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Biochemistry

Histone 1 is proximal to histone 2A and to A_{24}

(chromatin/nucleosomes/crosslinking/water-soluble carbodiimide)

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ABSTRACT **Water-soluble carbodiimide crosslinks histones 1A and 1B to histone 2A and to semi-histone A_{24} in chromatin from mouse cells. The identities of the histone species present in the crosslinked dimers were determined by fingerprinting. The molar ratio of H1–A_{24} to H1–H2A is the same as the molar ratio of A_{24} to H2A in these cells. The H1–H2A crosslinks form equally well in whole nuclei, lysed nuclei, and H1-containing mononucleosomes isolated from a sucrose gradient. These results suggest that there exist major H1 interactions within the nucleosome.**

Four major histone species are found in the nucleosome, a subunit that imposes the first level of compaction on the DNA double helix (1). A fifth major histone, H1 (H1 and H5 in some cells), is also found in chromatin, but its structural role is less clear. After brief nuclease digestion a fraction of the nucleosomes contains H1 (2–5); upon further digestion the H1 is released along with a piece of DNA 25 base pairs long (6). Although these studies have localized H1 near the histones of the nucleosome core, the modes of association, if any, between H1 and the core histones have not been elucidated. Indirect evidence for H1–H2B, H1–H3, and H1–H4 associations using cleavable crosslinkers has been reported (7–9) as has evidence that H1 can be crosslinked to form H1 homopolymers (7, 10, 11).

We report here that water-soluble carbodiimide condenses H1 with H2A and A_{24} in nuclei from mouse L1210 cells. Similar crosslinked species are also found in chicken erythrocyte nuclei.

METHODS

Cells. Static cultures of L1210 mouse leukemia cells were maintained in RPMI medium 1630 supplemented with 20% fetal calf serum without antibiotics. At weekly intervals, subcultures were initiated, and the cells were propagated in suspension culture in the presence of penicillin and streptomycin. Cell densities were kept between 0.25 and 1.0×10^6/ml; doubling time was 12–14 hr.

Chicken erythrocytes were obtained from Truslow Farms (Chestertown, MD) and were maintained in Alsever's solution at 4°C for use within 24 hr.

Labeling. L1210 cells were labeled overnight with L-[*U*-^{14}C]leucine (New England Nuclear) at 1 μCi (1 Ci = 3.7×10^{10} becquerels)/ml in leucine-free RPMI 1630 with 20% fetal calf serum, which contained sufficient leucine for normal cell growth.

Isolation of Nuclei. L1210 nuclei were isolated at 0–2°C by a detergent method (12). Pellets containing up to 1.5×10^8 cells were washed once with 10 ml of buffer A (1 mM potassium phosphate/1 mM $CaCl_2$/0.32 M sucrose, pH 6.7) and centrifuged for 5 min at $170 \times g$. The cells were suspended in 1 ml of buffer A and lysed by the addition of 9 ml of buffer A containing 0.3% Triton N-101 (Sigma). Nuclei were pelleted at $675 \times g$ for 5 min and washed once with buffer A/Triton N-101.

Chicken erythrocytes were washed in 0.15 M NaCl/0.015 M sodium citrate, pH 7, and then in buffer B (5 mM Tris·HCl/5 mM $MgCl_2$/0.25 M sucrose, pH 7) and lysed in buffer B containing 0.5% Nonidet P-40. Nuclei were pelleted at $675 \times g$ for 5 min and washed once with buffer B/0.5% Nonidet P-40.

Crosslinking. Nuclei (1 mg of DNA per ml) were dialyzed against 2.5 mM potassium phosphate (pH 6.7) at 4°C overnight and then incubated with 1 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce) for 1 hr at 37°C. 2-Mercaptoethanol was added (2 μl/ml) to stop the reaction.

Sample Preparation. Nuclei were extracted for 1 hr at 0°C with 0.2 M H_2SO_4/50 mM $NaHSO_3$. Insoluble material was pelleted; the supernatant was mixed with 4 vol of ethanol and kept overnight at −20°C. The ethanol precipitate was sedimented at $5000 \times g$ for 10 min and washed once with cold ethanol. The dried pellet was dissolved in 8 M urea containing 0.002% phenolphthalein and 50 mM dithiothreitol. NH_4OH was added until the solution turned a faint pink. After a 5-min incubation at 37°C, the solution was made 1 M in acetic acid.

Gel Electrophoresis. Samples were loaded onto acetic acid/8 M urea gels (13) prepared in forms 15 cm wide × 27 cm long × 0.15 cm thick. For analytical work, 5-mm slots of a gel prepared with 15% acrylamide/0.09% bisacrylamide were loaded with 20 μl of solution containing approximately 100 μg of histone. For preparative work, 200 μl of solution containing approximately 2 mg of histone was loaded onto a 5-cm slot of a gel prepared with 10% acrylamide/0.13% bisacrylamide.

After electrophoresis, staining, and destaining, samples were cut from the gel, soaked for 3 hr in 62 mM Tris·HCl, pH 6.8/5% 2-mercaptoethanol, and loaded on top of sodium dodecyl sulfate ($NaDodSO_4$) gels prepared in forms 15 cm wide × 27 cm long × 0.22 cm thick (9).

For analytical work, a 1-cm-wide strip cut from a 15% acrylamide/acetic acid/8 M urea gel was placed on top of a 5-cm $NaDodSO_4$ stacking gel and embedded with 1% agarose/62 mM Tris·HCl, pH 6.8/0.1% $NaDodSO_4$. For preparative work, a 3- to 5-cm-wide strip cut from a 10% acrylamide/acetic acid/8 M urea gel was placed on top of a 10-cm $NaDodSO_4$ stacking gel. In both cases, the $NaDodSO_4$ resolving gel was 15% acrylamide/0.09% bisacrylamide (14, 15).

Fingerprinting. Radioactive proteins were recovered from stained one- or two-dimensional gels, trypsinized, dansylated, and chromatographed on 10 × 10 cm silica gel plates according to the fingerprinting method of Zanetta *et al.* (16) as modified by Oskarsson *et al.* (17). Radioactive spots were detected by the fluorography method of Bonner and Stedman (18). This method consists of dipping the dried thin-layer plate in a solution of 0.4% 2,5-diphenyloxazole in 2-methylnaphthalene at 37°C. Plates were then exposed to flashed film (19) at −70°C (20).

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

RESULTS

Purification and Characterization of H1–H2A Crosslinks. When nuclei were treated with the carbodiimide, many new spots appeared on two-dimensional gels (Fig. 1 *upper*). Spot α has been identified as H3–H4 (11). The identification of spots A–D is the subject of this paper.

Fingerprinting analysis of spots A and B showed that spot A contained H1A and H2A (Fig. 2). Spot B contained H1B and H2A, possibly with some H2B. Fig. 3 identifies the spots on the peptide maps. Fifteen spots from H2A are apparent in both maps. Eleven H1 spots, including three H1A-specific spots, are seen in the map of spot A. Ten H1 spots, including one H1B-specific spot, are seen in the map of spot B. The relative intensities of the H1 and H2A spots were close to those expected for a H1–H2A crosslink. Fig. 4 shows an experiment in which an equimolar mixture of H1A and H2A was fingerprinted and compared to the fingerprint of putative H1A-H2A (spot A). The two patterns were similar with two exceptions. The first is that a few H2A spots (dotted circles in Fig. 4*E*) present in the mixture were not present or were present in much lesser amount in spot A. This result is not unexpected because a [^{14}C]leucine-labeled peptide that participates in a crosslink might be expected to have an altered mobility.

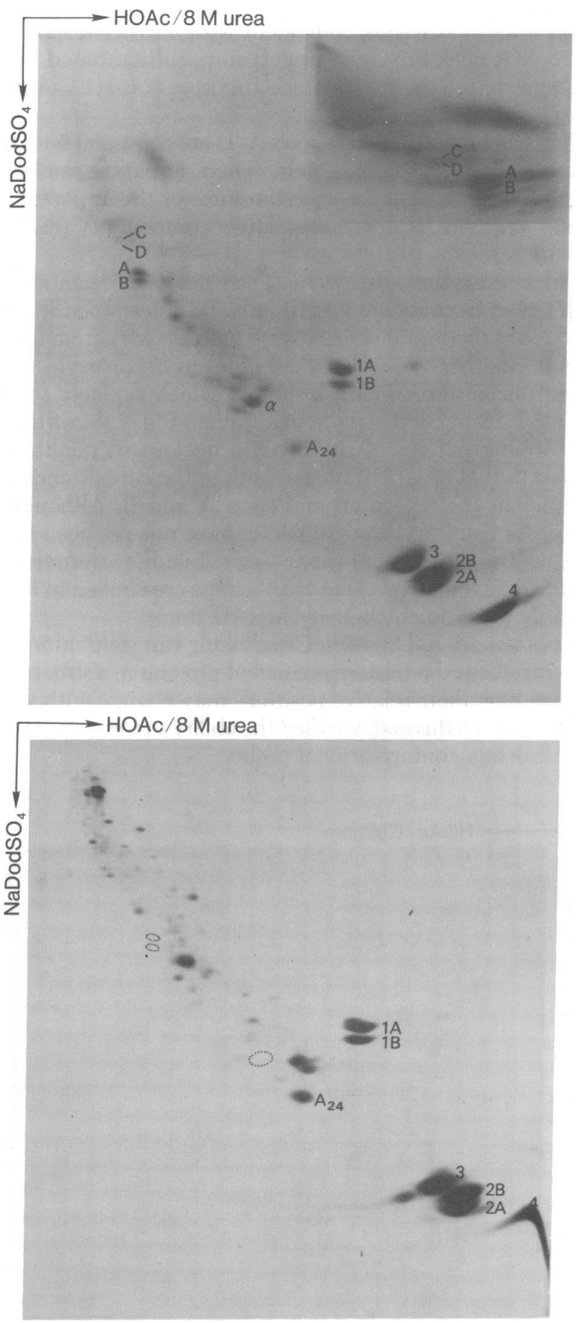

FIG. 1. Two-dimensional gels of carbodiimide-crosslinked L1210 nuclei (*Upper*) and control nuclei (*Lower*). Gels were 15% acrylamide/acetic acid/8 M urea in the first dimension and 15% acrylamide/NaDodSO$_4$ in the second dimension. (*Inset*) Gel was 10% acrylamide/acetic acid/8 M urea in the first dimension; it was overloaded with respect to A and B, so that C and D show up well. Dotted circles on the control (*Lower*) show where α, A, and B would migrate.

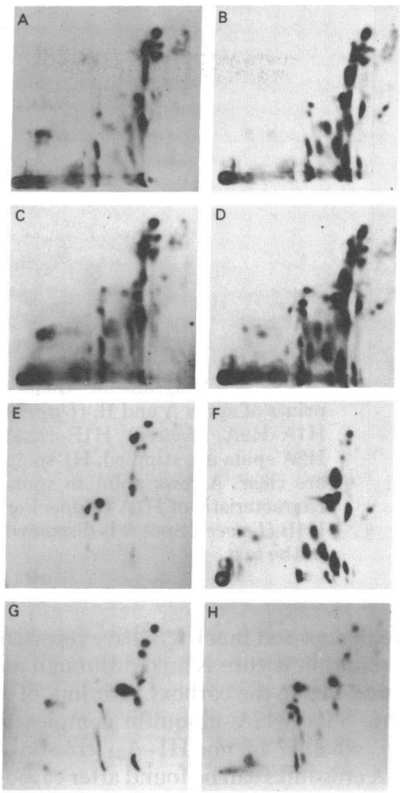

FIG. 2. Fluorograms of fingerprints of [^{14}C]leucine-labeled spots A and B and standard histones. (*A*) H1A; (*B*) spot A; (*C*) H1B; (*D*) spot B; (*E*) H2B; (*F*) H2A; (*G*) H4; (*H*) H3.

The second difference is that the fingerprints of the putative H1–H2A spots, particularly the fingerprint of H1B–H2A, contained a peptide (⊗ in Fig. 3) that could be the major H2B peptide (four of the six H2B leucines are in one tryptic peptide). By comparing the radioactivity in peptide ⊗ to various H2A peptides in the fingerprints of spots A and B and fingerprints of equimolar mixtures of H2A and H2B, one can determine the extent of possible H2B contamination. For spot A the H2B/H2A molar ratio was found to be 0.04; for spot B was 0.18.

Because of possible contamination with other crosslinks containing H2B, the sensitivity of spots A and B to CNBr was determined. H1 and the major H2A species H2A.1 (21) lack methionine and should therefore be resistant to CNBr. (A second H2A species, H2A.2, amounting to approximately 20% of the H2A in mouse L1210 cells contains a methionine at position 51.) Because the other histones, H4, H2B, and H3, all contain one or more methionines, CNBr should cleave any material containing H4, H2B, or H3 but leave most of H1–H2A uncleaved. Spots A and B were mainly resistant to CNBr (Fig. 5). H1–H2A.2 should be cleaved by CNBr at H2A position 51, which may account for spots A′ and B′. One would then expect 80% of spots A and B, if pure, to be resistant to CNBr. Upon CNBr treatment of purified [^{14}C]leucine-labeled A and B, 79% of A and 70% of B was resistant, substantiating the assignment from fingerprinting and ruling out the participation of H4, H2B, or H3 in the major components of spots A and B.

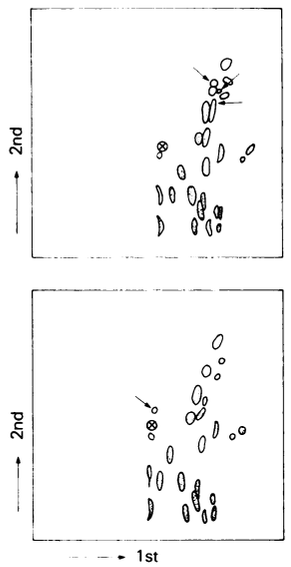

FIG. 3. Diagram of fingerprints of spots A and B. (*Upper*) H1A–H2A. (*Lower*) H1B–H2A. H2A spots are stippled; H1 spots are clear. Arrows point to spots characteristic of H1A (*Upper*) or H1B (*Lower*). Spot ⊗ is discussed in the text.

H1–A_{24} Crosslinks. Goldknopf and Busch (23) have reported that up to 20% of H2A found in nature is linked through an isopeptide linkage at lysine-119 to the carboxy terminus of a protein called "ubiquitin." The H2A–ubiquitin complex is called "A_{24}." Therefore, whether or not H1–A_{24} crosslinks analogous to the H1–H2A crosslinks can be found after carbodiimide treatment is of interest. Spots C and D (Fig. 1 *upper*) are close to the position predicted for H1–A_{24} crosslinks. Upon fingerprinting, both spots were found to contain H1 and A_{24}.

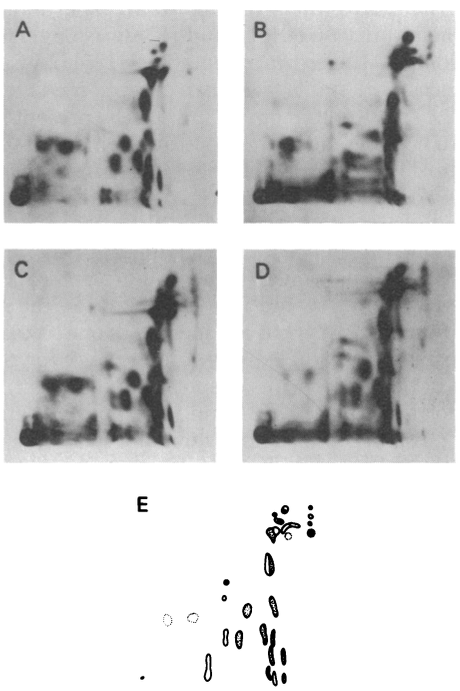

FIG. 4. Mixing experiment for H1A–H2A: [^{14}C]leucine fingerprints of H2A (*A*), H1A (*B*), equimolar mixture of H1A and H2A (assuming 10 leucines for H1A and 16 for H2A) (*C*), and spot A (*D*). (*E*) Diagram of spot A fingerprint. H1A spots are clear; H2A spots are stippled. Several spots present in *C* but absent or present in decreased amount in *D* are shown by dotted circles. There are some differences between the patterns here and in Fig. 3, particularly in the upper right quadrant of each fingerprint. This is because the migration of those spots with a high R_F in the second solvent is quite sensitive to small changes in solvent polarity.

Fig. 6 shows the fingerprints of spot C, various standards, and an equimolar mixture of H1A and A_{24}. A_{24} (Fig. 6*H*) has two spots that are absent from H2A (Fig. 6*G*) and two spots where shape is altered from that in H2A. These four spots are shown with hatching in Fig. 6*F*. All four of the A_{24} spots are present in the fingerprint of spot C (Fig. 6 *A* and *B*). In addition, several other A_{24} and H1A spots were found.

The major form of A_{24} (H2A.1–ubiquitin) does contain one methionine in the ubiquitin moiety, but it is in the NH_2-terminal position and its reaction with CNBr does not alter the mobility of A_{24} on NaDodSO$_4$ gels. Therefore, H1–(H2A.1–ubiquitin) complexes, which should be about 80% of the H1–A_{24}, would be expected to have the same mobility on NaDodSO$_4$ gels after CNBr treatment: 70% of CNBr-treated spot C was found to migrate coincident with untreated spot C on NaDodSO$_4$ gels. From the fingerprints, substantiated by the resistance to CNBr, we conclude that spot C is H1A–A_{24} and spot D is H1B–A_{24}.

The structures assigned to spots A–D are consistent with their mobilities on NaDodSO$_4$ gels. Their apparent molecular weights agree within 2% with the sums of the apparent molecular weights of their respective components (data not shown).

Chicken Erythrocyte Nuclei. The work described above was with nuclei from mouse L1210 cells. To determine the extent to which carbodiimide crosslinking patterns vary from one cell type to another, we crosslinked chicken erythrocyte nuclei. These nuclei also contain histone 5 which replaces some of histone 1 during erythrocyte maturation. Fig. 7 shows that the crosslinking pattern of erythrocyte nuclei was qualitatively similar to that of L1210 nuclei, both in the core dimer region (α) and in the region around spots A and B. Although the crosslinks from erythrocyte nuclei have not yet been fingerprinted, the similarity of the two crosslinking patterns leads us to conclude tentatively that H2A can be crosslinked to H1 and possibly to H5 in chicken erythrocyte nuclei.

Conformational Studies. Crosslinking can yield information not only about the relative position of proteins in a structure but also on how their relative positions may change with various conditions. To this end, we used the ability to form an H1–H2A crosslink as a conformational probe.

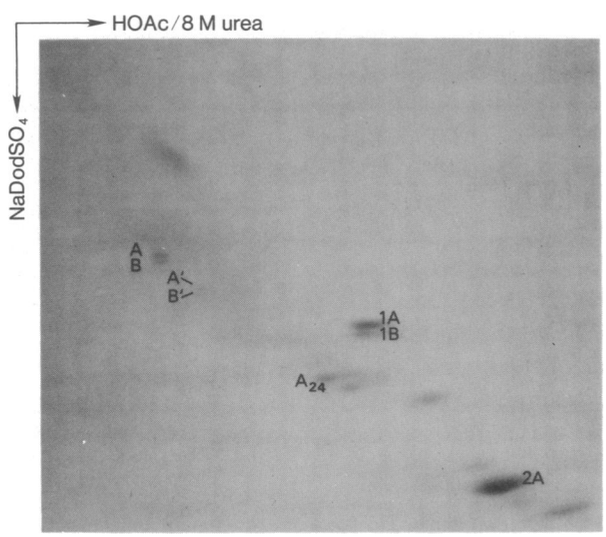

FIG. 5. CNBr resistance of spots A and B. After ethanol precipitation, the dried pellet (500 μg of histone) was dissolved in 0.25 ml of 70% formic acid, mixed with freshly sublimed CNBr (22) (25 μl of 40 mg/ml solution in 70% formic acid), and incubated for 6 hr at 37°C. The reaction mixture was lyophilized and then dissolved in sample buffer for the acetic acid/8 M urea gels.

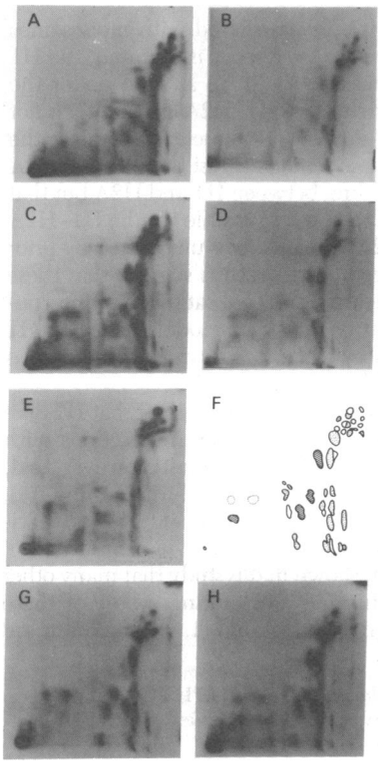

FIG. 6. Fluorograms of fingerprints of [^{14}C]-leucine-labeled spot C, standard histones, and mixture of H1A and A_{24}. (*A* and *B*) Spot C; 10-day and 3-day exposures. (*C* and *D*) Equimolar mixture of H1A and A_{24} (assumed, 10 leucines for H1A and 25 leucines for A_{24}); 10-day and 3-day exposures. (*E*) H1A. (*F*) Diagram of spot C fluorogram; H1A spots are clear, spots common to H2A and A_{24} are stippled, and characteristic A_{24} spots are hatched. (*G*) H2A. (*H*) A_{24}.

Renz *et al.* (24) and Renz and Day (25) have studied the effects of monovalent and divalent ions on the binding of H1 to DNA and chromatin. Below 20 mM NaCl or 0.8 mM $MgCl_2$, H1 binds randomly to DNA or chromatin fibers irrespective of size. Above these concentrations, H1 binds preferentially to longer DNA and chromatin fibers.

The ability to form the H1–H2A crosslink was compared under these two conditions. Intact nuclei (dialyzed overnight in 2.5 mM potassium phosphate, pH 6.7/1 mM $CaCl_2$) or lysed nuclei (dialyzed overnight in 2.5 mM potassium phosphate, pH 6.7/1 mM Na_2EDTA) were crosslinked and analyzed on two-dimensional gels (data not shown). Spots A and B were formed in the same proportions in both cases; the gels were essentially identical to the one in Fig. 1 *upper* except that there were some differences in the spot pattern around α (H3–H4). These results indicate that, although lysis of the nuclei may lead to differences in the crosslinks present in the core, the formation of the H1–H2A crosslink is not affected. The formation of H1–H2A is also unaffected when nuclei are isolated and crosslinked at physiological ionic strength. However, when nuclei are crosslinked in 0.7 M NaCl, which dissociates H1 but not the core histones from chromatin, the H1–H2A crosslink is not formed. Because of the smaller amounts of H1–A_{24} crosslinks formed, it is not yet known whether their formation is affected by these factors.

Varshavsky *et al.* (2) reported that, after micrococcal nuclease digestion, some of the monosomes contained H1 and others did not. Renz *et al.* (24) suggested that nuclease digestion may lead to redistribution of H1 in chromatin. One question of interest then is whether H1 present in monosomes has the same relationship to the nucleosome as does H1 in whole chromatin. We tested this point with respect to the H1–H2A crosslink. Monosomes were isolated from a sucrose gradient (data not presented) and their DNA was analyzed on gels. Only monomer DNA was found in the monosome peak, indicating that the monosomes are not contaminated by nucleosome dimers or oligomers (data not presented). When isolated monosomes were crosslinked, H1A–H2A and H1B–H2A were formed in the same proportion relative to H1 as when whole chromatin was crosslinked (Fig. 8). This result indicates that micrococcal nuclease digestion of chromatin does not alter the relationship between H1 and H2A, as long as the H1 remains on the nucleosome.

FIG. 7. Two-dimensional gel of carbodiimide crosslinked chicken erythrocyte nuclei. Putative H1 and H5-H2A crosslinks are indicated by brackets.

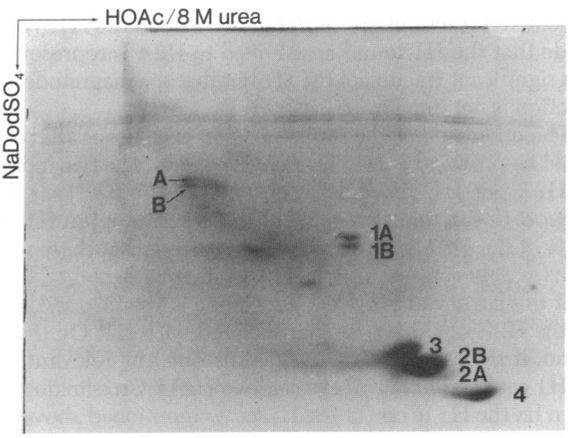

FIG. 8. H1–H2A in monosomes. L1210 nuclei (1 mg of DNA per ml) in 2.5 mM potassium phosphate, pH 6.7/1 mM $CaCl_2$ were digested for 15 min with micrococcal nuclease (Worthington; 40 μg/ml). Na_2EDTA was added to 5 mM to stop the reaction. The sample was dialyzed against 2.5 mM potassium phosphate, pH 6.8/1 mM Na_2EDTA and then centrifuged in an SW 27 rotor through a 5–15% sucrose gradient containing the same medium. Centrifugation was for 18 hr at 25,000 rpm and 4°C. Tubes were fractionated and the DNA profile was determined by measuring A_{260}. The monosome peak was collected in a dialysis bag, concentrated against dry Sephadex G-150, and dialyzed against 2.5 mM potassium phosphate, pH 6.7/1 mM Na_2EDTA. (The spots around the H3–H4 crosslink are not clearly visible because less material was loaded onto the gel.) When di- and oligonucleosomes were crosslinked, H1–H2A dimers were also formed (data not presented).

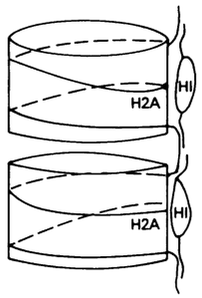

FIG. 9. Possible arrangement of H1s in chromatin, showing intranucleosomal binding to spacer DNA.

DISCUSSION

This study shows that in chromatin a fraction of the H1 molecules is close enough to a fraction of the H2A molecules so that carbodiimide, a zero-length condensing agent, can crosslink them. Whether or not all the H1 can be crosslinked to H2A is a question that dimer analysis cannot answer, because dimers are kinetic intermediates to trimers and higher oligomers. Further crosslinking leads to a decrease in the amount of H1–H2A and an increase in the amount of higher oligomers. Only a small fraction of the H1 may be present as H1–H2A dimers at any one time, even though all the H1 may be associated with H2A. In addition, if the H2A or the H1 is already part of a dimer or higher oligomer when the H1–H2A crosslink is formed, the H1–H2A association will not show up as a dimer but will immediately be among the higher oligomers.

We have studied the yields of various crosslinked species from [^{14}C]leucine-labeled L1210 cells. In a typical experiment performed for maximal resolution and not maximal yield, 5% of the H1 is in H1–H2A, 1% of the H1 is in H1–A_{24}, and another 3% of the H1 is found in trimers built on H1–H2A (data not presented). Because these crosslinked species are kinetic intermediates, the 9% of the H1 found associated with H2A and A_{24} is representative of a considerably larger fraction of the H1, but the size of this fraction is not known; it is possible that these crosslinked fractions are representative of all the H1. We conclude that the H1 found crosslinked to H2A is representative of a significant fraction of the H1 but that the magnitude of this fraction is not known.

The other side of the same question is whether all the H2A could be proximal to H1. There are thought to be two molecules of H2A per H1 molecule in chromatin (26, 27). Our results support this in that, when 5% of the H1 is found in H1–H2A, 2.2% of the H2A is found there, suggesting that there was approximately twice as much H2A to start with.

If this particular H1–H2A association is a static one, then half of the H2A could not be so associated with H1. On the other hand, if the association is a dynamic one, the relevant region of H1 could equilibrate between two H2As. Crosslinking would then fix the H1 to one of the H2As. As mentioned above, 1% of the H1 is found in H1–A_{24} compared to 5% in H1–H2A. In these cells the ratio of A_{24} to H2A is also close to 1:5. The similarity of these two ratios indicates that the ubiquitin moiety of A_{24} does not affect the crosslinking of A_{24} to H1. It further suggests that H1 may associate with the H2A portion of A_{24} in the same way it associates with unmodified H2A.

One question of interest is whether there is binding between H1 and H2A/A_{24} or whether H1 and H2A/A_{24} are maintained in a proximal configuration by binding at other more distant sites. There is no evidence for a strong association between H1 and H2A or any other core histone, whereas close associations between core histones are often reflected by strong associations in solution (28). Also, H1 is only associated with monosomes containing spacer DNA and, as the spacer is digested, the H1 is liberated from the monosome (2–6). So at least under the conditions of these experiments, the H1–H2A association is not strong enough to retain the H1 on the nucleosome after the spacer DNA is digested. These results suggest that the H1–H2A association is not due to binding between H1 and H2A but that H1 binding to the spacer DNA is responsible for the H1–H2A proximity. Because H1–H2A crosslinking is unaffected by prior micrococcal nuclease digestion, the results suggest that these sites of H1 binding are intranucleosomal rather than internucleosomal. This interpretation supports models in which H1 binds to two regions of spacer DNA in the same nucleosome as shown in Fig. 9.

The above interpretation is not meant to suggest that H1 does not have other interactions—for example, interactions with distant or adjacent nucleosomes. It does suggest, however, that, in the fraction of H1 that can be crosslinked to H2A and A_{24}, these other interactions are less persistent under the experimental conditions used in these studies.

It is clear from the results shown in this study that many other species arise from carbodiimide crosslinking. Many of these other spots, some of which also contain H1, will be discussed elsewhere.

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