Histone–Histone Interactions within Chromatin. Preliminary Location of Multiple Contact Sites between Histones 2A, 2B, and 4†

Harold G. Martinson, Rod True, Chu Kwan Lau, and Margarete Mehrabian*

ABSTRACT: The contact-site cross-linkers tetranitromethane, UV light, formaldehyde, and a monofunctional imido ester have been used to generate a collection of histone–histone dimers and trimers from nuclei and chromatin. Four different H2B–H4 dimers have been isolated. Preliminary CNBr peptide mapping has shown that all are cross-linked at different positions that are apparently clustered within the C-terminal regions of these histones. Similarly, two different H2A–H2B dimers and two different H2A–H2B–H4 trimers have been partially characterized. The data suggest a functional map for H2B in which the N-terminal third interacts with DNA, the middle third interacts with H2A, and the C-terminal third interacts with H4. We hope, by pursuing this type of analysis, to develop a detailed understanding of each histone–histone binding interaction through saturation cross-linking of the binding sites.

It is now known that chromatin is constructed of subunits termed nucleosomes. Many general aspects of nucleosome structure have been elucidated (Felsenfeld, 1978). Its structure is based upon a complex of eight histones (two each of H2A, H2B, H3, and H4) which are tightly bound to each other, forming a globular core. On the outside of this histone core are firmly bound about 144 base pairs of DNA arranged in tight superhelical coils. The resulting structure is termed the "nucleosome core" and is connected to other nucleosome cores in chromatin by a variable length of linker DNA. Those portions of the histone molecules which constitute the central globular core are extraordinarily well conserved evolutionarily. It follows that the histone–histone binding interactions must be of great biological significance. We are engaged in studies directed at characterizing the binding surfaces of the histones within the nucleosome core.

In order to identify points of contact between histones, we are using a number of chemically diverse cross-linking agents of a type we call "contact-site cross-linkers". We define contact-site cross-linkers, in the context of macromolecular structure, as cross-linking agents which give rise to such short covalent linkages that the linked atoms, in their prior noncovalent state, must be capable of virtual contact in order to become joined.

Obvious members of this class of cross-linkers are the zero-length cross-linkers: tetranitromethane [$C(NO_2)_4$], UV light, and the carbodiimides (Martinson & McCarthy, 1976; Martinson et al., 1976; Bonner & Pollard, 1975). These give rise to direct covalent joining of residues with no interposed bridge. Moreover, their chemical specificities are such that the residues which become cross-linked are likely to be involved in thermodynamically important noncovalent binding interactions prior to the cross-linking (Martinson & McCarthy, 1976; Martinson et al., 1976). They are therefore also binding site probes.

Another class of contact-site cross-linkers are those which themselves become incorporated into the covalent linkage. Examples of this type are formaldehyde (HCHO) and various monofunctional alkyl imidates. HCHO can link lysine to tyrosine, arginine, histidine, or another lysine via a methylene bridge (Feldman, 1973). The monofunctional imidates give rise probably to diamidino cross-links between lysines (Sweadner, 1977; Wyns et al., 1978). Both types of cross-linking involve a condensation reaction in which a 1-carbon bridge derived from the reagent is incorporated into the linkage. Since the internuclear distance of the atoms joined by the 1-carbon bridge is equivalent to their closest possible van der Waals approach prior to cross-linking, HCHO and the monofunctional imidates are contact-site cross-linkers. Note, however, that these cross-links (e.g., lysine–lysine) often may not reflect prior noncovalent *attraction* between the affected residues. In such cases the locations of these cross-linked sites may represent regions of noninteractive contact at the periphery of the actual thermodynamic binding sites between the histones.

We have shown previously that $C(NO_2)_4$ induces the linkage of H2B to H4 within their C termini (Martinson & McCarthy, 1976). We have also shown that irradiation with UV light induces the linkage of H2A to H2B within their N-terminal portions (Martinson & McCarthy, 1976). More recently we have found that it is tyrosine-37, -40, or -42 of H2B that becomes linked to proline-26 of H2A (De Lange et al., 1979). We have also presented data which permitted the inference, based on sequential cross-linking by UV and then $C(NO_2)_4$, that H2B was simultaneously and specifically bound to both H2A and H4 in chromatin (Martinson & McCarthy, 1976).

In this paper we will first confirm by CNBr peptide mapping that sequential UV and $C(NO_2)_4$ treatment does indeed yield an H2A–H2B–H4 trimer and that the cross-links in this trimer are the same as those previously characterized for the UV-induced H2A–H2B and the $C(NO_2)_4$-induced H2B–H4 dimers. We will then show that not only $C(NO_2)_4$ but also UV induces an H2B–H4 dimer and that the UV-induced H2B–H4 dimer is cross-linked at a position close to but different from that of the $C(NO_2)_4$ H2B–H4 dimer. As would be predicted on the basis of this finding, we show that the UV-induced trimer which appears after long periods of irradiation (Martinson et al., 1976) also is an H2A–H2B–H4 trimer. As expected, however, this trimer bears a different H2B–H4 cross-link from that in the H2A–H2B–H4 trimer induced by $C(NO_2)_4$ treatment of mildly irradiated nuclei. We also present the characterization of two additional H2B–H4 dimers produced by formaldehyde whose cross-links map to positions close to those of the H2B–H4 dimers induced by UV and $C(NO_2)_4$. Finally, we present preliminary characterization of a new H2A–H2B dimer produced by methyl acetimidate.

Materials and Methods

Cross-Linking Procedures. Calf-thymus nuclei were prepared in 75 mM NaCl, 24 mM EDTA, pH 8, as described

† From the Division of Biochemistry, Department of Chemistry, and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received September 1, 1978; revised manuscript received December 6, 1978.* This work was supported by Grant PCM 76-00712 from the National Science Foundation.

0006-2960/79/0418-1075$01.00/0

(Martinson et al., 1979b). UV irradiation was conducted for 4 h on 1-L suspensions of nuclei prepared from about 50 g of calf thymus also as described (Kunkel & Martinson, 1978) except that a Corex (Martinson et al., 1976) rather than a Vycor filter was used. Cross-linking with $C(NO_2)_4$ was carried out in a similar manner. In both cases, scrupulously anaerobic conditions were required in order to minimize side reactions. For $C(NO_2)_4$ cross-linking, nuclei were resuspended in phosphate buffer (112 mM NaCl, 20 mM sodium phosphate, pH 7.5) at an A_{260} of about 500 (determined by dilution of an aliquot into 2 M NaCl, 5 M urea). About 100 mL of such a suspension was stirred in a stoppered Erlenmeyer flask under a steady stream of N_2 for about 20 min. The total volume was then brought to about 1 L by the anaerobic addition of 900 mL of N_2-purged phosphate buffer. The reaction was initiated with 0.25 mL of $C(NO_2)_4$ (Sigma). The flask was closed off, and the reaction was allowed to proceed under the N_2 atmosphere with stirring for about 30 min at room temperature. The nuclei harvested from both the UV and $C(NO_2)_4$ cross-linking reactions were washed with 0.35 M salt and extracted for histones with acid as described (Martinson et al., 1979b).

Successive cross-linking by UV, followed by $C(NO_2)_4$ (Martinson & McCarthy, 1976), was conducted by initiating UV irradiation in the normal way (in saline–EDTA, pH 8), but irradiation was carried out for only 2 h instead of 4 h. The UV light source was then turned off and $C(NO_2)_4$ was introduced into the reaction vessel. The nuclear suspension was stirred for another 30 min under nitrogen, the nuclei were harvested, and the histones were extracted as described. During the $C(NO_2)_4$ cross-linking under these conditions, the pH of the suspension decreased from 8 to 7.6 as would be expected (Martinson & McCarthy, 1975); however, when $C(NO_2)_4$ cross-linking was conducted in phosphate buffer (above), no drop in pH occurred. For more recent experiments, therefore, we have converted to the use of phosphate-buffered saline–EDTA for preparation of nuclei.

Formaldehyde cross-linking was conducted on nuclei resuspended at an A_{260} of about 40 in 5 mM phosphate, pH 7.5. HCHO (Mallinckrodt) was added to a final concentration of 0.2% and cross-linking was carried out at 0 °C for 5 h. After cross-linking, one-tenth volume of 3.5 M NaCl was added and the chromatin was harvested by centrifugation. The histones were acid-extracted in the usual way.

Purified H2A and H2B were cross-linked with formaldehyde to form an H2A–H2B dimer by a low-pH procedure based on a suggestion made to us by Dr. Harold Weintraub. H2A and H2B monomers were purified to about 90% homogeneity by column chromatography and then mixed at about 0.5 mg/mL each in 1.3 mL of 3 mM HCl. Then 2.7 mL of 2.9 M NaCl, 50 mM sodium acetate, pH 5, was added with mixing. Formaldehyde, adjusted to pH 5 with Na_3PO_4, was then added to a final concentration of 0.5%, and cross-linking was carried out for about 2 h at room temperature. The reaction was stopped by the addition of sulfuric acid to 0.4 N, and the solution was then dialyzed against 0.4 N H_2SO_4 overnight in the cold by use of Spectrapor 1 dialysis tubing. The histones were collected for electrophoresis by acetone precipitation.

Methyl acetimidate cross-linking was carried out on nuclei prepared as described except that phosphate-buffered saline–EDTA (30 mM sodium phosphate, 35 mM NaCl, 24 mM EDTA, pH 7.5) was used. Nuclei (45 mL) suspended at an A_{260} of about 90 were mixed with 5 mL of 0.79 M methyl acetimidate (0.79 M methyl acetimidate hydrochloride, Pierce Chemical, in 0.35 M NaOH). The nuclei were reacted at 0 °C for 1 h, collected by centrifugation, and then resuspended in 40 mL of 0.1 mM phosphate, pH 7, for completion of the cross-linking. After 20 h at 5 °C the histones were prepared by acid extraction. Specific dimer formation occurred under a wide range of conditions and was not limited to this protocol. Yields were about the same as those for HCHO and $C(NO_2)_4$ cross-linking.

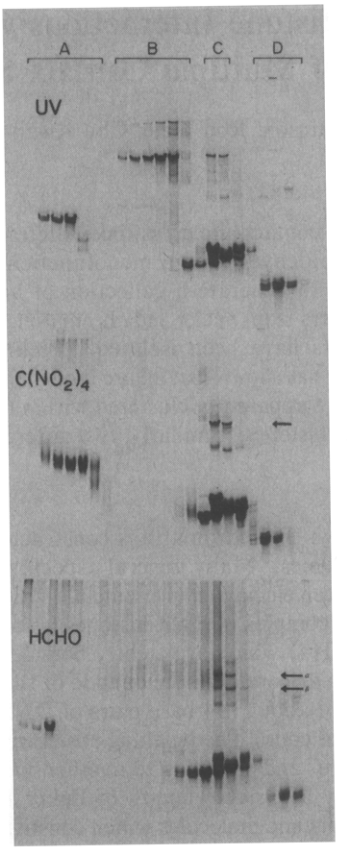

FIGURE 1: Bio-Gel P30 column chromatography of histones from cross-linked nuclei. Total histones extracted from nuclei cross-linked with UV, $C(NO_2)_4$, or HCHO were chromatographed at 4 °C on three 2.3 × 95 cm columns attached in series. Approximately 50 mL of histone dissolved at about 10 mg/mL in water containing 10% β-mercaptoethanol was loaded, and fractions of 8–9 mL were collected. Aliquots of 5–10 μL from every fourth fraction were analyzed for histone on acid–urea gels.

Purification of Cross-Linked Histone Species. Histones isolated from cross-linked nuclei were subjected to fractionation on columns of Bio-Gel P30 in 10 mM HCl, 10 mM β-mercaptoethanol (Martinson et al., 1979b; Martinson & McCarthy, 1976). The results of such a fractionation of histones from UV-cross-linked, $C(NO_2)_4$-cross-linked, and HCHO-cross-linked nuclei are shown in Figure 1. The figure shows the results of acid–urea gel electrophoretic analysis of aliquots taken from fractions across the column elution profile. Histones 1 and 4 appear in regions A and D, respectively. H2A–H2B dimers and H2A–H2B–H4 trimers elute in region B. H2B–H4 dimers elute in region C along with poorly separated H2A, H2B, and H3. We have found that the exact relative elution positions of the histone monomers depend to some extent on the age of the column and the concentration and amount of sample loaded. In the column profiles shown, for example, H3 elutes in two regions of intensity, one at the beginning of region C and the other between regions C and D. This phenomenon appears to be concentration dependent and is presumably the result of the tendency of H3 to aggregate. Further purification of the histone monomers and

dimers involved concentration of appropriate pools by lyophilization, followed by rechromatography on P30 as described in the legend to Figure 1. Additional purification by one or two cycles of chromatography on Sephadex G-200 was also employed as required (see Martinson & McCarthy, 1976). The Sephadex G-200 step was carried out in the cold as for P30 by use of either the HCl eluant or 0.1 M acetic acid, 10 mM β-mercaptoethanol. Preparative acid–urea gel electrophoresis (Martinson et al., 1979b) usually was used as a final purification step.

CNBr Cleavage of Histones. Prior to reaction with CNBr, any oxidized methionine in the purified histones was reduced by treatment with 40% thioglycolic acid (Mallinckrodt). In general, protein samples (usually 50–200 μL) of widely varying concentration, some of which contained 5 or 6 M urea to promote solubility, were mixed with the appropriate amount of 98% thioglycolic acid and incubated under nitrogen at room temperature for 10–16 h (Alfageme et al., 1974). After reduction the histone was freed of the thioglycolic acid by precipitation at −20 °C for several hours with a vast excess of acetone in the presence of sulfuric acid (500 parts acetone, 1 part 5 N H_2SO_4). The sulfuric acid was necessary to convert the histones to the less soluble sulfate form. The precipitates were collected by centrifugation, freed of solvent under a stream of nitrogen, dissolved in a small volume (e.g., 30 μL) of 10 mM HCl, and then reprecipitated with the acetone–sulfuric acid mixture (e.g., 5 mL) in order to remove the last traces of thioglycolic acid. The histone pellets were dissolved in a small volume (e.g., 20 μL) of 80% formic acid to which was added an equal volume of freshly distilled CNBr dissolved in 80% formic acid at 32 mg/mL. Incubation was carried out at room temperature in the dark under nitrogen for 12–16 h. The solvents and CNBr were then removed by lyophilization over pellets of NaOH. The digested protein was then dissolved in a small volume of 5–8 M urea containing 10% β-mercaptoethanol and analyzed directly on either acid–urea or $NaDodSO_4$–polyacrylamide gels.

Polyacrylamide Gel Electrophoresis. Fifteen percent acid–urea (Martinson & McCarthy, 1976) and 18% $NaDodSO_4$ (Bonner & Pollard, 1975) polyacrylamide gel electrophoresis was carried out as described. Second-dimension $NaDodSO_4$ electrophoretic analyses of lanes from acid–urea gels were carried out following incubation of the appropriate gel strip for about 1 h in 1% $NaDodSO_4$, 3 M urea, 0.125 M Tris, pH 7, and imbedding the strip across the top of the stacking gel of the $NaDodSO_4$ dimension by use of 0.75% agarose, 0.125 M Tris, pH 6.8. Both the first- and second-dimension gels were 0.8-mm thick. Staining and destaining of the acid–urea dimension gave rise to swelling, however, so that it was not possible to insert the gel slice between the slab gel plates of the second dimension unless a thicker gel was to be run (which was done occasionally). We preferred, however, to place the gel slice on the ledge of the cutout portion of the inside gel plate which normally allows contact of the upper buffer reservoir with the stacking gel. Direct contact of the buffer with the face of the strip sitting on the ledge was prevented by use of Saran wrap. Thus electrical contact was made only along the upper edge of the strip, allowing the proteins in the bands of the first dimension to be stacked into narrow zones for good resolution in the $NaDodSO_4$ dimension. This procedure was used for analysis of lanes cut from both freshly run acid–urea slabs and slabs which had been dried onto filter paper under vacuum at 100 °C. In the latter case, it was necessary to rehydrate the strips for 1 day in water. In the former case, it was necessary to incubate the gel slices in several changes of the Tris buffer in order to remove completely the acetic acid used for destaining. Prior staining of the gels with Coomassie Blue had no effect on the $NaDodSO_4$ dimension, and, in fact, the Coomassie Blue front provided a good indication of the progress of electrophoresis. Staining and destaining of all gels were as described (Martinson & McCarthy, 1975).

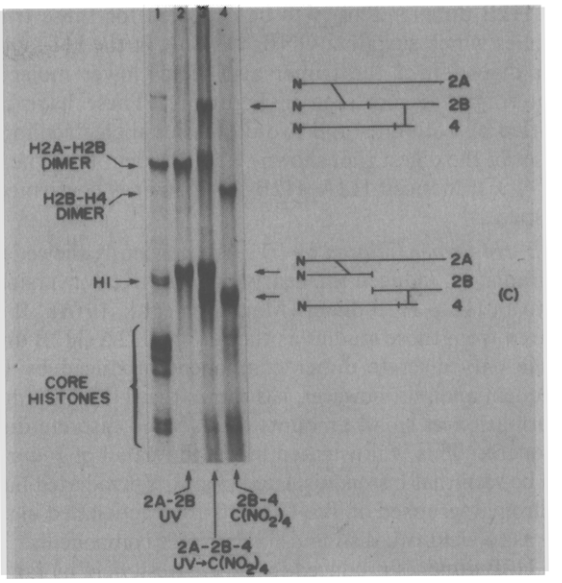

FIGURE 2: Characterization by CNBr peptide mapping of the H2A–H2B–H4 trimer induced by sequential UV and $C(NO_2)_4$ treatment. Nuclei were treated sequentially with UV and $C(NO_2)_4$ as described under Materials and Methods. The histones were extracted with acid and fractionated by P30 column chromatography as described for Figure 1. Fractions enriched in the UV-induced H2A–H2B dimer, the $C(NO_2)_4$-induced H2B–H4 dimer, and the composite trimer H2A–H2B–H4 were rechromatographed on P30 and then on Sephadex G-200. Final purification was by preparative gel electrophoresis (Martinson et al., 1979b). The purified dimers and trimer were treated with CNBr and then analyzed on an acid–urea gel. The gel was run for a relatively long period of time so as to display clearly the relative mobilities of the higher molecular weight incomplete CNBr cleavage products (see text). The smaller fragments therefore do not appear on this gel. The parenthetical "c" on the right-hand side of the figure refers to the peak of the same designation in Figure 3. Lane 1 is a reference pattern of total histones isolated from UV-irradiated nuclei.

Results

H2A–H2B–H4 Trimer Induced by UV and $C(NO_2)_4$. Calf-thymus nuclei were treated sequentially with UV and then $C(NO_2)_4$ [henceforth designated UV → $C(NO_2)_4$]. The dimer characteristic of each cross-linking agent as well as a trimer were produced as previously described (Martinson & McCarthy, 1976). Each of these cross-linked components was purified and characterized by CNBr cleavage. The results are shown in Figure 2. In our previous report (Martinson & McCarthy, 1976) we identified the various CNBr cleavage products derived from the UV-induced H2A–H2B dimer and the $C(NO_2)_4$-induced H2B–H4 dimer. The highest molecular weight cleavage product of each dimer has a mobility similar to that of H1 as can be seen in lanes 2 and 4 of Figure 2. These cleavage products are identified diagrammatically at the right of the figure. It is clear that an H2A–H2B–H4 trimer which would be induced by UV → $C(NO_2)_4$ treatment should also yield these two fragments among the products of a partial CNBr cleavage reaction. The bands of appropriate mobility which appear in the trimer digest (lane 3) demonstrate that this is so. In addition, the trimer digest of lane 3 yields a cleavage product of approximately the mobility of intact

H2A–H2B dimer. This is to be expected for those trimer molecules which sustain a CNBr cut only in the H4 moiety. CNBr digestion of the trimer also yields lower molecular weight fragments not seen in Figure 2. These have been identified by both one- and two-dimensional electrophoretic analyses of the digest (not shown), and the identity of the UV → $C(NO_2)_4$ induced H2A–H2B–H4 trimer is confirmed in all respects.

H2B–H4 Dimer Induced by UV. We previously showed that UV irradiation induced the nearly quantitative conversion of H2B to an H2A–H2B dimer (Martinson et al., 1976). It also appeared from those studies as though the H2A–H2B dimer was the only discrete dimer component produced by UV. Subsequent analysis, however, has shown that UV also induces the formation of small amounts of a second discrete dimer component. This is illustrated in the top panel of Figure 1. It can be seen that histones isolated from UV-irradiated nuclei and chromatographed on Bio-Gel P30 are fractionated in such a way as to yield two distinguishable dimer components. The H2A–H2B dimer is produced in the greatest yield by far and elutes in region B of the Bio-Gel P30 profile as previously shown (Martinson & McCarthy, 1976). In addition, a minor component of identical acid–urea gel electrophoretic mobility can be seen to elute from the columns in region C. This is the region in which the H2B–H4 dimer induced by $C(NO_2)_4$ elutes (see middle panel of Figure 1; also Martinson & McCarthy, 1976).

The putative H2B–H4 dimer induced by UV was digested with CNBr in parallel with a sample of H2B–H4 dimer induced by $C(NO_2)_4$, and the two digests were analyzed by acid–urea polyacrylamide gel electrophoresis as shown in Figure 3. It can be seen that although the electrophoretic mobilities of the two dimers themselves differ, they both, nevertheless, yield the same N-terminal fragements of H4 and H2B (see Martinson & McCarthy, 1976). This not only confirms the identity of the H2B–H4 dimer induced by UV but also shows that the cross-link in this dimer, like that of the $C(NO_2)_4$ dimer, is located within the C-terminal CNBr fragments of the histones 2B and 4. The fact that the two H2B–H4 dimers are homologous is further emphasized by the overall similarity of the CNBr digest patterns (peaks a, b, and c in Figure 3 are labeled for comparison with Figure 4 of Martinson & McCarthy, 1976). However, a very important feature of Figure 3 is the systematic shift to lower mobilities for all fragments bearing the cross-link in the UV dimer as compared to the $C(NO_2)_4$ dimer. This demonstrates that, although the dimers are homologous, the cross-links are nevertheless different. Acid–urea gels are known to allow distinction between isomeric cross-linked histones (Panyim et al., 1971).

H2A–H2B–H4 Trimer Induced by UV Irradiation Alone. We have shown that UV irradiation induces the formation of a trimer (Martinson et al., 1976). The production by UV of both an H2A–H2B and an H2B–H4 dimer suggests that the trimer is H2A–H2B–H4. This UV trimer coelutes with the UV → $C(NO_2)_4$ H2A–H2B–H4 trimer in region B from columns of P30 (not shown), strengthening this inference. Moreover, the UV-induced trimer has a lower electrophoretic mobility than the UV → $C(NO_2)_4$ induced trimer on acid–urea gels (Martinson & McCarthy, 1976) just as the H2B–H4 dimer induced by UV also has a lower mobility on acid–urea gels than the one induced by $C(NO_2)_4$ (Figure 3). A CNBr analysis of the UV-induced trimer, similar to that shown for the UV → $C(NO_2)_4$ trimer in Figure 2, was carried out (not shown) and was consistent with the H2A–H2B–H4 assignment. However, as was discussed in connection with Figure 3, the CNBr fragment labeled c, which is produced in digests of both dimer (Figure 3) and trimer (Figure 2), has a lower mobility in the case of the UV-induced H2B–H4 cross-link than for the $C(NO_2)_4$-induced cross-link (Figure 3). Thus the two diagnostic "half-trimer" CNBr cleavage fragments which are clearly resolved for the UV → $C(NO_2)_4$ trimer of Figure 2 are poorly resolved in the case of the UV-induced trimer (not shown). We therefore turned to two-dimensional electrophoretic analysis in order to confirm positively the assignment.

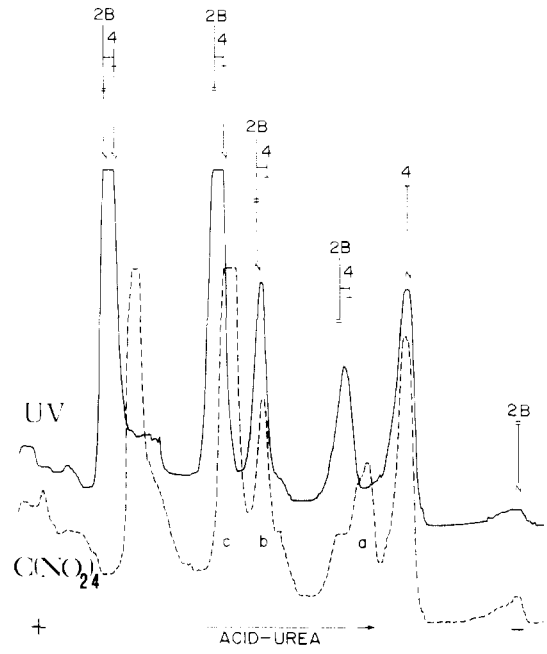

FIGURE 3: CNBr cleavage patterns of two different H2B–H4 dimers. Batches of nuclei were cross-linked separately with UV and $C(NO_2)_4$ and the histones isolated. Chromatography of the histones on P30 gave the patterns shown in the top two panels of Figure 1. Dimer-containing fractions from region C for both lots of cross-linked histone were pooled and purified as described for Figure 2. CNBr cleavage was carried out and the samples were analyzed by acid–urea gel electrophoresis. The gels were scanned by use of a Joyce Loebl densitometer. Peaks corresponding to uncleaved dimer as well as the liberated H4 and H2B N-termini are indicated. The peaks labeled a, b, and c correspond to the similarly labeled peaks for the C-$(NO_2)_4$-induced H2B–H4 dimer in Figure 4 of Martinson & McCarthy (1976). The shoulders on peaks a and b of the $C(NO_2)_4$ profile have been identified by two-dimensional electrophoresis as monomeric H4 and H2B, respectively. The $C(NO_2)_4$ cross-link is subject to a low level of reversal. These products of reversal are not evident in fresh preparations of dimer (Martinson & McCarthy, 1976) but can become quite prominent upon storage. The UV-induced H2B–H4 cross-link has not been found to be subject to reversal. Two-dimensional electrophoresis shows that peak a of the UV profile contains no monomer H4 (see Figure 4). Except for reversal, the two-dimensional patterns for the CNBr digests of both the UV and $C(NO_2)_4$ H2B–H4 dimers are equivalent.

The two-dimensional electrophoretic patterns of CNBr digests of the UV-induced H2A–H2B and H2B–H4 dimers are shown in Figure 4. The two-dimensional electrophoretic pattern of the digest of the UV-induced trimer is shown in Figure 5. A detailed comparison of these two figures shows that both H2A–H2B and H2B–H4 are component linkages of the UV trimer. Many spots can be seen and the identities of some are discussed in detail in the figure legends. However, features worthy of particular note are discussed here. First of all, the two major spots homologous to the diagnostic half-trimer fragments shown for the UV → $C(NO_2)_4$ induced trimer in Figure 2 are now clearly resolved. Secondly, fragments a and c and the N-terminal fragment of H4 are seen

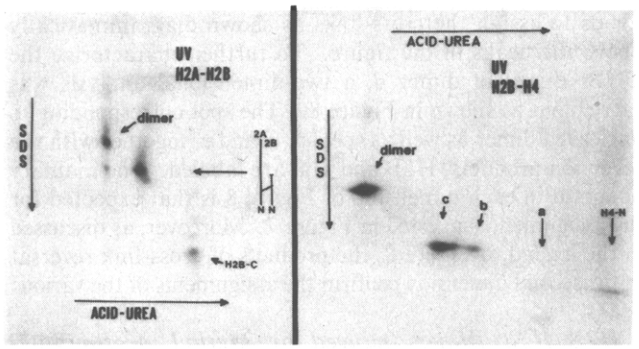

FIGURE 4: Two-dimensional CNBr peptide maps of the two UV-induced histone dimers. The left panel shows the pattern for the UV-induced H2A–H2B dimer. The gel slice taken for the second dimension was rehydrated from a dried acid–urea gel. The spots corresponding to residual uncleaved dimer as well as the two cleavage products are labeled. When the second dimension is run on rehydrated rather than fresh first-dimension gel slices, satellite spots corresponding to the appropriate CNBr cleavage fragments appear under certain of the primary spots in the figure. Thus spots corresponding to both of the CNBr fragments of the H2A–H2B dimer can be seen directly below the spot corresponding to the intact dimer. Apparently some of the uncleaved protein which remains after our CNBr treatment is actually the precleavage cyanosulfonium addition product of CNBr action. The high temperature and acidic conditions associated with drying of the acid–urea gel presumably serve to complete the reaction sequence, giving rise to additional cleavage in situ during gel drying. The cleaved products are then observed in the second dimension as satellite spots of greater mobility than the parent spot. We have run a great number of two-dimensional gels on both fresh and dried first-dimension gel slices for this and other CNBr digests, and all lead to spot assignments consistent with the above interpretation. The H2B–H4 panel on the right of the figure is the result of a second dimension run on a fresh slice from an acid–urea gel. The spots are labeled to correspond to the peaks shown in Figure 3. The N-terminal fragment of H2B, which can be seen in Figure 3, was too faint to be visible in the second dimension and therefore does not appear. Absolute mobilities of spots cannot be compared between the two panels because electrophoresis for the H2B–H4 panel was for longer periods of time in both dimensions than for the H2A–H2B panel. Moreover, for many of the gels shown in this and subsequent figures, the acid–urea origin is not seen because the first-dimension slice was trimmed to fit the apparatus.

to occupy the same relative positions in both the trimer digest of Figure 5 and the H2B–H4 dimer digest of Figure 4 as would be expected. Moreover, we have verified by direct comparison in both acid–urea and $NaDodSO_4$ one-dimensional slab gels that the mobilities of these three fragments are identical whether they are derived from dimer or trimer (not shown). Thirdly, as for the UV → $C(NO_2)_4$ trimer, there is a fragment produced which is little more than an H2A–H2B dimer. A spot of appropriate mobility is seen clearly in the two-dimensional pattern of Figure 5. The mobility of this fragment has been found to be very slightly less than that of authentic UV H2A–H2B dimer by direct comparison on both $NaDodSO_4$ and acid–urea one-dimensional slab gels (not shown). Finally, the diagnostic features just enumerated for the two-dimensional peptide map of the UV-induced H2A–H2B–H4 trimer are exhibited also in the peptide map of the UV → $C(NO_2)_4$ H2A–H2B–H4 trimer (not shown). All slight differences in mobility which exist between spots in the two patterns are ascribable in a consistent way to the difference in the H2B–H4 linkage [UV vs. $C(NO_2)_4$ induced] which exists between the two trimers. The UV-induced trimer is thus unambiguously identified as an H2A–H2B–H4 trimer.

H2B–H4 Dimers Induced by Formaldehyde. In our hands, HCHO cross-linking of chromatin gives rise to two dimers, termed α and β, as the most prominent components on acid–urea gels. They elute from Bio-Gel P30 in the C region

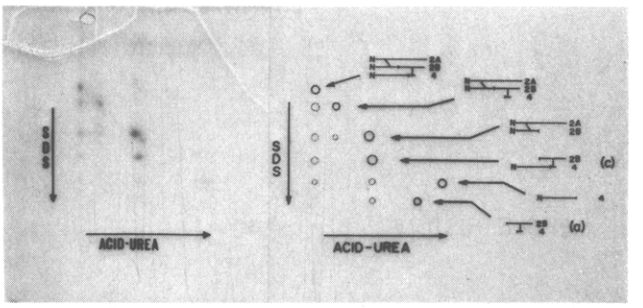

FIGURE 5: Two-dimensional CNBr peptide map of the UV-induced H2A–H2B–H4 trimer. The trimer was purified from histones extracted from UV-irradiated nuclei by several column chromatographic steps, followed by preparative gel electrophoresis as described. As for the H2A–H2B panel of Figure 4, the second dimension was run on a rehydrated strip cut from a dried acid–urea gel. Consequently, satellite spots (dotted circles) corresponding to additional CNBr cleavage events occurring during gel preparation can be seen (see legend to Figure 4). To the left is a photograph of the gel, and to the right is an explanatory diagram identifying the various spots. Satellite spots which are visible are designated by dotted circles. The designations a and c in the figure correspond to the equivalently labeled peaks and spots in Figures 3 and 4. Note particularly that the spot corresponding to H2A linked to the N-terminal half of H2B has no visible satellites, whereas the spot corresponding to H4 linked to the C-terminal part of H2B has below it two appropriate satellite spots.

(Figure 1). This is the region where H2B–H4 dimers elute (see above). In attempting to characterize these dimers, we found that the formaldehyde cross-link was susceptible to slow reversal under our conditions of storage and handling. [The $C(NO_2)_4$ H2B–H4 dimer is also subject to reversal but less so than the HCHO ones (see legend to Figure 3).] While this made CNBr peptide analysis difficult to interpret, the reversal phenomenon itself permitted identification of the dimers with respect to their composition. Knowledge of the composition of the dimers, together with awareness of the reversal phenomenon, permitted interpretation of the CNBr patterns (see below).

Figure 6 shows that reversal of the cross-link allowed us to confirm that both α and β were H2B–H4 dimers. The bottom panel of Figure 6 shows the $NaDodSO_4$ electrophoretic patterns of the dimers following their preparative electrophoretic purification on acid–urea gels. Note that the mobilities of α and β are different from each other also in $NaDodSO_4$ and are in fact the reverse of their relative mobilities in acid–urea gels. Figure 6 shows that for both dimers partial reversal of the cross-link gives rise to the same monomer histones: H2B and H4. This demonstrates that both dimers are H2B–H4. We have no explanation for the lower mobility material in the bottom panel. Perhaps it arises by some sort of covalent rearrangement related to reversal. Note, however, that the material is of the same mobility for both dimers consistent with their compositional homology.

For comparison, a mixture of purified H2A and H2B was cross-linked with HCHO. The middle panel of Figure 6 shows that the HCHO-induced H2A–H2B dimer is of lower mobility than the H2B–H4 dimers as would be expected on the basis of the mobilities of the component monomers. Thus mobility in $NaDodSO_4$ gels, elution position from Bio-Gel P30 columns, and the products of reversal all show that both HCHO dimers, α and β, are H2B–H4. Their distinct electrophoretic mobilities, however, indicate that they nevertheless differ with respect to the point of cross-linkage.

A preliminary analysis of the sites of cross-linkage in the two dimers was undertaken by CNBr peptide mapping. Figure 7 shows the acid–urea gel electrophoretic profile for the CNBr digests of electrophoretically purified α and β HCHO dimers.

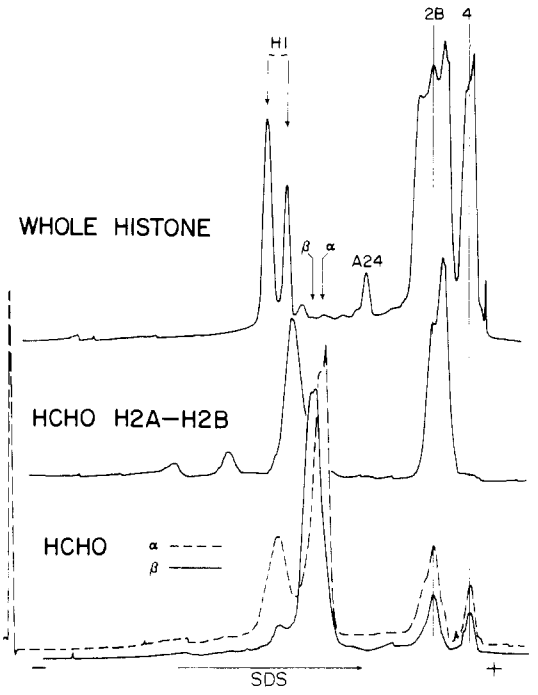

FIGURE 6: Compositional analysis of the HCHO H2B–H4 dimers α and β by reversal of the cross-link. Chromatin was cross-linked with formaldehyde, acid extracted, and fractionated by column chromatography. Dimers α and β were purified by preparative gel electrophoresis. The electrophoretically "pure" dimers were then analyzed by $NaDodSO_4$ gel electrophoresis as illustrated in the bottom panel of the figure. The top panel is the electrophoretic pattern of histones from uncross-linked nuclei. The middle panel is the pattern of an H2A–H2B histone mixture which has been partially cross-linked with HCHO.

Knowledge of the reversal phenomenon allows identification of the peaks as shown in the figure and as discussed in the legend. Dimer α can be seen to be homologous to the H2B–H4 dimers induced by UV or $C(NO_2)_4$. Two-dimensional analysis (not shown) confirms the homology. Peaks b and c are labeled for comparison with Figure 3.

Dimer β gives a very different electrophoretic pattern for its CNBr digest in the low-mobility region of the acid–urea gel. Knowing that dimer β is an H2B–H4 dimer, it is possible for us to assign the cross-links as shown diagrammatically above the peaks in the figure. To further characterize the CNBr digest of dimer β, a two-dimensional analysis was carried out as shown in Figure 8. The spot corresponding to uncleaved dimer as well as spots d, c, and e, together with the reversion products, H2B and H4, are labeled. The mobility of spot d in each dimension of Figure 8 is that expected for the assignment suggested in Figure 7. Moreover, as discussed in the legend to Figure 8, the products of cross-link reversal in the second dimension confirm the assignments of the various peaks.

H2A–H2B Dimer Induced by Methyl Acetimidate. Treatment of chromatin with monofunctional alkyl imidates has been shown to give rise to cross-linked histones (Tack & Simpson, 1977; Wyns et al., 1978). We have prepared and purified the major dimer produced by methyl acetimidate and find that it elutes in region B from columns of Bio-Gel P30. This confirms (Wyns et al., 1978) that it is compositionally an H2A–H2B dimer. CNBr analysis of pure methyl acetimidate dimer, in comparison with monomer histones isolated from the same preparation, has further confirmed the H2A–H2B assignment. However, unlike the UV-induced H2A–H2B dimer (Martinson & McCarthy, 1976), the acetimidate-induced H2A–H2B dimer yielded the N-terminal rather than the C-terminal fragment of H2B upon CNBr cleavage. This was determined by analysis on both acid–urea and $NaDodSO_4$ gels as shown in Figure 9. Such an analysis is quite convincing because the relative mobilities of the N- and C-terminal portions of H2B are reversed in the two gel systems. This has been demonstrated by two-dimensional analysis of the CNBr cleavage products of H2B (not shown). Moreover, the C-terminal fragment of H2B stains much more intensely than the N-terminal fragment. Therefore, the complete absence of C-terminal material coupled with the presence of weakly staining N-terminal bands demonstrates unambiguously that the cross-link is in the C-terminal half of H2B. This assignment is at variance with that of Wyns et al. (1978). However, they based their inference upon rather indirect evidence.

Discussion

We have generated several histone–histone cross-linked dimers and trimers using contact-site cross-linkers. Char-

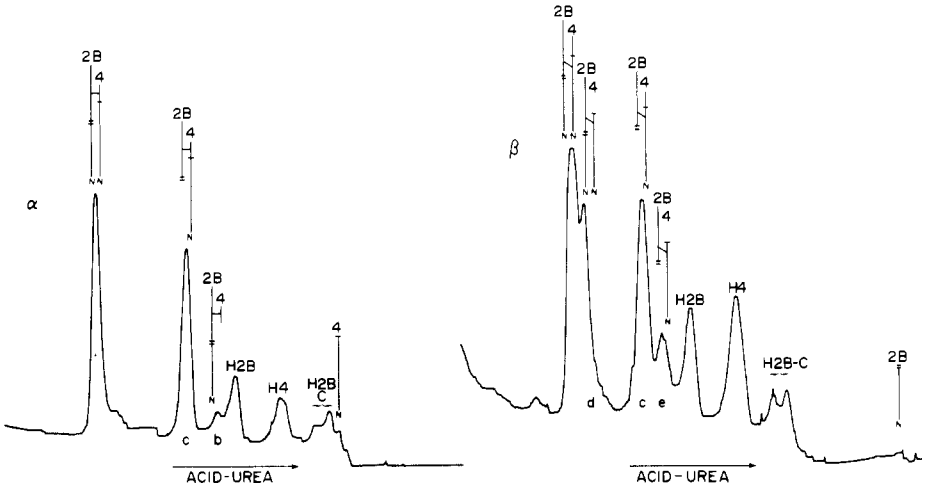

FIGURE 7: CNBr peptide analysis of HCHO dimers α and β. CNBr peptide analysis was carried out as for Figure 3. The letters b and c identify peaks which are homologous to peaks in Figure 3. Note that reversal of cross-linking gives rise to peaks of free H2B and H4 as well as peaks corresponding to the C-terminal half of H2B. The N-terminal portion of H2B does not stain well in this system (Martinson & McCarthy, 1976) and is therefore only barely visible or absent altogether. A peak corresponding to a in Figure 3 is not evident in the electrophoretic profile of the dimer α CNBr digest. This presumably is the result of a combination of low yield owing to cross-link reversal and the complexity of the electrophoretic pattern in that region of the gel.

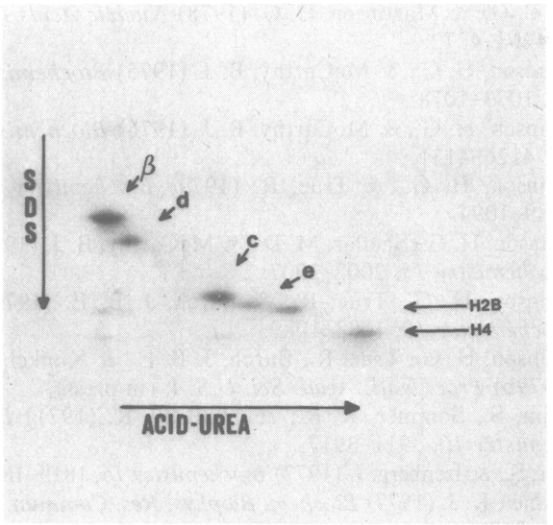

FIGURE 8: Two-dimensional CNBr peptide map of the HCHO H2B–H4 β dimer. Two-dimensional electrophoresis of a CNBr digest of the HCHO β dimer was carried out just as for the UV H2B–H4 dimer of Figure 4. The spots in the figure are labeled to correspond to the peaks shown for the first-dimension analysis of the β dimer in Figure 7. The first-dimension gel was not dried prior to second-dimension analysis, and therefore no satellite spots corresponding to residual cleavages at methionine appear. However, there *are* satellite spots and these correspond rather to the reversal of the HCHO cross-link which we are unable to avoid. The major H2B and H4 spots indicated in the figure arose from reversal prior to first-dimension analysis. The satellite spots correspond to reversal occurring subsequent to the first-dimension analysis. Thus spot β, corresponding to intact dimer, has below it two satellite spots corresponding to H2B and H4. Similarly, spot d has a satellite (barely visible) corresponding to H2B, while spot c has a satellite corresponding to H4.

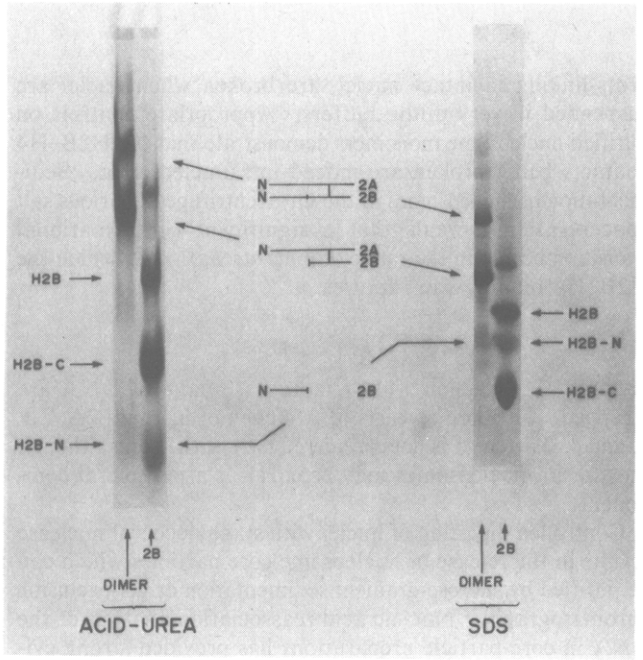

FIGURE 9: CNBr peptide analysis of the methyl acetimidate induced H2A–H2B dimer. Chromatin was cross-linked with methyl acetimidate, and the dimer was purified by chromatography on Bio-Gel P30 and preparative gel electrophoresis. The products of CNBr digestion were analyzed in one dimension on both acid–urea and $NaDodSO_4$ gels.

acterization of these cross-linked products has begun to yield the outlines of the histone–histone binding surfaces within chromatin. Of particular note is the fact that four isomeric H2B–H4 dimers have been characterized, each cross-linked at a different site with cross-linkers of different chemical specificities. Yet all the cross-links clearly are clustered in specific regions of the histone molecules. All cross-links are to the C-terminal half of H2B, and all but one are to the C-terminal 18 amino acid residues of H4. In the case of the one exception, dimer β induced by HCHO, the cross-link may well be close to the CNBr cleavage site at methionine-84 of H4.

The contacts on H2B for H2A are also probably clustered. We have shown previously that UV induces a cross-link from H2A to the N-terminal half of H2B (Martinson & McCarthy, 1976). More recently we have localized this cross-link to within residues 37 to 42 of H2B (De Lange, et al., 1979). On the other hand, the data presented above show that methyl acetimidate induces a cross-link from H2A to the C-terminal half of H2B. Spiker & Isenberg (1977) have shown that, in solution, the central methionines of H2B are essential to the H2A–H2B interaction but are of little importance to the H2B–H4 interaction. It therefore seems likely that the acetimidate cross-link is in the central portion of H2B (perhaps to an arginine on the C-terminal side of methionine-62), while the cross-links to H4 are in the C-terminal third of H2B much as for H4 itself.

Thus a functional map for H2B is beginning to emerge. The N-terminal third interacts with DNA (Whitlock & Simpson, 1977; Kato & Iwai, 1977), the C-terminal third interacts with H4, and the middle third interacts primarily with H2A. A more complicated pattern and additional interactions are of course not excluded.

The identification and characterization of trimers produced by contact-site cross-linkers are important for the ultimate interpretation of the contact-site data. Thus the very fact that contact-site H2A–H2B–H4 trimers can be produced at all shows that the precursor dimers to these trimers do not by virtue of their cross-links perturb significantly the structure of the nucleosome. Especially significant is the fact that the trimers contain the specific cross-links characteristic of the particular cross-linker used. Thus the cross-link between H2B and H4 in the H2A–H2B–H4 UV trimer is the same as that in the UV-induced H2B–H4 dimer, while the cross-link between H2B and H4 in the H2A–H2B–H4 UV $\rightarrow C(NO_2)_4$ trimer is the same as the one in the $C(NO_2)_4$-induced H2B–H4 dimer. We have shown previously (Martinson & McCarthy, 1975), and in greater detail more recently (Martinson et al., 1979a), that the integrity of the H2B–H4 interaction as probed by contact-site cross-linkers is particularly sensitive to perturbations of structure in the nucleosome.

We should point out that, with the exception of the UV-induced H2A–H2B dimer, the other cross-linked products which we have described are produced in relatively low yield. Considering the various chemical mechanisms involved, these low yields are not at all surprising, and we presume that they are the result of chemical inefficiency such as competing side reactions. It is possible, however, that the clustered cross-links which we are characterizing represent not different points within the same binding surface but rather different contact arrangements within nucleosomes of different conformations. This is obviously an interesting possibility, but we consider it unlikely because acid-extracted histones, renatured into 2.5 M NaCl in the absence of DNA, give cross-linking yields similar to those of intact nuclei (Martinson & True, 1979). Complete mixing of the histones in this way in the absence of constraints imposed by DNA or the nonhistones would not be expected to give rise to conformational subpopulations characteristic of native chromatin. Nevertheless, we have

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

We thank Shirley Phillips for preparation of the manuscript and Therese Ruettinger for helpful criticisms.

References

Alfageme, C. R., Zweidler, A., Mahowald, A., & Cohen, L. H. (1974) *J. Biol. Chem. 249*, 3729–3736.
Bonner, W. M., & Pollard, H. B. (1975) *Biochem. Biophys. Res. Commun. 64*, 282–288.
De Lange, R. J., Williams, L. C., & Martinson, H. G. (1979) *Biochemistry* (in press).
Feldman, M. Y. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 1–49.
Felsenfeld, G. (1978) *Nature (London) 271*, 115–122.
Kato, Y., & Iwai, K. (1977) *J. Biochem. 81*, 621–630.
Kunkel, G., & Martinson, H. G. (1978) *Nucleic Acids Res. 5*, 4263–4272.
Martinson, H. G., & McCarthy, B. J. (1975) *Biochemistry 14*, 1073–1078.
Martinson, H. G., & McCarthy, B. J. (1976) *Biochemistry 15*, 4126–4131.
Martinson, H. G., & True, R. (1979) *Biochemistry 18*, 1089–1094.
Martinson, H. G., Shetlar, M. D., & McCarthy, B. J. (1976) *Biochemistry 15*, 2002–2007.
Martinson, H. G., True, R., & Burch, J. B. E. (1979a) *Biochemistry 18*, 1082–1089.
Martinson, H. G., True, R., Burch, J. B. E., & Kunkel, G. (1979b) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Panyim, S., Sommer, K. R., & Chalkley, R. (1971) *Biochemistry 10*, 3911–3917.
Spiker, S., & Isenberg, I. (1977) *Biochemistry 16*, 1819–1826.
Sweadner, K. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 962–969.
Tack, L. O., & Simpson, R. T. (1977) *Biochemistry 16*, 3746–3753.
Whitlock, J. P., Jr., & Simpson, R. T. (1977) *J. Biol. Chem. 252*, 6516–6520.
Wyns, L., Lasters, I., & Hamers, R. (1978) *Nucleic Acids Res. 5*, 2345–2358.

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

Harold G. Martinson, Rod J. True, and John B. E. Burch*

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

† From the Division of Biochemistry, Department of Chemistry, and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received September 1, 1978; revised manuscript received December 6, 1978.* This work was supported by Grant PCM 76-00712 from the National Science Foundation and by the Research Committee of the Academic Senate, UCLA.

0006-2960/79/0418-1082$01.00/0