococcal nuclease results in the release of nucleosome core particles which can be purified by sucrose-gradient sedimentation or gel-exclusion chromatography. Nucleic acid reassociation analysis of the DNA in core particle preparations has provided strong evidence that all chromosomal DNA sequences, including those actively engaged in transcription, are packaged as nucleosomes (Reeves, 1976; Garel & Axel, 1976; Garel et al., 1977; Panet & Cedar, 1977; Levy et al., 1979). Nucleosomes can also be visualized in the electron microscope, and such studies suggest that regions of chromatin actively engaged not only in transcription (Foe et al., 1976) but also in replication (McKnight & Miller, 1977) remain packaged in nucleosomal form. Moreover, both replication and transcription can be carried out in vitro on SV40 minichromosomes apparently

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without loss of the nucleosomal histones from the template (Su & DePamphilis, 1976; Green & Brooks, 1977). These results imply a requirement for some nucleosomal unfolding mechanism which would facilitate the passage of the DNA and RNA polymerases. Indeed, transcriptionally active chromatin is known to be of a different conformation than transcriptionally inactive chromatin (Garel et al., 1977; Garel & Axel, 1976; Flint & Weintraub, 1977; Wallace et al., 1977; Gottesfeld & Butler, 1977; Gottesfeld & Partington, 1977; Weintraub, 1975; Weintraub & Groudine, 1976; Foe et al., 1976; Reeves, 1976; Panet & Cedar, 1977; Levy et al., 1979).

Weintraub et al. (1976) have suggested a specific way in which nucleosomes may unfold preparatory to or in response to replication or transcription by opening into two symmetrical half-nucleosomes. Recent evidence in favor of the half-nucleosome model has been reported by Oudet et al. (1977). They found by electron microscopy that, at very low ionic strength, nucleosomes do indeed apparently split into half-nucleosomes. Recent physical-chemical studies on purified nucleosomes in solution also demonstrate unfolding at low ionic strengths (Gordon et al., 1978).

In the experiments described below, we have initiated a detailed biochemical characterization of the nucleosome unfolding which occurs at low ionic strength. The experiments are based on the earlier observation that an H2A–H2B–H4 trimer could be induced by UV light in nuclei under physiological conditions but not in chromatin at low ionic strengths (Martinson et al., 1976, 1979b). We now show, using several contact-site cross-linking probes (Martinson et al., 1979b), that lack of cross-linking at low ionic strength reflects the rupture of a major intranucleosomal contact between H2B and H4. We also show that another major intranucleosomal contact, that between H2A and H2B, is unaffected by low ionic strength. We are able to correlate our high and low ionic strength cross-linking results from nuclei with a physical conformational transition in nucleosomes measured hydrodynamically. Our results do not yet provide evidence for or against specific proposals such as the half-nucleosome model of Weintraub et al. (1976).

Materials and Methods

Preparation of Nuclei and Histones. Calf-thymus nuclei were prepared in phosphate-buffered saline–EDTA, pH 7.4, as described (Martinson et al., 1979a,b). About 5 g of frozen thymus was blended at high speed in 800 mL of buffer, filtered, and centrifuged. The nuclei were washed by homogenization in 200 mL of buffer, collected by centrifugation, and resuspended for use as described in the text. The concentration was determined from the A_{260} value of an aliquot diluted 1:10 or more with a solution of 2 M NaCl, 5 M urea.

Histones were prepared from nuclei by extraction with 0.3–0.5 N H_2SO_4. If the nuclei were not already in solutions of low salt concentration (e.g., 20 mM NaCl or less), they were sedimented and resuspended in distilled water before the addition of acid. The histones were recovered by acetone precipitation.

Cross-Linking. Large-volume irradiations were conducted as described (Martinson et al., 1979b) in a Pyrex reaction vessel into which a 450-W Hanovia medium-pressure mercury lamp was lowered. The dimensions of the reaction vessel were chosen so as to accomodate about 1.1–1.2 L of sample in such a way that the lamp was completely surrounded by the sample. Irradiation was begun by turning on the lamp and was conducted with stirring under a constant stream of nitrogen. The irradiation times specified in the text include the time taken for the lamp to reach full intensity after being turned on. Irradiation times were selected empirically, and no attempt has been made to calculate irradiation dosages. Small samples (less than 3 mL) were irradiated in 7-mm internal diameter quartz tubes as described (Martinson et al., 1979a).

Tetranitromethane [$C(NO_2)_4$] cross-linking at low ionic strength required considerable care because of the limitation on buffer concentration when the salt concentration must be kept low. Protons are released during the $C(NO_2)_4$ reaction, causing the pH of the medium to drop. Consequently, because the reaction itself is pH dependent, it is necessary to limit the production of protons. This was accomplished by reducing the chromatin concentration to very low levels. Thus in a typical experiment employing 0.1 mM phosphate as buffer, nuclei were suspended to give an A_{260} value of no greater than 0.3. About 0.1 mL/L of $C(NO_2)_4$ was then added, and the reaction was allowed to proceed for 4 h at room temperature. Under these conditions the pH does not change significantly during the reaction. When the system is well purged with nitrogen, the cross-linking reaction reaches a limit sometime between 1 and 4 h, and little further change is observed even if the reaction is allowed to proceed overnight with further addition of $C(NO_2)_4$ [cf. Martinson & McCarthy (1975), where purging was not used]. To facilitate collection of the chromatin by centrifugation, one-tenth volume of 10 times concentrated phosphate-buffered saline–EDTA, pH 5.5, was added. The increased salt concentration induces aggregation, while the low pH stops any further action of the $C(NO_2)_4$ on the aggregated chromatin.

For cross-linking with formaldehyde (HCHO), nuclei were suspended in the appropriate concentration of NaCl at an A_{260} of 2.5 by use of 0.1 mM phosphate, pH 7.5, as buffer. The reaction was conducted at room temperature with HCHO (Mallinckrodt) at 0.05%. The HCHO was neutralized with 0.25 M Na_3PO_4 just prior to use. Cross-linking reactions conducted in NaCl solutions of 10 mM or less were terminated directly by the addition of acid, and the histones were then prepared as usual. No cross-linking occurs during the course of acid extraction. Cross-linking conducted in NaCl solutions of 150 mM was terminated by rapidly centrifuging the nuclei and resuspending them in distilled water for acid extraction.

Preparation of Nucleosomes. The chromatin monomers used in our experiments contained from 140 to 180 base pairs of DNA. Most of them were thus larger than core particles but smaller than a complete chromatin repeating unit. For convenience we refer to them as nucleosomes. They were prepared as before (Martinson et al., 1979a) but with the following trivial variations. No Triton was used in the preparation of nuclei. Digestion was conducted with micrococcal nuclease (Sigma) at 2.4 units/mL for 50 min to give 35% acid solubility of the DNA. The nuclear digest was then resuspended in 0.6 M NaCl, 1 mM EDTA, 3 mM sodium azide, 1 mM Tris, pH 7.5, and fractionated on Sepharose 4B by use of the same buffer as eluant.

Polyacrylamide Gel Electrophoresis. Column-fraction aliquots from nucleosome preparations were analyzed for DNA size on 4% polyacrylamide–NaDodSO$_4$ gels (by use of 0.5 μL/mL of TEMED and with 0.75 mg/mL of ammonium persulfate added after degassing) as described by Todd & Garrard (1977). A set of sequenced restriction fragments served as standards. Histones were analyzed on 18% polyacrylamide–NaDodSO$_4$ stacking gels as described by Bonner & Pollard (1975).

Acid–urea slab gels were run according to Panyim & Chalkley (1969) as previously described (Martinson et al., 1976). Histone samples were dissolved in 5–8 M urea, 10%

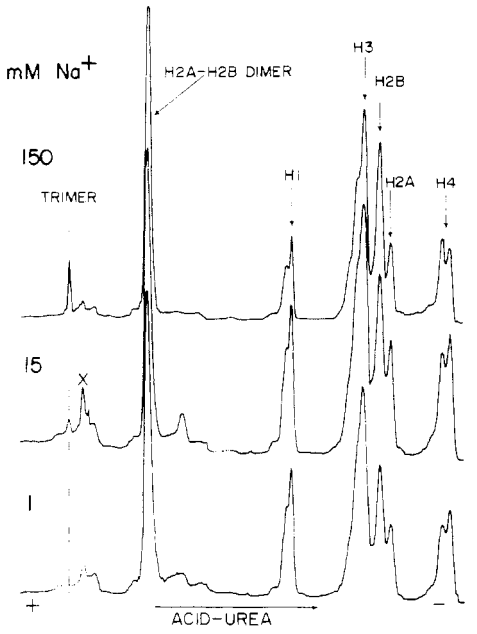

FIGURE 1: Effect of salt concentration on H2A–H2B–H4 trimer production by UV light. Nuclei (13 mL) at an A_{260} of 15.4 were centrifuged at 2000*g* for 15 min. The surface of the resulting nuclear pellet was rinsed with a buffer containing 150, 15, or 1 mM Na^+ as indicated in the figure. The pellet was resuspended in about 1100 mL of the same buffer to give an A_{260} of about 0.18 and then irradiated for 15 min. The pH remained between 7.4 and 7.5 throughout the procedure. The irradiated chromatin was collected by centrifugation at 2000*g* (the 15- and 1-mM samples were supplemented with NaCl to facilitate aggregation and sedimentation). The pellet was resuspended at an A_{260} of 18 with distilled water. The histones were prepared by acid extraction and electrophoresed on an acid–urea gel. The arrow represents the base line for the bottom scan which exhibits the highest background of the three. Irradiation at 1 mM Na^+ reduces the yield of acid-extractable histone by about twofold, perhaps because of enhanced protein–DNA cross-linking. The amounts of histone loaded per gel slot were chosen to give approximately the same amount of dimer. The buffers used were as follows: 150 mM Na^+–phosphate-buffered saline–EDTA; 15 mM Na^+–10 mM sodium phosphate; and 1 mM Na^+ (approximately)–0.5 mM sodium phosphate.

β-mercaptoethanol and were loaded as such on either acid–urea or $NaDodSO_4$ gels. Staining of the protein gels with Coomassie Blue and destaining with acetic acid were as previously described (Martinson & McCarthy, 1975). The gels were scanned with a Joyce Loebl densitometer.

Results

"Labile" H2A–H2B–H4 UV Trimer. It was noted previously in the course of characterizing the H2A–H2B dimer produced by UV light that a histone trimer also was produced (Martinson et al., 1976). This trimer has since been identified as H2A–H2B–H4 (Martinson et al., 1979b). However, trimer production occurred only when cells were irradiated directly, not when isolated chromatin in 1 mM EDTA was irradiated (Martinson et al., 1976). In order to determine whether the lack of trimer production in chromatin was a consequence of the low salt concentration or of the chromatin isolation procedure, we have irradiated calf-thymus nuclei suspended directly in buffers of various ionic strengths. Following irradiation, the histones were isolated and subjected to acid–urea polyacrylamide gel electrophoresis. Figure 1 shows that nuclei suspended in 150 mM Na^+ give rise to trimer production upon irradiation, whereas nuclei lysed by resuspension in 1 mM Na^+ do not (for an electrophoretic profile of uncross-linked histones, see the top scan of Figure 6). Nuclei suspended in 15 mM Na^+ yield intermediate levels of trimer upon irradiation. The irradiations at all three salt concentrations were conducted by the same method on the same nuclear preparation. Thus the inability of UV light to produce a cross-linked trimer at low salt concentrations appears to be a direct consequence of that parameter itself.

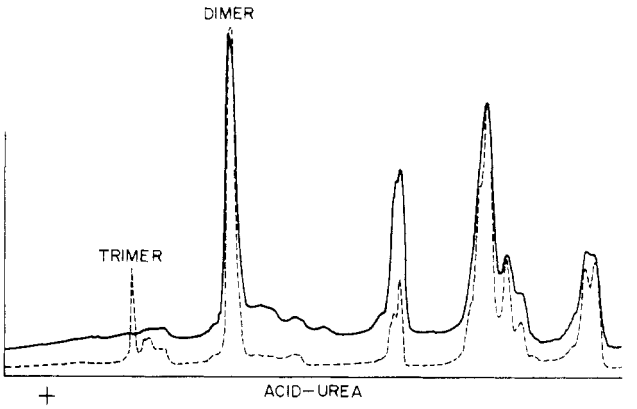

FIGURE 2: Effect of irradiation time on trimer production. Nuclei were resuspended in either 150 mM Na^+ (dashed line) or 1 mM Na^+ (solid line) and irradiated as described for Figure 1, except that the irradiation time was nearly twice as long. Electrophoresis in an acid–urea gel was also conducted as for Figure 1. The origin is indicated at the left and migration was from left to right. The curves are superimposed relative to the same base line as indicated. The amount of protein loaded on the gel was adjusted to give approximately equal amounts of dimer. As can be seen from the tracings, approximately twice as much protein from the low-salt chromatin was loaded on the gel, much of which shows up as background.

Nevertheless, the very phenomenon of nuclear lysis, no matter how gentle, inevitably involves a certain amount of shear. This is probably not the explanation of these results, since we have often (but not always) been able to regenerate trimer-forming capacity in lysed nuclei by mere readdition of salt. More importantly, we can consistently obtain the trimer cross-linking pattern from nucleohistone reconstituted from acid-extracted histones using a salt–urea protocol (Martinson et al., 1979a). When this reconstituted material is dialyzed to low salt concentrations, the trimer is not formed upon irradiation.

The effect of low salt on trimer production is independent of irradiation time. Figure 2 shows the results of doubling the irradiation time for nuclei suspended in 150 mM Na^+ (dashed line) or 1 mM Na^+ (solid line). More extensive time-course studies have confirmed that no trimer can be formed in chromatin at very low salt concentrations even if irradiation is continued until all histones become nonextractable by acid.

Stable H2A–H2B Contact. Figures 1 and 2 also demonstrate that, despite the effect on the trimer, the yield of the H2A–H2B dimer is not influenced by decreasing the salt concentration. This shows that it is not the H2A–H2B contact of the trimer that is labile in low salt. Moreover, this illustrates that the inability to produce the trimer is not the result of some generalized interference with cross-linking at the low ionic strength and suggests that the loss of trimer production is a specific effect of low salt concentration on the integrity of the H2B–H4 contact, not on the chemistry of cross-linking.

This interpretation is strengthened by the observation that the yield of the cross-linked product labeled X, which migrates slightly ahead of the trimer in Figure 1, varies in yet a different way as a function of salt concentration. Thus while the yield of the more slowly migrating trimer decreases steadily with salt concentration, the yield of product X first increases at intermediate concentrations of salt and then decreases. Only suspension of the nuclei in distilled water can cause this component to disappear completely. Component X is probably

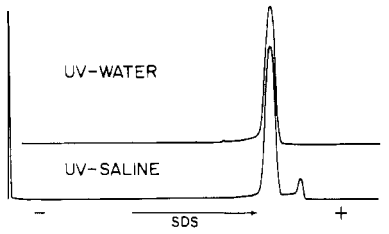

FIGURE 3: Effect of salt concentration on H2B–H4 dimer production by UV light. UV cross-linking was conducted as described for Figure 1 on nuclei resuspended either in water or in phosphate-buffered saline–EDTA. The histones were acid-extracted and electrophoresed on an acid–urea gel. After staining and destaining, the dimer bands were excised and equilibrated briefly in 1% $NaDodSO_4$, 3 M urea, 0.12 M Tris, pH 7. A 1.5-mm thick $NaDodSO_4$ gel was then overlayed with 0.1% agarose, 0.125 M Tris, pH 6.8. The excised gel bands were positioned in this agarose layer on top of the stacking gel with their long axes vertical. The gel was run, stained, and destained in the normal way, and each lane was scanned. The background for each scan was nearly base line and therefore is not represented by the arrow in this figure. The H2B–H4 dimer is the small peak of slightly greater mobility.

also an H2A–H2B–H4 trimer. We will return to this observation in the Discussion section.

Labile UV H2B–H4 Contact. The great stability of the H2A–H2B contact in chromatin allows the surmise that failure to produce the H2A–H2B–H4 trimer in low salt concentration is the result of rupture of the H2B–H4 contact. In order to prove this point directly, the effect of low salt concentration on production of the UV-induced H2B–H4 dimer (Martinson et al., 1979b) was investigated. This dimer has the same mobility as the H2A–H2B dimer in our acid–urea gels (Martinson et al., 1979b) so a second-dimension electrophoresis in $NaDodSO_4$ was used for its analysis.

Nuclei were suspended either in 150 mM Na^+ or distilled water and irradiated. The histones from each sample were isolated and electrophoresed in separate lanes on an acid–urea slab gel as usual. After staining the gel, the single dimer band in each of the two lanes was excised and the dimer composition analyzed on an $NaDodSO_4$ gel. Figure 3 shows that, just as with the trimer, the H2B–H4 dimer is not formed upon UV irradiation in water (UV-water) but is formed upon irradiation of nuclei at physiological salt concentrations (UV-saline).

H2A–H2B and H2B–H4 Contacts Are Intranucleosomal. The contacts which give rise to the cross-linked trimer could occur either within the nucleosome or between adjacent nucleosomes along the chromatin fiber. In order to determine whether the low-salt labile contact is intra- or internucleosomal, we purified both mono- and oligonucleosomes from staphylococcal nuclease digested calf-thymus nuclei. The mono- and oligosomes were then irradiated and their histone cross-linking patterns compared by $NaDodSO_4$ gel electrophoresis. Acid–urea gels were not used for this experiment because of interference by small fragments of DNA which coisolate with the histones.

The top scan of Figure 4 shows the protein content of untreated mononucleosomes. Oligosomes give the same pattern. The monosomes and oligosomes were purified by chromatography in the presence of 0.6 M NaCl and therefore lack H1 and most nonhistones. The second and third scans of Figure 4 show that, upon irradiation, both the mono- and oligosomes give rise to prominent high molecular weight peaks corresponding to cross-linked products. Among these is the UV-induced H2A–H2B–H4 trimer which, like all of the other cross-linked components, appears to be produced in essentially identical stoichiometry for both monosomes and oligosomes. We therefore conclude that the contacts which give rise to the

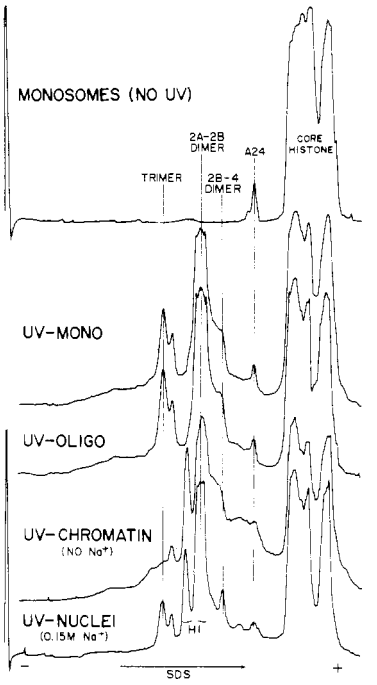

FIGURE 4: UV cross-linking of mononucleosomes. Samples (0.6 mL) of mono- and oligonucleosomes at an A_{260} of 7.3 were irradiated for 15 min in the small quartz tubes. The $NaDodSO_4$ gel electrophoretic patterns of the histones from unirradiated monosomes, irradiated monosomes, and irradiated oligosomes are compared to the patterns obtained from chromatin irradiated in the absence of salt and from nuclei irradiated in phosphate-buffered saline–EDTA. The positions of migration of H3, H2B, H2A, and H4 are designated collectively as "core histone" in the figure. The mono- and oligosomes for this experiment were taken from a stock prepared as described under Materials and Methods, except that the nuclei were prepared in phosphate-buffered saline–EDTA and digested at an A_{260} of approximately 100 in 1 mM CaCl, 0.25 M sucrose, 10 mM Tris, pH 7.5. Digestion was to 25% acid solubility of the DNA with 3 units/mL of nuclease. Two column fractions were selected for cross-linking analysis. Fraction 103 contained predominantly 140–170 base pair length DNA and a small amount of shorter material with no disome-length DNA detectable even on heavily overloaded DNA gels. Fraction 81 consisted primarily of trisome- and tetrasome-length DNA with no detectable disome DNA. The oligosome fraction was concentrated by use of a Millipore immersible separator from an A_{260} of about 2 to the A_{260} of 7.3 used during irradiation. The monosome fraction which was taken from near the peak of the elution profile was diluted with column elution buffer from an A_{260} of 40 to the A_{260} of 7.3. Cross-linking was done directly in the 0.6 M NaCl buffer, and the salt was removed from the samples after irradiation by dialysis against 0.4 N H_2SO_4. Following centrifugation the histones were collected by acetone precipitation. The bottom scan of the figure (UV-nuclei) is the electrophoretic profile in $NaDodSO_4$ of the same sample as shown in the top scan of Figure 1. The next to the bottom scan (UV-chromatin) is the electrophoretic profile of histones extracted from nuclei which were suspended in distilled water for irradiation.

H2A–H2B–H4 trimer are intranucleosomal.

The identity of the trimer band indicated in Figure 4 with that shown previously on acid–urea gels was determined by two-dimensional gel electrophoresis (not shown). Further demonstration that the trimer of Figure 4 is the same as that of Figure 1 is given by the bottom two scans of Figure 4, which allow comparison of the electrophoretic profiles of chromatin cross-linked in the absence and in the presence of Na^+. The trimer component is absent from the histones of chromatin irradiated in the absence of salt. The trimer is absent also from H1-free oligosomes irradiated in the absence of salt (not shown).

The H2B–H4 dimer region of the monosome and oligosome profiles is obscured by heterogeneous material which is produced and which prevents direct visualization of the

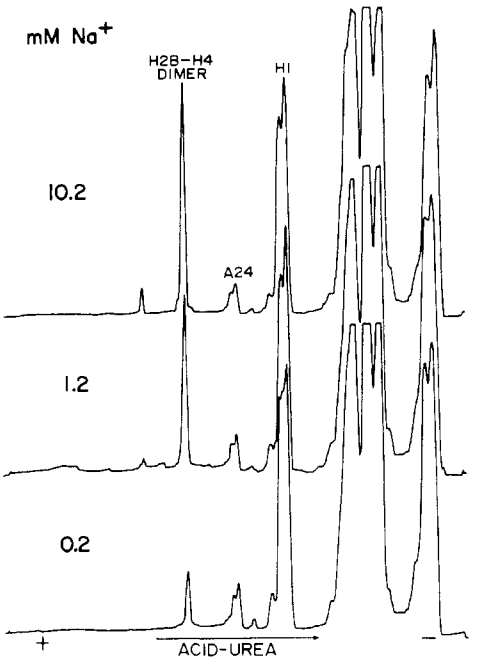

FIGURE 5: Effect of salt concentration on the production of H2B–H4 dimer by $C(NO_2)_4$. Cross-linking with tetranitromethane at low ionic strength was carried out as described under Materials and Methods. After cross-linking, the chromatin was collected as described, and the histones were extracted and run on an acid–urea polyacrylamide gel. The 0.1-mM phosphate buffer used in the cross-linking reaction was either unsupplemented (bottom scan) or supplemented with 1 mM NaCl (middle scan) or 10 mM NaCl (top scan). The approximate resulting Na^+ concentrations are shown in the figure. As can be seen, slightly more histone was loaded in the gel slot represented by the bottom scan in order to demonstrate convincingly the reduced dimer yield at the low salt concentration. The backgrounds were about the same for all three of the scans. The actual base line for the bottom scan is represented by the arrow.

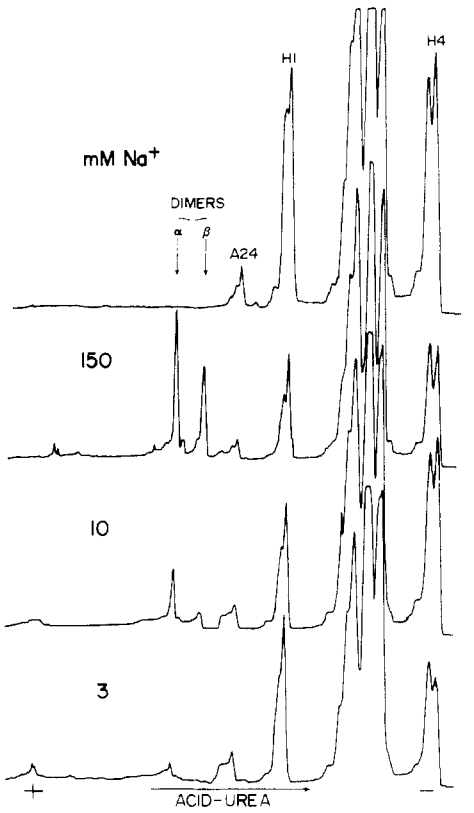

FIGURE 6: Effect of salt concentration on the production of H2B–H4 dimers by formaldehyde. Cross-linking was carried out for 1 h as described under Materials and Methods. The 0.1-mM phosphate cross-linking buffer was supplemented with the indicated concentrations of NaCl. All samples were acid-extracted and electrophoresed on acid–urea polyacrylamide gels. The top tracing is the electrophoretic profile of acid-extracted proteins from uncross-linked nuclei. The actual base line was not determined for this set of scans.

H2B–H4 dimer. We do not know what this material is but the important point is that the monosome and oligosome profiles are nevertheless indistinguishable in this regard. Bonner (1978) has shown that isolated nucleosomes differ somewhat from chromatin in their cross-linking properties.

$C(NO_2)_4$ H2B–H4 Contact Also Is Labile. $C(NO_2)_4$ induces an H2B–H4 cross-link at a contact different from but close to that cross-linked by UV (Martinson et al., 1979b). The $C(NO_2)_4$ susceptible contact should therefore also be labile at low salt concentrations. Since cross-linking of H2B to H4 by use of $C(NO_2)_4$ was first demonstrated employing chromatin in 1 mM EDTA [Martinson & McCarthy (1975), Figure 1], it seemed unlikely that this H2B–H4 contact would prove to be as labile as the one fixed by UV. In salt-dependence experiments similar to those of Figure 1, this expectation was borne out. Nevertheless, as shown in Figure 5, between 10 and 0.2 mM Na^+ a substantial decrease in dimer yield is observed, most of which occurs between 1.2 and 0.2 mM Na^+. Based on what is known of the mechanism of $C(NO_2)_4$ cross-linking (Martinson & McCarthy, 1975), it is difficult to imagine that a decrease in 1 mM of Na^+ concentration could so dramatically affect the chemistry of cross-linking. As for UV, therefore, the most reasonable explanation for the reduction in cross-linking efficiency lies in a conformational transition.

As was done for UV cross-linking, the $C(NO_2)_4$ reaction has also been carried out on mono- and oligonucleosomes (not shown). Again the cross-linking pattern is more complicated than for nuclei or chromatin. Nevertheless, mono- and oligosomes from a given preparation always yield identical patterns of cross-linking, and dimer formation is abolished at low ionic strength.

HCHO α and β H2B–H4 Contacts Also Are Labile. Like UV and $C(NO_2)_4$, HCHO also cross-links H2B to H4 within the same major region of contact (Martinson et al., 1979b). We therefore investigated the effects of salt concentration on HCHO cross-linking. The top two scans of Figure 6 show the acid–urea electrophoretic profiles for histones of uncross-linked and HCHO-cross-linked nuclei at physiological salt concentration. The bottom two scans show that as the Na^+ concentration is reduced to 3 mM both the α and β H2B–H4 contacts apparently are broken. Although in this case we cannot argue a priori [as for the UV and $C(NO_2)_4$ situations] that a conformational change rather than cross-linking chemistry is the best explanation of the results, all things considered, it seems to us that this is so (see below). As for UV cross-linking, we have verified that the α and β dimers can be produced in similar yield in mono- and oligonucleosomes and therefore represent intranucleosomal contacts.

Van Lente et al. (1975) have reported that HCHO cross-linking does not vary with salt concentration down to 0.2 mM EDTA. However, they used much higher concentrations of chromatin than we normally do. It therefore seems likely that the actual salt concentrations in their preparations were much higher than they reported. This interpretation is supported by our observation (not shown) that we can reproduce the results of Figure 6 using their methods provided the chromatin is dialyzed against low ionic strength buffer prior to cross-linking. In addition, Van Lente et al. (1975) have reported that only one of two major HCHO-cross-linked

products is an H2B–H4 dimer, the other being an H2A–H2B dimer. This does not necessarily conflict with our HCHO-cross-linking results [Figure 6 and Martinson et al. (1979b)] in which the two major cross-linked dimers were both shown to be H2B–H4. We have made no specific attempt to reconcile our observations, but we note the following. First, there is the obvious difference in tissue used, chick erythrocyte vs. calf thymus. Second, there is the difference in the polyacrylamide gel system used. Our primary analysis was on acid–urea gels in order to avoid interference with the dimer region of the gel by H1 and to allow distinction of configurational isomers; their analysis was on an $NaDodSO_4$ gel. Thus their cross-linked dimer band X-1 may, in fact, correspond to both dimers α and β which comigrate in their gel system. Furthermore, our HCHO reaction does produce multiple dimer components which elute from P30 columns in the H2A–H2B dimer region (see Figure 1 of Martinson et al., 1979b). Perhaps their cross-linked product X-2 corresponds to several isomeric H2A–H2B dimers which comigrate in their $NaDodSO_4$ system but not in acid–urea.

Nucleosome Unfolding Accompanies Disruption of the H2B–H4 Contact. To examine more directly the interpretation that nucleosomes are actually unfolding at very low concentrations of salt, we carried out hydrodynamic measurements (Gordon et al., 1978) on purified calf-thymus nucleosomes in very dilute phosphate buffer containing various amounts of added salt. Figure 7A shows the relationship between sedimentation coefficient and sodium ion concentration for a preparation of mononucleosomes. The results show that the nucleosomes undergo a decrease in sedimentation coefficient below 5 mM Na^+. In a detailed study of chicken erythrocyte nucleosomes, Gordon et al. (1978) have shown that this change in sedimentation coefficient reflects a reversible conformational change and is blocked completely by cross-linking with HCHO. One to five millimolar Na^+ is within the range of salt concentrations where the cross-linking changes discussed above were observed (see Figure 7B), confirming that the changes in the cross-linking pattern are accompanied by significant changes in overall conformation.

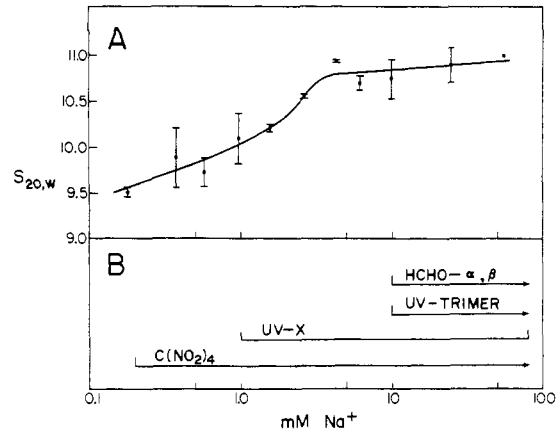

FIGURE 7: (A) Effect of salt concentration on the sedimentation coefficient of nucleosomes. Nucleosomes were prepared as described under Materials and Methods. Four fractions were pooled, concentrated from 32 to 2.7 mL by use of a Millipore immersible separator, and then dialyzed to 0.5 mM Tris, 0.1 mM EDTA, pH 7.6, and an A_{260} of approximately 125. This sample contained predominantly 140–180 base pair length DNA with no disome-length DNA detected even on heavily overloaded gels. Two buffers (0.1 mM $NaPO_4$, 0.01 mM EDTA, pH 7.2, and 0.1 mM phosphate, 0.01 mM EDTA, 50 mM NaCl, pH 7.2) were combined in varying proportions to provide solutions at different salt concentrations to which aliquots of the nucleosomes were added to yield a final A_{260} of 0.55 for each sample. A 160-μL aliquot of each sample was loaded directly into a double sector cell, and a 220-μL aliquot in which a grain of sucrose had been dissolved was underlaid to reduce convection. These samples were centrifuged to 40 000 or 44 000 rpm in a Beckman Model E ultracentrifuge, and the boundaries were monitored by use of a photoelectric scanner. The sedimentation coefficients obtained were corrected for density and temperature to water at 20 °C. The correlation coefficient derived from a linear least-squares fit of each set of data plotted as ln r vs. t was greater than 99.9% with only one exception. Two measurements were made on different days for each salt concentration except the highest. The range of values obtained is indicated in the figure. (B) Arrows and brackets are drawn indicating the approximate range of salt concentrations over which the indicated dimers and trimers are prominent products of cross-linking. UV-X refers to the principal UV-induced trimer seen in the middle scan of Figure 1.

Discussion

The unfolding of nucleosomes at low ionic strength has been studied by cross-linking of nuclei and chromatin and by cross-linking and sedimentation analysis of isolated nucleosomes. Three different contact-site cross-linking agents (Martinson et al., 1979b) were used (UV light, $C(NO_2)_4$, and HCHO), and six different cross-linked products were studied. Three of the cross-linked products were UV induced: the H2A–H2B dimer (Martinson et al., 1976; Martinson & McCarthy, 1976), an H2B–H4 dimer, and an H2A–H2B–H4 trimer (Martinson et al., 1979b). The $C(NO_2)_4$ cross-linked product studied was the previously characterized H2B–H4 dimer (Martinson & McCarthy, 1975, 1976). The other cross-linked products were two different H2B–H4 dimers induced by HCHO.

Of these six cross-linked products, five contain a cross-link between H2B and H4. The cross-linking yields of these five products were drastically reduced or completely eliminated upon expansion of nuclei in buffers of very low salt concentration. In contrast, the production of the H2A–H2B dimer was unaffected by salt concentration. The cross-linking experiments were done primarily on nuclei subjected to minimal manipulation to avoid disruption by shearing. Additional experiments were done on isolated nucleosomes in order to verify the intranucleosomal location of the cross-linked products.

For several reasons, we believe that our cross-linking data demonstrate rupturing of a major H2B–H4 contact as a result of nucleosome unfolding at low ionic strength. First, the cross-linking yield at one major contact, that between H2A and H2B, is unaffected by reducing the salt concentration despite the fact that the same cross-linking agent (UV light) provides evidence of rupture at another major contact, the H2B–H4 site. Second, the chemistry of UV and $C(NO_2)_4$ cross-linking is such that small changes in salt concentration would not be expected to have an effect on the cross-linking reaction per se. Third, the observed dependence of cross-linking on salt concentration is similar for three chemically diverse types of cross-linker. Fourth, these three cross-linkers all serve to probe loci which are clustered within or around the same major H2B–H4 binding surface (Martinson et al., 1979b), arguing that their similar response to salt concentration is more than mere coincidence. Finally, analysis of the sedimentation properties of purified nucleosomes as a function of salt concentration demonstrates directly the occurrence of a significant conformational change within the range of salt concentrations over which the cross-linking changes are observed. We therefore conclude that, as chromatin expands with decreasing salt concentration, a sufficiently low concentration eventually is reached at which the nucleosome itself unfolds, accompanied by rupture of a major binding interaction between histones 2B and 4.

We feel that the cross-linking approach to studying nu-

cleosome unfolding is a particularly meaningful one. Cross-linking experiments designed to demonstrate the existence of contacts between proteins are often difficult to interpret. This is because not only stable permanent contacts but also relatively unstable and short-lived contacts may be cross-linked if appropriate amino acid side chains are favorably disposed. In the present series of experiments, however, we have focused our attention on the disappearance of contacts. It is significant, for example, that even after 40 min of irradiation, no trimer is produced by UV at very low salt concentrations (Figure 2). The $C(NO_2)_4$ and HCHO cross-linking reactions were carried out for even longer periods of time. The unfolded form of the nucleosome at low ionic strength must be a very stable, perhaps rigid, structure in which previously adjacent amino acid residues are prohibited from approaching one another to any significant extent over long periods of time.

Although our results do not yet allow us to construct a detailed model for nucleosome unfolding, they do suggest that the unfolding phenomenon is an orderly process. The approximate ranges of Na^+ concentrations over which the various dimers and trimers are prominent products of cross-linking are indicated in Figure 7B. From Figure 7 it is immediately obvious that some contacts, for example, those represented by the HCHO α and β dimers, are broken prior to the onset of the hydrodynamically measurable conformational transition, whereas other contacts, in particular that of the $C(NO_2)_4$ dimer, are not broken significantly before the completion of the hydrodynamic transition. This suggests that the nucleosomes unfold in a stepwise manner by means of a sequential breakage of individual contacts within the large H2B–H4 binding domain.

Consideration of the UV-X component (Figures 1 and 7B) suggests that specific rearrangements of histone–histone contacts may take place in addition to the simple rupture of contacts during nucleosome unfolding. Preliminary CNBr peptide mapping results suggest strongly that X also is an H2A–H2B–H4 trimer. As can be seen in Figure 1, this component is much less prominent than the normal H2A–H2B–H4 trimer at physiological salt concentrations, but its yield increases until it becomes the most prominent trimer when the salt concentration is decreased to about 15 mM. Thereafter, its production falls off as the salt concentration is further reduced until, in distilled water, it usually cannot be produced at all. This behavior of the X component has been reproduced in many experiments. An attractive explanation of the phenomenon is that as the nucleosome begins to unfold, but before the gross hydrodynamically measurable transition occurs, some minor conformational changes take place which involve the breakage of some contacts and the establishment of some new ones in the H2B–H4 region such that a fairly well-defined transition complex results. It is this structure then that opens during the hydrodynamic transition.

An interesting, but unlikely (Martinson et al., 1979b), alternative explanation of the data, suggested by the low efficiencies of H2B–H4 cross-linking, is that different subpopulations of nucleosome may differ slightly in their stability to low salt. The UV-X contact of the putative transition complex would still be a feature in such an explanation but would apply only to a subpopulation of nucleosomes. Experiments are underway currently to distinguish between the ordered transition and multiple transition models.

Our experiments do not provide sufficient information to determine the manner in which the nucleosomes unfold. All we can say is that a major H2B–H4 contact is broken. If this contact is *between* heterotypic tetramers (Weintraub et al., 1975), the transition could reflect the opening of nucleosomes to form half-nucleosomes (Weintraub et al., 1976). Alternatively, the contact could be within a heterotypic tetramer arranged in such a way that it is stable only in the intact nucleosome but not in symmetrical half-nucleosomes. Finally, the broken H2B–H4 contact could represent a disproportionation of the symmetrical halves of the intact nucleosome (Kornberg & Thomas, 1974; Weintraub et al., 1975; Martinson & True, 1979) to yield, for example, separated H2A–H2B and H3–H4 tetramers bound to DNA. All of these possibilities would be consistent with the appearance of half-nucleosomes in the electron microscope at low ionic strength (Oudet et al., 1977). However, it seems likely that our data may relate more directly to the solution conformation of nucleosomes than would visualization of chromatin bound to an electron microscope grid.

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On the Mechanism of Nucleosome Unfolding†

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ABSTRACT: We have studied the relative stabilities to urea denaturation of histone–histone binding interactions as they occur both in chromatin and in histone complexes free in solution. We have used the two zero-length contact-site cross-linking agents, tetranitromethane and UV light, to measure the relative degree of H2B–H4 and H2A–H2B association under various conditions. The two interactions were disrupted coordinately when nuclei were treated with increasing concentrations of urea. In contrast, when histone complexes in 2 M NaCl were treated with urea, the H2B–H4 interaction was found to be much less stable than the H2A–H2B interaction. We have shown previously that nucleosomes unfold at low ionic strengths such that the H2B–H4 but not the H2A–H2B interaction is broken in the process. We speculate that the preferential rupture of the H2B–H4 contact is of physiological significance.

In eukaryotes, chromosomal DNA is packaged tightly into small subunits called nucleosomes (Kornberg, 1977; Felsenfeld, 1978). Nucleosomes consist of a compact histone core composed of two each of histones 2A, 2B, 3, and 4 about which are wrapped nearly two turns of DNA 144 base pairs long. A fundamental problem which arises in considering the genetic functions of DNA in chromatin concerns the mechanism by which DNA packaged so tightly can be utilized as a template in replication and transcription. Presumably the nucleosome must unfold in some way to facilitate these functions.

In the preceding paper (Martinson et al., 1979a), we presented studies directed at characterizing the mode of nucleosome unfolding in vitro in response to low ionic strength. Those studies were done in the absence of denaturants and may therefore reflect conformational transitions which occur in vivo. We found that nucleosome unfolding resulted in the disruption of one major histone–histone binding interaction within the nucleosome core (H2B–H4) but that another of the major interactions (H2A–H2B) was unaffected (Martinson et al., 1979a). This result would not have been predicted based on the equivalent pairwise affinities of these histones for each other in solution (Van Holde & Isenberg, 1975). However, it is not surprising that the complex interrelationships of components within the native nucleosome should influence the binding parameters of individual histone pairs. We therefore decided to investigate the effects of various parameters on the relative stabilities of the H2B–H4 and H2A–H2B interactions as they occur in complex systems such as chromatin or complete mixtures of the histones.

We have approached this problem by studying the cross-linking patterns of histones and chromatin as a function of salt and urea concentrations, using the highly specific binding site cross-linkers, tetranitromethane [$C(NO_2)_4$] and UV light (Martinson & McCarthy, 1975, 1976; Martinson et al., 1976). Numerous studies on the destabilization of chromatin and nucleosomes by salt and urea have been carried out (e.g., Whitlock & Simpson, 1976; Carlson et al., 1975; Olins et al., 1977; Weintraub & Van Lente, 1974; Chang & Li, 1974; Hardison et al., 1977). However, these studies do not provide information on specific histone–histone interactions. The predominant cross-linked product induced by $C(NO_2)_4$ in nuclei is an H2B–H4 dimer. The main product induced by UV irradiation is an H2A–H2B dimer. In addition, UV, like $C(NO_2)_4$, induces the specific formation of an H2B–H4 dimer in which the cross-link, however, is at a slightly different position from the one in the homologous $C(NO_2)_4$ dimer (Martinson et al., 1979b). The use of highly selective probes of this type permits data on specific histone–histone binding interactions to be obtained directly in complex systems and obviates the need for prior purification of individual components.

Our results show that urea-induced denaturation of the nucleosome does not follow a sequential pathway involving specific initial rupture of the H2B–H4 contact as we had expected based on our results on unfolding in low salt (Martinson et al., 1979a). Instead we find that the H2B–H4 and H2A–H2B binding interactions in nucleosomes are denatured by urea coordinately. However, if the histone core in 2 M salt (free of DNA) is denatured by urea, a coordinate effect is no longer observed, and the H2B–H4 interaction is found now to be considerably less stable than the H2A–H2B interaction. We speculate that the H2B–H4 interaction is

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