Amino Acid Contacts between Histones Are the Same for Plants and Mammals. Binding-Site Studies Using Ultraviolet Light and Tetranitromethane†

Harold G. Martinson* and Rod J. True

ABSTRACT: Leek chromatin has been cross-linked by UV light and tetranitromethane. The same major H2A–H2B and H2B–H4 cross-linked dimers are formed as in mammalian chromatin. CNBr peptide mapping shows that the cross-links occur in the same regions of the histone sequence for both plants and mammals. Interspecies complexes formed between leek and calf H2A and H2B can be cross-linked by UV light with the same specificity as intraspecies H2A–H2B complexes. We conclude that certain geometric features of histone–histone binding sites are conserved precisely during evolution despite large changes in the overall histone sequence. Moreover, our data show that identification of cross-linked amino acids using binding-site probes such as UV light and tetranitromethane can yield significant information about thermodynamically important contacts within histone–histone binding sites.

The structure of all eukaryotic chromatin is apparently based upon the fundamental unit now generally called the nucleosome. This chromosomal subunit consists of an octamer of histones (two each of the so-called "core histones", H2A, H2B, H3, and H4) around which is wrapped between one and two turns of DNA about 140 base pairs long [see Felsenfeld (1978) for a review].

One approach to the study of the internal structure of the nucleosome involves the use of contact-site cross-linkers (Martinson & McCarthy, 1976; Martinson et al., 1979b). These cross-linkers, by definition (Martinson et al., 1979b), cross-link only amino acid residues that are sterically capable of contact in their prior noncovalent state. Obviously, there is a reasonable probability that such amino acids may be part of thermodynamically important binding sites prior to covalent linkage. Reagents with specificity for an enzyme-active site are called active-site probes. Similarly, any contact-site cross-linkers which show a preference for amino acids that are likely to exist within binding sites we refer to as binding-site probes. We have argued previously that UV light and tetranitromethane [$C(NO_2)_4$] are binding-site probes (Martinson & McCarthy, 1976). Protein–protein binding contacts may be expected to be particularly resistant to evolutionary change in much the same way as enzyme-active sites. In this paper, we show, by comparison of plant and mammalian chromatin cross-linking properties, that the histone–histone contacts which are cross-linked by UV and $C(NO_2)_4$ are much more highly conserved evolutionarily than are histone sequences in general.

These data, in combination with previous arguments concerning the chemical nature of the cross-links induced, argue that UV and $C(NO_2)_4$ can be used as true binding-site probes in the study of nucleosome structure. We have recently identified the amino acids involved in the UV-induced H2A–H2B cross-link [tyrosine-37, -40, or -42 of H2B and proline-26 of H2A; DeLange et al. (1979)]. The data presented in the present paper leave little doubt that the same residues are joined by UV in leek as in mammalian chromatin and that the special tyrosine–proline steric relationship which facilitates UV cross-linking is part of a fundamentally important binding interaction between H2A and H2B within nucleosomes.

Materials and Methods

Leek was chosen as our plant material because it is readily purchased and easily stored. Chromatin was prepared through two washings in 50 mM Tris as described by Nadeau et al. (1974). Chromatin from 2 kg of tissue was then cross-linked with $C(NO_2)_4$ or UV light essentially as described previously (Martinson et al., 1979b), except that, in the case of UV irradiation, the exposure time was 1 h instead of 4 h. After cross-linking, the chromatin was recovered by centrifugation, washed with 0.35 M NaCl, and extracted of histones essentially as described (Martinson et al., 1979c). Smaller samples of chromatin, as well as samples of purified histone, were cross-linked by UV light in a manner similar to that described previously (Martinson et al., 1979c). Histone purification by column chromatography and preparative gel electrophoresis was carried out as before (Martinson et al., 1979b,c). Our methods of CNBr cleavage, as well as one- and two-dimensional gel electrophoresis, have also been described (Martinson et al., 1979b).

Results

Cross-Linking of Leek Chromatin. Leek chromatin was cross-linked by UV and $C(NO_2)_4$ according to standard procedures. Figure 1 shows that UV and $C(NO_2)_4$ each induce the production primarily of one major dimer component (indicated by arrows). As for mouse L cell chromatin (Martinson & McCarthy, 1976), the $C(NO_2)_4$ dimer has a greater mobility on acid–urea gels than the UV-induced dimer, suggesting that these dimers are H2B–H4 and H2A–H2B, respectively, just as in mammalian chromatin.

In view of the unusually low mobility of the UV cross-linked component, the two-dimensional gel in Figure 2 was run to rule out the possibility that it may be a trimer. That the cross-linked component is, in fact, a dimer can be seen by comparison of its mobility with that of authentic H3 dimer. (It may be noted that H1 displays three components in Figure 2 but only two in Figure 1. We frequently observe selective loss of particular members of the H1 group. Other features of Figure 2 are discussed in the legend.)

Fractionation of Cross-Linked Histones on Bio-Gel P30. As the first step in the preparation of the cross-linked dimers for further characterization, the histones were chromato-

† From the Division of Biochemistry, Department of Chemistry, and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024. *Received October 9, 1978; revised manuscript received January 19, 1979.* This work was supported by Grant PCM 79-00712 from the National Science Foundation.

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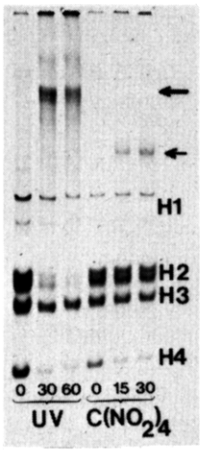

FIGURE 1: Histone–histone dimers from UV or $C(NO_2)_4$ cross-linked leek chromatin. UV cross-linking was conducted for the indicated times (in minutes) on 0.5 mL samples of leek chromatin at a DNA concentration of about 0.6 mg/mL. $C(NO_2)_4$ cross-linking was conducted on leek chromatin at a DNA concentration of about 0.4 mg/mL in 35 mM sodium phosphate, pH 7.5. Approximately 4 mL of chromatin was stirred gently under a stream of humid N_2 for 30 min. Cross-linking was initiated by the anaerobic addition of 10 μL of $C(NO_2)_4$. At the indicated times, 0.75-mL aliquots were withdrawn. The histones from both the UV and $C(NO_2)_4$ cross-linked chromatin were acid extracted and electrophoresed on a 15% acid–urea–polyacrylamide slab gel.

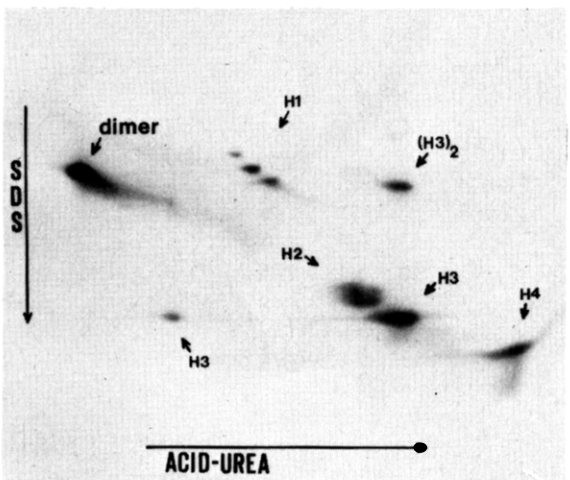

FIGURE 2: Two-dimensional gel pattern of histones from UV cross-linked leek chromatin. Cross-linking and gel electrophoresis were carried out as described. This particular sample was from a preparation which was irradiated in distilled water. Thus, this figure demonstrates that the H2A–H2B dimer of leek can be formed at very low ionic strength just as is the case for the H2A–H2B dimer–but not other dimers—of calf (Martinson et al., 1979a). Two H3 spots and an H3 dimer spot [$(H3)_2$] are indicated in the figure. The lower right H3 spot is at the normal position for H3 in this system. The lower left spot arose from H3, which ran as dimer in the acid–urea dimension but which was reduced by exposure of the first-dimension gel slice to mercaptoethanol prior to electrophoresis in the second dimension. The upper right-hand H3 dimer spot arose by oxidative dimerization of the H3 monomer following first-dimension electrophoresis. In fact, we presume that most H3 becomes oxidized during staining, etc., following the acid–urea dimension. Incomplete reduction prior to second-dimension analysis then gives rise to the appearance of residual dimer.

graphed on Bio-Gel P30. Aliquots from the fractions collected were assayed for histone content by acid–urea–polyacrylamide gel electrophoresis as shown in Figure 3. These elution profiles further substantiate that $C(NO_2)_4$ induces an H2B–H4 dimer, while UV light induces an H2A–H2B dimer. Figure 3 shows that the $C(NO_2)_4$ dimer of leek elutes between the H2 and the H3 region of the profile, whereas the UV-induced dimer of leek elutes between the H1 and the H2 region of the profile. Bio-Gel P30 fractionates histones on the basis of affinity as well as molecular weight. Previous studies involving mammalian histones have shown that H2B–H4 dimers elute between H2B and H3 on Bio-Gel P30, whereas H2A–H2B dimers elute between H1 and H2A (Martinson & McCarthy, 1976; Martinson et al., 1979b).

CNBr Peptide Mapping of Cross-Linked Leek Histones. The dimer identifications and the general locations of the cross-links were determined directly by CNBr peptide mapping. Appropriate fractions from the column runs of Figure 3 were pooled and purified further by column chromatography and preparative gel electrophoresis prior to cleavage with CNBr. The results are shown in Figure 4. The salient features of this figure, together with the major conclusions derived from it, will be outlined briefly below. A more detailed analysis of Figure 4, together with additional CNBr mapping data, will then be covered in the next section.

Lanes 6 and 7 of Figure 4 show the results of CNBr treatment of the two principal members of the H2 histones of leek (see Figure 1). The upper member (lane 6), which is not cleaved, must contain no methionine and is therefore identified as H2A by analogy with the predominant H2A of mammalian cells [e.g., see Spiker & Isenberg (1977)]. The lower member of the leek H2 doublet (lane 7) *is* cleaved by CNBr to yield a pattern similar to that of CNBr-treated calf thymus H2B (lane 4) and is therefore identified as H2B. This is in agreement with Hayashi et al. (1977) who have provided evidence from peptide analysis that the methionine-containing region of pea H2B is identical with that of calf thymus.

Lane 5 of Figure 4 shows that the UV-induced leek histone dimer is an H2A–H2B dimer. The gel pattern of the CNBr-cleaved leek UV dimer is fundamentally the same as that obtained for the H2A–H2B dimer of mammalian chromatin (Martinson & McCarthy, 1976). There is a faint band of low mobility which corresponds to residual, uncleaved dimer. The very dark band below it corresponds to intact H2A linked to the NH_2-terminal half of H2B. Finally, a prominent band of high mobility corresponding to the COOH-terminal half of H2B cleaved from the dimer by CNBr can be identified by comparison with the H2B standard of lane 7. No band corresponding to the NH_2-terminal half of H2B appears (although a faint band of unknown composition can be seen at a position of slightly lower mobility; see legend to Figure 4). We conclude that UV light induces specific H2A–H2B dimer formation in leek chromatin and that the cross-link is located in the NH_2-terminal half of H2B just as for mammalian chromatin.

The CNBr cleavage products of the $C(NO_2)_4$-induced leek histone dimer are displayed in lane 2 of Figure 4. Comparison with the cleavage products of H4 and H2B, shown in the adjacent lanes (1 and 3), confirms that the $C(NO_2)_4$-induced dimer of leek is an H2B–H4 dimer and that the position of the cross-link is homologous to that for mammalian chromatin (see below).

With the exception of an unidentified minor component of high mobility (see legend to Figure 4), the gel pattern of CNBr-cleaved H4 (land 1) is the same as that which we obtain from mammalian chromatin (Martinson & McCarthy, 1976; Mehrabian and Martinson, unpublished experiments) as would be expected (DeLange et al., 1969; Nadeau et al., 1974). The 18 amino acid COOH-terminal fragment is seen only on heavily loaded gels such as those of Figure 4. The CNBr peptides of H2B from $C(NO_2)_4$ cross-linked chromatin are displayed in lane 3. This pattern is obviously the same as that

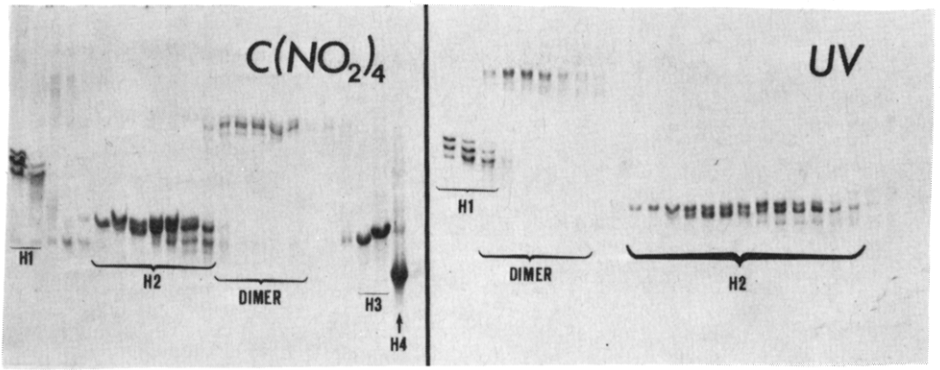

FIGURE 3: Bio-Gel P30 fractionation of leek histones from cross-linked chromatin. About 100 mg of histone was chromatographed on Bio-Gel P30 in 10 mM HCl as described. Aliquots from alternate [$C(NO_2)_4$] or every (UV) column fraction were concentrated by lyophilization and analyzed by acid–urea–polyacrylamide gel electrophoresis. Note that only the first half of the elution profile is shown in the case of histones from UV-irradiated chromatin.

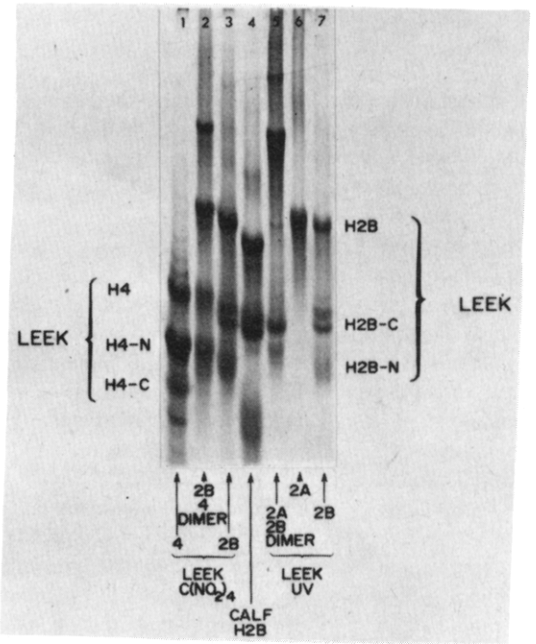

FIGURE 4: CNBr peptide analysis of cross-linked leek histones. Histones were purified from leek chromatin which had been cross-linked either with $C(NO_2)_4$ or UV light as indicated in the figure. Each species was subjected to cleavage by CNBr, and the products of the reaction were displayed on an acid–urea–polyacrylamide gel. The cleavage products for H4 (lane 1) and H2B (lane 7) are indicated in the figure. "N" and "C" refer to the NH_2- and COOH-terminal CNBr fragments of the designated histones. The center lane in the figure shows the electrophoretic pattern of H2B from calf thymus cleaved with CNBr. All of the histones except H4 and the H2A–H2B dimer were electrophoretically purified prior to analysis. The faint, unidentified bands in lanes 1 and 5 (see text) probably arise from contaminants in the preparations.

for H2B from UV-treated chromatin, demonstrating that the cross-linking procedures do not influence the subsequent CNBr cleavage characteristics of the histone monomers.

Comparison of the CNBr pattern of the $C(NO_2)_4$ dimer (lane 2) with that for H4 and H2B shows that CNBr cleavage releases peptides from the dimer which correspond to the NH_2-terminal CNBr fragments of both H4 and H2B. No peptides migrate at the positions of the H2B and H4 COOH-terminal fragments. Thus, just as with calf thymus (Martinson et al., 1979b) and mouse L cell chromatin (Martinson & McCarthy, 1976), it must be the COOH-terminal portions of H2B and H4 which are covalently joined by the cross-link. This cross-linked peptide, released from the dimer by CNBr cleavage, migrates in the position of intact H4 on acid–urea gels (see below).

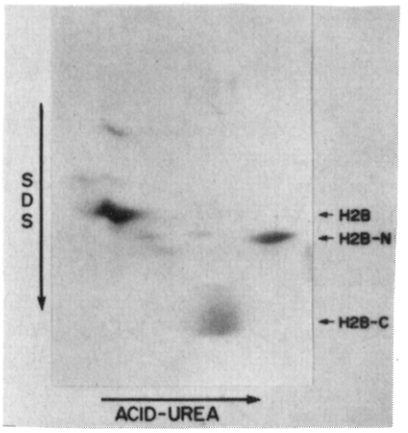

FIGURE 5: Two-dimensional CNBr peptide map of leek H2B. An aliquot of the sample shown in lane 3 of Figure 4 was electrophoresed as shown in that figure. The lane was excised from the gel after staining and destaining, equilibrated with sodium dodecyl sulfate (SDS) loading buffer, and then electrophoresed in the second dimension as shown. The main spots were identified by comparison with a similar two-dimensional map of calf H2B CNBr peptides (see text).

Detailed Analysis of the CNBr Cleavage Data. The essential similarity of the CNBr peptide maps for leek and calf H2B has been noted above (compare lanes 3 and 7 with lane 4 of Figure 4). Differences do exist, however, but these are easily rationalized on the basis of current knowledge concerning plant and mammalian H2B. Thus, the COOH-terminal CNBr doublet of leek H2B [there is a doublet because either or both of two closely spaced methionines may be cleaved; Martinson & McCarthy (1976)] has a similar mobility to that of calf, while the NH_2-terminal doublet of leek H2B is of considerably lower mobility. This is consistent with the finding that plant H2B has a higher molecular weight than mammalian H2B and that most of this increase resides in the NH_2-terminal region (Hayashi et al., 1977).

In order to confirm further the CNBr-fragment assignments for leek H2B, a two-dimensional gel electrophoretic analysis was carried out. We have reported previously that the relative mobilities of the NH_2- and COOH-terminal halves of calf thymus H2B are reversed in sodium dodecyl sulfate relative to acid–urea gel electrophoresis (Martinson et al., 1979b). That this is true also for the homologously assigned leek H2B CNBr fragments was shown by two-dimensional gel electrophoresis as illustrated in Figure 5. There it can be seen that the less positively charged and more hydrophobic COOH-terminal half of leek H2B migrates more slowly in acid–urea but more rapidly in sodium dodecyl sulfate com-

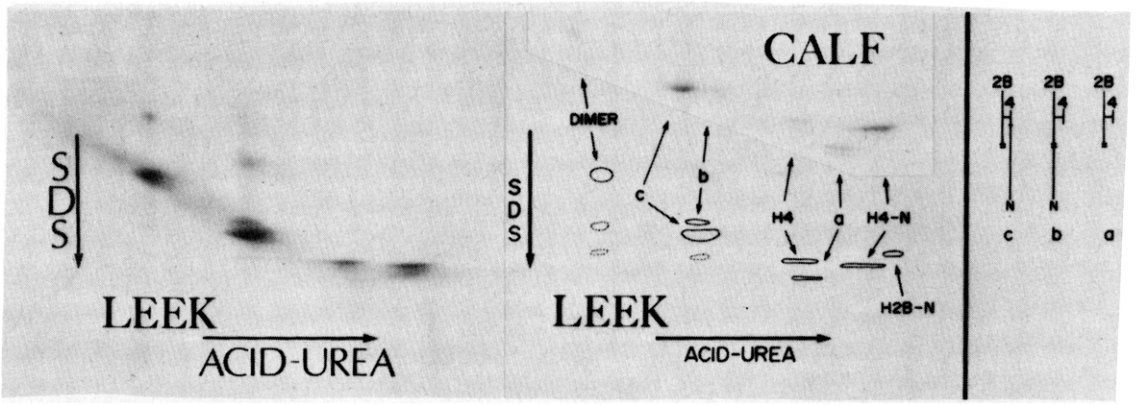

FIGURE 6: Two-dimensional CNBr map of the $C(NO_2)_4$ cross-linked H2B–H4 dimer from leek. The left-hand panel shows the map obtained from an aliquot of the sample shown in lane 2 of Figure 4, which was electrophoresed in two dimensions as described in the legend to Figure 5. The middle panel is a diagrammatic representation of the leek peptide map. The "calf" inset of the middle panel is the relevant portion of a two-dimensional map for the $C(NO_2)_4$-induced H2B–H4 dimer of calf. The designations a, b, and c of the middle panel are explained by means of a diagrammatic representation of partially or fully cleaved H2B–H4 dimer at the extreme right [see Martinson & McCarthy (1976) or Martinson et al. (1979b)].

pared to the NH_2-terminal half. Two-dimensional gels of CNBr-cleaved calf thymus H2B are very similar in appearance, particularly with respect to the diffuse nature of the COOH-terminal H2B spot. We therefore conclude that our identification of leek H2B (and H2A), as well as our analysis of the UV-induced leek H2A–H2B dimer (see above), is correct.

Complete analysis of the CNBr products produced by cleavage of the leek $C(NO_2)_4$ H2B–H4 dimer is more complicated than for the H2A–H2B dimer for several reasons. First, the H2B–H4 dimer yields a larger number of CNBr fragments (Martinson & McCarthy, 1976; Martinson et al., 1979b). Second, the $C(NO_2)_4$-induced cross-link is subject to slow reversal during handling and storage (Martinson et al., 1979b). Finally, in the case of leek, there is a greater incidence of comigration of the various products of CNBr cleavage and cross-link reversal.

In order to confirm the cross-linking assignments deduced from the acid–urea data of Figure 4, the two-dimensional gel analysis illustrated in Figure 6 was carried out. The left-hand panel of Figure 6 shows a two-dimensional gel of a CNBr digest of the leek $C(NO_2)_4$ H2B–H4 dimer. In the center panel of Figure 6, this two-dimensional electrophoretic pattern of the cleaved dimer is represented diagrammatically and compared to a two-dimensional display of fragments from a similar digest of the $C(NO_2)_4$ H2B–H4 dimer from calf thymus. The spots labeled a, b, and c correspond to the histone compositions illustrated in the right-hand panel of Figure 6 and are the same designations as used previously (Martinson & McCarthy, 1976; Martinson et al., 1979b). The dotted outlines correspond to spots resulting from H2B and H4 which apparently are produced by continued reversal of the cross-link within the first-dimension gel prior to second-dimension analysis (see Martinson et al., 1979b).

The middle panel of Figure 6 relates the two-dimensional pattern for the CNBr-cleaved leek $C(NO_2)_4$H2B–H4 dimer to the two-dimensional CNBr cleavage pattern of the homologous and previously characterized mammalian $C(NO_2)_4$ H2B–H4 dimer (Martinson & McCarthy, 1976; Martinson et al., 1979b). The overall homology of the patterns is evident, and, where they differ, the changes are predictable. Thus, spot c should display roughly the same mobility for both calf and leek, because it corresponds to H4 linked to the COOH-terminal half of H2B, both highly conserved entities [Hayashi et al. (1977) and see above]. In contrast, spot b has markedly reduced mobilities in both dimensions for leek as compared to calf, as would be expected for a product comprised, in part, of the higher molecular weight plant H2B molecule [Hayashi et al. (1977) and see above]. By use of the H4 spot as a point of reference [the H4 is a product of cross-link reversal; see Martinson et al. (1979b)], it can be seen that spot a has a slightly lower mobility in the case of leek than for calf thymus. But the effect is small in keeping with the relatively small difference in mobility on acid–urea of the COOH-terminal halves of H2B from leek and calf (Figure 4, lanes 3 and 7 vs. lane 4). Thus, a direct comparison of the two-dimensional electrophoretic gel patterns of CNBr digests of leek and calf H2B–H4 dimers shows that $C(NO_2)_4$ acts on both types of chromatin with comparable specificity to yield equivalent cross-linked products.

Interspecies Cross-Complexing between Leek and Calf H2A and H2B. The highly specific nature of the UV and $C(NO_2)_4$ cross-linking reactions and the apparent preservation during evolution of the appropriate steric relationships of the cross-linkable residues suggested that leek and calf histones would be competent to interact specifically to form interspecies complexes of equivalent binding geometry. In a preliminary experiment, this has been tested for the case of the UV-induced H2A–H2B dimer.

Leek histones 2A (i.e., H2A + H2B) were copurified on Bio-Gel P30 as shown in Figure 3. They were then irradiated in solution either alone or in combination with calf H2A or H2B under conditions which give rise only to cross-linking of specific H2A–H2B complexes (Martinson et al., 1979c). Figure 7 shows that irradiation of the leek H2A + H2B mixture gives rise to the usual leek UV H2A–H2B dimer (lane 1). However, inclusion of calf H2A in the mixture (lane 2) gives rise to a new dimer of mobility intermediate to the intraspecies leek or calf H2A–H2B dimers. This new dimer is presumably the hybrid, H2A(calf)–H2B(leek). Inclusion of calf H2B in the mixture rather than calf H2A gives rise to yet another new dimer (lane 3), which we presume to be the interspecies dimer H2A(leek)–H2B(calf). We note that when either calf H2A or H2B is mixed with the leek histones, calf–leek dimer formation is favored over leek–leek dimer formation. We have no explanation for this effect. Mixing all four histones (i.e., H2A and H2B from calf as well as H2A and H2B from leek) gives rise to all four possible H2A–H2B dimers, both intraspecies and interspecies, although calf–calf dimer formation is favored (lane 4). Thus the histone–histone binding parameters are apparently so well preserved evolutionarily that even the minute details of geometry that permit

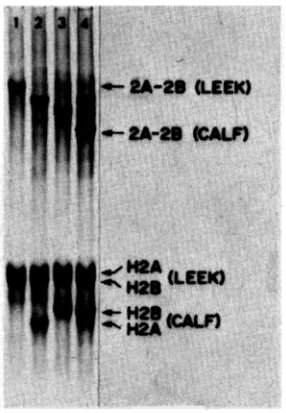

FIGURE 7: Interspecies cross-complexing of leek and calf H2A and H2B. Acid-extracted histones from *un*cross-linked leek chromatin were fractionated by column chromatography as shown in Figure 3. The H2-containing fractions were pooled and lyophilized. Leek H2 was then mixed with calf H2A (lane 2), calf H2B (lane 3), calf H2A + H2B (lane 4), or no calf histones (lane 1) as evident in the figure. Cross-linking by UV irradiation in 2 M NaCl and 2 M urea was carried out exactly as described previously [see Figure 4 and Materials and Methods of Martinson et al. (1979c)].

precise positioning of the cross-linkable residues [see Discussion as well as DeLange et al. (1979)] are maintained with remarkable fidelity. We anticipate that similar evolutionary conservation would be observed for interspecies $C(NO_2)_4$ cross-linking, since H4 is virtually identical in sequence between plant and mammal (DeLange et al., 1969).

Discussion

The results which we have presented demonstrate that UV light and $C(NO_2)_4$ exhibit the same selectivity of cross-linking in leek chromatin as in mammalian chromatin. For each agent and each type of chromatin, primarily one species of cross-linked product is formed. In addition to equivalent selectivity, these cross-linking agents also exhibit the same specificity of cross-linking in plant and mammalian chromatin. The same histones become cross-linked, and the cross-links are located within the same regions of the histone sequences. Thus, for both types of chromatin, UV light induces the formation of an H2A–H2B dimer with a cross-link located within the NH_2-terminal half of H2B, and $C(NO_2)_4$ induces the formation of an H2B–H4 dimer in which the cross-link joins the COOH-terminal portions of the two histones. These results serve to confirm and extend the study of Spiker & Isenberg (1977) in which they showed that plant (pea) histones exhibit the same specific histone–histone binding interactions in solution as do calf histones.

Our results on interspecies complexing between the histones 2A and 2B of leek and calf suggest that, despite the substantial differences in molecular weight and sequence between these histones, the binding surfaces are nevertheless so well preserved that even the details of geometry necessary for UV-induced cross-linking to occur are maintained. As explained below, we feel that this is of considerable significance with respect to the use of contact-site cross-linkers for the study of histone–histone binding interactions.

UV is known to induce cross-linking through the activation of tyrosine (Martinson et al., 1976; Martinson & McCarthy, 1976; DeLange et al., 1979). Yet, despite the fact that each of the core histones contains several tyrosines, only one of these tyrosines gives rise to a high yield of cross-links upon absorption of UV light. We have shown that this tyrosine is residue-37, -40, or -42 of H2B (DeLange et al., 1979). Apparently, the special nature of this tyrosine is preserved through evolution.

The remarkable specificity of the UV-induced H2A–H2B cross-linking event is further illustrated by the unusual chemical nature of the cross-link. To our knowledge, all previously characterized UV-induced cross-links in other systems involve the formation of bityrosyl bridges (see Martinson & McCarthy, 1976). However, we have shown recently that the H2A–H2B dimer involves a tyrosine(H2B) → *proline*(H2A) linkage (DeLange et al., 1979). In order for a proline to be covalently receptive to an activated tyrosine, we expect that it must be positioned very precisely indeed. Apparently the conformation of the H2A–H2B binding site is so exactly preserved throughout evolution that the precise positioning of the proline–tyrosine pair is maintained even for interspecies complexes. This is dramatically consistent with the prediction of Spiker & Isenberg (1978) based on thermodynamic arguments that a change between plants and mammals of even one residue at the H2A–H2B binding interface was unlikely. They showed that the interspecies and intraspecies binding constants for plant and mammalian H2A and H2B were the same within experimental error.

The interspecies cross-linking results which we have reported support the original suggestion (Martinson et al., 1976) that the UV-induced cross-link lies within the H2A–H2B binding site and that the noncovalent relationship between the residues prior to cross-linking is of significant thermodynamic importance to the binding interaction. The use of various contact-site cross-linkers (Martinson et al., 1979b) to study histone–histone interactions thus offers the possibility of obtaining particularly significant information when the studies are combined with evolutionary comparisons in order to identify those contacts which are most likely to be of fundamental importance to the structure and function of the nucleosome.

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