Conclusion

On the basis of kinetic measurements for the folding/unfolding of RNase A with/without an extrinsic cross-link, we conclude that RNase A unfolds *sequentially* and that the 41 N-terminal residues must be unfolded in the transition state of the unfolding pathway. Also, the 41 N-terminal residues make no conformational contribution in the folding pathway.

Added in Proof

P. C. Weber, D. O. Ohlendorf, B. Finzel, S. Sheriff, and F. R. Salemme (private communication) have determined the crystal structure of this cross-linked protein to 2-Å resolution, refined by restrained least-squares methods to an R factor of 0.19. The structure is essentially that of native RNase, with a root mean square deviation on all backbone atoms of 0.52 Å and over all side-chain atoms of 1.34 Å. The *small* differences observed between these two structures are comparable to coordinate differences typically observed for identical proteins crystallized in different crystal forms.

Acknowledgments

We are indebted to J. J. Osterhout for technical assistance in the stopped-flow measurements and to P. W. Mui for help with the analysis of the stopped-flow kinetic data. We also thank P. W. Mui, C. A. McWherter, and M. E. Denton for helpful discussions and comments on the manuscript.

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Contact Site of Histones 2A and 2B in Chromatin and in Solution†

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ABSTRACT: Irradiation of isolated nuclei or of a complex of histones 2A (H2A) and 2B (H2B) with ultraviolet light produces a covalent cross-link between H2A and H2B. Sequence analysis of the peptides isolated from the H2A–H2B dimer formed in solution and in nuclei demonstrated that both dimers are produced through the covalent linkage of Tyr-40 of H2B and Pro-26 of H2A. Tyrosyl residues proximal to Tyr-40 did not produce a cross-link with H2A, thereby indicating that strict conformational parameters are required for production of the H2A–H2B cross-link. We conclude that the precise juxtaposition of Tyr-40 of H2B and Pro-26 of H2A in this region of the H2A/H2B contact site is not altered upon interaction of these histones with H3 and H4 (tetramer), DNA, or other chromosomal components during nucleosome assembly.

Chromatin is composed of DNA associated with a heterogeneous protein component, of which the histones are the most prevalent class. The substructure of chromatin includes the nucleosome, a histone octamer wrapped in superhelical DNA associated with histone 1. A limited nuclease digest of chromatin produces a core particle of 146 base pairs of DNA surrounding core histones 2A, 2B, 3, and 4 [for reviews, see Kornberg (1977) and McGhee & Felsenfeld (1980)]. Histones 3 and 4 can form a tetramer that interacts with short strands

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of DNA to produce a structure with hydrodynamic features similar to a core particle. Histones 2A and 2B have been shown to associate into a heterodimer and may then combine with H3–H4 tetramer and DNA to form a true core particle (Ruiz-Carrillo et al., 1979; Daban & Cantor, 1982; Laskey & Earnshaw, 1980).

In an attempt to understand better the physiology of the core particle, much work has been focused on the organization of the histone octamer and its relationship to the surrounding DNA. Electron microscopy (Klug et al., 1980), DNA–protein cross-linking (Mirzabekov et al., 1978), and protein–protein cross-linking (Thomas & Kornberg, 1975a,b; Bonner & Pollard, 1975; Hardison et al., 1975; Van Lente et al., 1975; Carter et al., 1980) have all been utilized to generate a map of how the histones and DNA are positioned with respect to one another. Methods have more recently been employed that do not introduce any linker arm when producing a covalent cross-link. These zero-length linker agents have revealed a H3–H3 contact by coupling of cysteine residues (Camerini-Otero & Felsenfeld, 1977) and H2B contact sites with H4 and H2A (Martinson & McCarthy, 1975, 1976), allowing for the juxtaposition of specific residues.

UV[1] irradiation can produce protein–protein cross-linking through activation of photoreactive amino acids (Schaich, 1980). A UV-induced cross-link between H2A and H2B has been shown to form in isolated nuclei (Martinson & McCarthy, 1976; Martinson et al., 1976, 1979) and involves proline-26 of H2A and tyrosine-37, -40, or -42 of H2B (DeLange et al., 1979).

In this paper we present evidence that only tyrosine-40 of H2B participates in cross-linking to proline-26 of H2A. This indicates that strict conformational requirements are necessary for a cross-link to be produced. These same residues are involved in the H2A–H2B cross-link when irradiation is performed on intact nuclei or purified H2A and H2B, associated in solution. Our results indicate that purified H2A and H2B can associate in such a manner as to reproduce precisely a region of the H2A/H2B binding site found in chromatin.

Experimental Procedures

Materials. Trypsin treated with TPCK, chymotrypsin, and carboxypeptidases A and B treated with DFP were obtained from Worthington. HPLC-grade acetonitrile and water were purchased from Mallinckrodt, TFA was from Pierce, and hydrochloric acid (Ultrex) for hydrolysis was from Baker. The μBondapak columns were obtained from Waters Associates, the Vydac column was from Western Analytical, and the Ultrasphere column was from Beckman. The HPLC system was a Beckman Model 110A, with gradient capability.

Nuclei Isolation. Frozen calf thymus (350 g) was homogenized in 2 L of cold 75 mM NaCl/24 mM EDTA, pH 8.0. This suspension was filtered through cheesecloth, and the resulting filtrate was centrifuged at 1500*g* for 30 min. The pelleted nuclei were resuspended in 1 L of buffer, and the centrifugation procedure was repeated twice.

Dimer Formation in Nuclei. The nuclei preparation (as above) was suspended in 1.2 L of NaCl/EDTA buffer and irradiated for 32 h while under a constant flow of nitrogen at 4 °C with mixing. Photolysis was achieved with a 450-W Honvia medium-pressure mercury lamp housed in a Corex filter (to remove lower wavelength transmissions; Martinson et al., 1976) surrounded by a quartz water jacket (outer diameter 6 cm). All irradiations were done at 4 °C.

Histone Isolation. Nuclei isolated as described above were placed in 1.5 L of cold 10 mM Tris-HCl, pH 8.0, and centrifuged at 12000*g*. This was repeated twice, after which the chromatin was suspended in 1 L of 10 mM Tris buffer and the DNA was sheared by homogenizing at full speed for 30 s in a Sorvall Omnimixer. A solution of 4 N H_2SO_4 was added dropwise with stirring to give a final concentration of 0.25 N H_2SO_4. This was stirred at 4 °C for 30 min, and the precipitate was removed by centrifugation prior to the addition of 4 volumes of cold ethanol (Bonner et al., 1968). The mixture was stored at −10 °C for 24 h, after which the precipitate was collected by centrifugation and lyophilized. The H2A/H2B complex was isolated from whole histone by gel filtration (Callaway et al., 1985).

Dimer Isolation. Histones isolated from UV-irradiated nuclei were suspended in 6 M guanidine hydrochloride to minimize aggregation, and 500-mg aliquots (4 mL) were chromatographed on a Bio-Gel P-60 column (3.8 × 150 cm) in 100 mM NaCl/10 mM HCl at a flow rate of 60 mL/h (Bohm et al., 1973). Fractions (collected at 10-min intervals) containing the H2A–H2B dimer as the major component were combined from 15 separate runs, concentrated, and rechromatographed (two separate runs) under the same conditions to produce a highly enriched H2A–H2B dimer.

Dimer Formation in Solution. Purified H2A/H2B complex from Sephadex G-100 chromatography (often stored in freezer), or redissolved from a lyophilized powder into the NaCl/Tris buffer, was irradiated in a quartz cylinder (diameter 3 cm, maximum volume 150 mL) 2 cm from the water jacket surrounding the light source. The irradiation lasted 4–5 min at 4 °C with constant purging of nitrogen and stirring.

Isolation of the Cross-Linked Peptides. The H2A–H2B dimer (150 mg) was modified at all lysine residues with maleic anhydride (Glazer et al., 1976), followed by treatment with trypsin (1% w/w) at a dimer concentration of 5–10 mg/mL for 2 h at 37 °C in 0.1 M NH_4HCO_3 (pH 8.0). The maleyl groups were removed by lowering the pH to 3 (acetic acid) and incubating at 37 °C for 72 h prior to lyophilization. Resulting peptides were dissolved in 2 mL of 99% formic acid and diluted with 2 mL of 30% acetic acid for chromatography on a Sephadex G-50 column (3.8 × 150 cm) in 30% acetic acid at a flow rate of 60 mL/h. The eluant was monitored at 280 nm, and pooled regions were concentrated by rotary evaporation.

The tryptic maleylated fragment of the cross-linked region was further purified by reverse-phase HPLC on a preparative (10-μm particle size, 0.8 × 30 cm) μBondapak C_{18} column. The starting buffer contained 2 g of TFA/4 L of H_2O, and the gradient was established with this buffer and solvent B, composed of 1.5 g of TFA/4 L acetonitrile; these solvents are used in all HPLC separations of peptides discussed in this paper. A gradient of 1%/min was initiated at a flow rate of 2 mL/min. Fractions were collected at 1-min intervals, and acetonitrile was removed (Savant Speed Vac concentrator) prior to lyophilization. Aliquots were removed for amino acid analysis.

Tryptic digestion of the demaleylated cross-linked peptide (200 nmol) was done at 37 °C for 2 h with 1% trypsin (w/w) in 1 mL of 0.1 M NH_4HCO_3, pH 8.0. Resulting peptides were rechromatographed by HPLC under the identical conditions described above, except the eluant was monitored at 220 nm. The tryptic fragment of the cross-linked peptide now elutes

[1] Abbreviations: UV, ultraviolet light; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DFP, diisopropyl fluorophosphate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

as two broad peaks from 32 to 34 min. This peptide fraction (from the dimer formed in solution or in nuclei) was used for chymotryptic and carboxypeptidase analyses.

For sequence analysis we chose to trypsinize the H2A–H2B dimer (50 mg) without prior modification of lysine residues (see procedure above). Resulting tryptic peptides were separated by HPLC on the preparative μBondapak C_{18} column. Aliquots of the tryptic cross-linked peptide were then subjected to HPLC on a Vydac C_{18} column (5-μm particle size, 0.4 × 25 cm) with the buffers described above at a flow rate of 0.5 mL/min. The gradient went to 30% acetonitrile in the first 5 min and then proceeded at 0.5%/min with the cross-linked tryptic fragment eluting at 30–32 min. This procedure resulted in separation of the incomplete cleavage forms encountered during purification of this peptide from the maleylated dimer.

Proteolytic Studies. Chymotryptic digestions of the cross-linked tryptic peptides (10–20 nmol) were done at 37 °C for 15 h with 4% (w/w) enzyme in 200 μL of 0.1 M NH_4HCO_3 (pH 8.0). The reaction was stopped by addition of glacial acetic acid, followed by lyophilization. The peptides were dissolved in HPLC starting buffer and centrifuged through a 0.2-μm cellulose filter prior to chromatography on the preparative μBondapak C_{18} column in the buffers described previously. A 1%/min gradient was initiated after 6 min, and the resulting eluant was monitored at 220 nm. Peaks were collected by hand and dried under vacuum prior to amino acid analysis.

Carboxypeptidase A and B digestion was performed on the cross-linked tryptic peptide (10–25 nmol) with 4% (w/w) of each enzyme in 200 μL of 200 mM NH_4HCO_3, pH 8.0, at 37 °C for 4 h. The reactions were stopped by addition of acetic acid and lyophilized to dryness. Amino acid analyses were done directly on the digested sample and a blank to determine amino acids cleaved. Release of the penultimate tyrosine was expressed as the percentage detected when compared with the terminal lysine; this was also done for the penultimate glycine, which was compared to the terminal arginine.

Sequence Analysis. The cross-linked tryptic peptide (60 nmol) was sequenced on a Beckman 890C sequencer in the presence of cytochrome *c* (200 nmol) and polybrene (3.5 mg). The PTH-amino acids were identified by reverse-phase HPLC on an Ultrasphere column (0.45 × 25 cm) in 0.1 M ammonium acetate (pH 4.1). A 0%–45% acetonitrile gradient was utilized at a flow rate of 1.5 mL/min, and the eluant was monitored at 254 nm. The second method of quantitation was to hydrolyze the PTH-amino acids at 150 °C in 1.5 mL of 5.7 N HCl (containing 3 drops of 5% phenol) overnight in vacuo and analyze the resulting amino acids. For steps 2 and 4, stannous chloride (0.1%) was added to the 5.7 N HCl (phenol excluded), and the hydrolysis was done for only 4 h at 150 °C.

Amino Acid Analysis. Peptides (1–10 nm) were hydrolyzed in 1.5 mL of 5.7 N HCl containing 3 drops of 5% phenol at 110 °C for 24 h in evacuated tubes (Glazer et al., 1976). The HCl was removed over solid NaOH in a heated desiccator at 60 °C under vacuum. The samples were then analyzed on a modified Beckman 121 M amino acid analyzer with single-column methodology, automated integrator, and greater sensitivity.

Gel Electrophoresis. The low ionic strength Tris/glycine system was utilized in the presence of SDS (Studier, 1973) with a 16% separating gel and an 8% stacking gel. The gels (12 × 18 cm; 0.75-mm thick) were run at constant current (15 mA) until the tracking dye was less than 1 cm from the bottom of the gel. Staining with Coomassie Blue and sample preparation were as described by Callaway & Bechtel (1981).

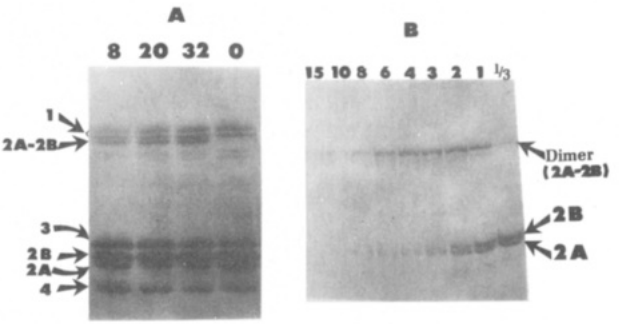

FIGURE 1: SDS–polyacrylamide gel electrophoresis of aliquots removed at specified intervals during UV irradiation of intact nuclei (panel A) and purified H2A/H2B complex (panel B). Nuclei isolated from calf thymus were irradiated for 0–32 h as designated above each lane (panel A), after which the histones were isolated prior to gel analysis (see Experimental Procedures). The H2A/H2B complex from the Sephadex G-100 column was irradiated in a quartz cylinder (see Experimental Procedures) for 0–15 min, as designated above each lane. Protein bands were visualized by Coomassie Blue staining.

RESULTS

Formation of the H2A–H2B Dimer. Exposure of nuclei to UV causes DNA–protein and protein–protein cross-linking. Figure 1A is a SDS–polyacrylamide gel of the histone fraction isolated from nuclei exposed to UV (wavelengths higher than 240 nm) for varying lengths of time. The only change is the appearance of a band moving slightly faster than H1. This band increased in intensity with longer UV exposure. Amino acid analysis of this band demonstrated that it was a product of H2A–H2B cross-linking. The opaque quality of the nuclei suspension caused extensive light scattering, which greatly reduced the rate of cross-link formation. More efficient conversion of H2A and H2B to H2A-H2B dimer was achieved when more dilute suspensions of nuclei were used (Martinson & McCarthy, 1976; Martinson et al., 1976). We elected to use large amounts of nuclei and produce a lower percentage of dimer to avoid other histone cross-linkages (Martinson et al., 1979), which were not detected under the conditions used to prepare our dimer.

When the H2A/H2B complex (as it elutes off the Sephadex G-100 column) is exposed to UV light (Figure 1B), a covalent H2A–H2B dimer is rapidly produced that migrates in SDS–polyacrylamide gel electrophoresis in an identical manner with the dimer formed in isolated nuclei. Quantitative conversion of H2A and H2B monomers to dimer occurs within 6 min and demonstrates that virtually all of the H2A and H2B associate in such a manner as to form the covalent cross-link upon UV irradiation. The disappearance of both monomer and dimer at later time points is attributed to photolytic degradation and polymerization. The Corex filter removes all UV light below 240 nm (Martinson et al., 1976), but light of 253.7 nm has been reported to cleave the peptide linkage (Schaich, 1980). To optimize dimer production and reduce the extent of UV damage, irradiation was carried out for 4–5 min. The more rapid production of H2A–H2B dimer in solution, as compared to nuclei, is probably due to the much more efficient transmission of UV light through the dilute protein solution as compared to the turbid nuclei suspension. H2A and H2B monomers were not removed prior to isolation of the cross-linked peptide from the dimer formed from the H2A/H2B complex.

Isolation of the H2A–H2B Dimer. Gel filtration was the method of choice for purification of the cross-linked H2A–H2B dimer formed in nuclei following isolation of whole histone and subsequent removal of H1 (by perchloric acid extraction). After elution of large histone aggregates (Figure 2, fractions

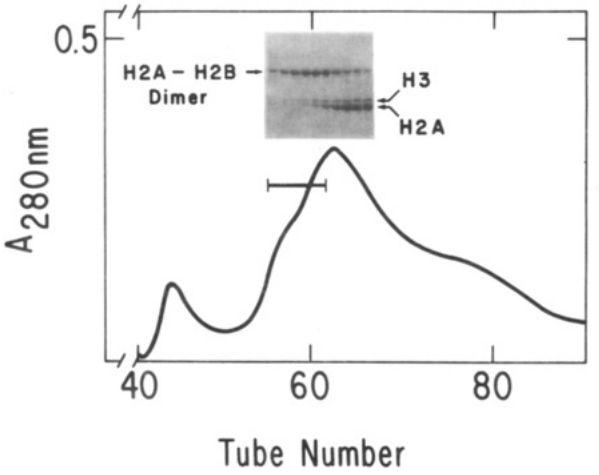

FIGURE 2: Elution profile of the histone fraction from UV-irradiated nuclei after perchloric acid extraction. Aliquots (about 400 mg) dissolved in 6 M guanidine hydrochloride were chromatographed on a Bio-Gel P-60 column (3.8 × 150 cm) equilibrated and eluted with 100 mM NaCl/10 mM HCl at 60 mL/h. The eluant was monitored at 280 nm and collected at 10-min intervals. The solid bar denotes the region collected (tubes 56–60) for rechromatography. Under these conditions, H2B elutes in fractions 95–110, and H4 elutes in fractions 135–150. The inset shows a SDS–polyacrylamide gel of chromatography fractions 56–65, stained with Coomassie Blue following SDS–polyacrylamide gel electrophoresis.

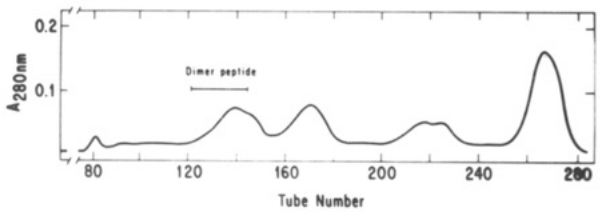

FIGURE 3: Elution profile of a tryptic digest of maleylated H2A–H2B dimer on a Sephadex G-50 column (3.8 × 150 cm) equilibrated and eluted in 30% acetic acid at 60 mL/h. The eluant was monitored at 280 nm, and fractions were collected at 10-min intervals. The solid bar denotes the location of the peptide containing the cross-linked region. The profile was the same for the digested dimer whether formed in nuclei or from the purified H2A/H2B complex.

40–50), the H2A–H2B dimer begins to elute but is quickly contaminated by self-associated H2A (Bohm et al., 1980). Fractions 56–60, which contained a predominance of dimer, were pooled from 15 separate runs, and the pooled dimer fraction was rechromatographed (two runs) under the same conditions. In this column profile, H3 elutes at the trailing edge of the H2A peak, H2B elutes in fractions 95–110, and H4 elutes in fractions 135–150.

Isolation of the Cross-Linked Peptide. If one first reversibly modifies all lysine residues with maleyic anhydride, tryptic digestion results in the cross-linked peptide being the largest fragment. Therefore, gel filtration was done in 30% acetic acid and monitored at 280 nm to detect the large tyrosine-containing cross-linked peptide. The leading shoulder of the first peak in Figure 3 was absent when a mixture of H2A and H2B monomers was maleylated and digested prior to Sephadex G-50 chromatography (data not shown). The shoulder region contained the H2A–H2B cross-linked peptide along with a H2A fragment. The Sephadex G-50 profile was the same for the digested dimer whether formed in nuclei or in solution.

The Sephadex G-50 fraction containing the cross-linked peptide was then chromatographed by reverse-phase HPLC on a preparative μBondapak C_{18} column, again monitoring at 280 nm to detect the cross-linked peptide. Edman sequencing and amino acid analysis confirmed that the broad peak eluting

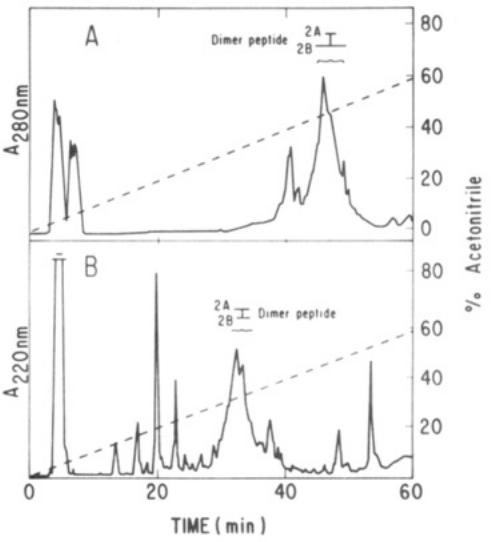

FIGURE 4: HPLC profile of the tryptic fragment of the maleylated H2A–H2B dimer (panel A) on a μBondapak C_{18} column (0.8 × 30 cm) eluted with a 2%/min gradient of acetonitrile (––) with 1.5 g of TFA/4 L, at a flow rate of 2 mL/min. The sample was partially purified on Sephadex G-50 (Figure 3) prior to chromatography. The eluant was monitored at 280 nm and collected in 1-min fractions. The brace denotes the region containing the cross-linked peptide as determined by amino acid analysis. This peptide (maleyl groups now removed) was digested with trypsin for 2 h, and the resulting peptides were purified by HPLC (panel B) as described above, except that the eluant was monitored at 220 nm. The brace denotes the tryptic fragment of the cross-linked region as determined by amino acid analysis. The HPLC profiles for the tryptic maleylated and tryptic fragments were the same for the digested dimer whether formed in nuclei or from the purified H2A/H2B complex.

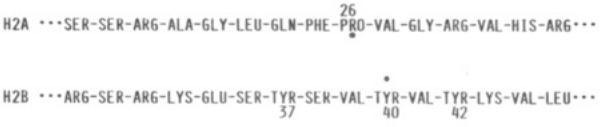

FIGURE 5: Amino acid sequence of H2A and H2B surrounding the region of the cross-link produced by ultraviolet irradiation. The astericks (*) denote the residues that are covalently cross-linked to produce the H2A–H2B dimer.

at 45 min in Figure 4A was the cross-linked peptide, containing residues 21–29 of H2A and 34–72 of H2B. Partial oxidation of the methionine residues of H2B was responsible for some of the peak broadening. Also, the sequence data (not given) show that the NH_2 terminus is heterogeneous with about 30% of the H2B portion starting at serine-32 and the remainder at the predicted lysine-34. Introduction of a negative charge of maleylation at lysine-34 may have inhibited cleavage at arginine-33 (see Figure 5). The HPLC profile, amino acid analysis, and NH_2-terminal heterogeneity were indistinguishable for the dimer formed in solution or in nuclei.

Figure 4B is a reverse-phase HPLC profile of the cross-linked peptide from Figure 4A following trypsin treatment. The maleyl groups protecting the lysines were removed (see Experimental Procedures) so that retrypsinization results in cleavage at lysine-43 of H2B. The trimmed cross-linked peptide elutes at about 33 min as two partially resolved peaks due to NH_2-terminal heterogeneity. The incomplete removal of lysine-34 is caused by the poor exopeptidase activity of trypsin and the presence of the negatively charged glutamic acid-35.

Amino acid analysis of the cross-linked peptide following retrypsinization and HPLC (Figure 4B) is shown for both the nuclei and solution dimer in Table I. These data show, as previously reported (DeLange et al., 1979), that tryptic

Table I: Amino Acid Analysis[a] of Cross-Linked Tryptic Peptides

	nuclei dimer	solution dimer		nuclei dimer	solution dimer
Lys	1.0 (1)	1.6 (1)	Gly	2.4 (2)	2.2 (2)
Arg	0.9 (1)	1.0 (1)	Ala	1.2 (1)	1.1 (1)
Asx	1.0 (0)	0.4 (0)	Val[b]	2.1 (3)	2.2 (3)
Thr	0.3 (0)	(0)	Ile	0.3 (0)	0.3 (0)
Ser[b]	1.9 (2)	1.6 (2)	Leu	1.2 (1)	2.0 (1)
Glx	2.1 (2)	2.1 (2)	Tyr[b]	1.1 (2)	1.5 (2)
Pro	0.3 (0)	0.4 (0)	Phe	0.7 (1)	0.9 (1)

[a] Hydrolysis in 5.7 N HCl at 110 °C for 24 h. Parentheses contain predicted number of residues for the cross-linked tryptic peptides. [b] Uncorrected values.

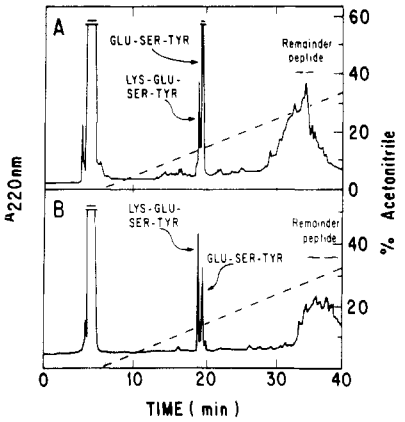

FIGURE 6: HPLC elution profile of chymotryptic digests of the tryptic cross-linked fragments from dimer formed in nuclei (panel A) or from the H2A/H2B complex (panel B) on a μBondapak C_{18} column (0.8 × 30 cm). Elution was achieved by a 1%/min gradient of acetonitrile (--) with 1.5 g of TFA/4 L, which was initiated after 6 min at a flow rate of 2 mL/min. The eluant was monitored at 220 nm; peaks were collected by hand and then identified by amino acid analysis.

fragments 21–29 of H2A and 34–43 of H2B are present with the exception of proline-26 of H2A and one of three tyrosines in this region of H2B. The analyses of the peptides from the cross-linked dimer formed in nuclei and the one formed from purified proteins in solution were quite similar. Contamination by H2B residues 100–116 was responsible for high leucine levels, as well as trace amounts of proline and threonine; aspartic acid and glycine are common contaminants of our HPLC-purified peptides. The low valine values may be due in part to steric hindrance of hydrolysis or possibly intermolecular cross-link formation, since the tyrosine values are low even after taking into account the loss of one residue due to cross-link formation. The solution dimer analysis contained more lysine due to the region of the HPLC peak (Figure 4B) used for analysis.

Proteolytic Analysis. To determine which of the three tyrosyl residues were not involved in the cross-link, retrypsinized cross-linked peptide was digested with chymotrypsin. The digests of the peptides isolated from the H2A–H2B dimer formed in nuclei (Figure 6A) or in solution (Figure 6B) were chromatographed by reverse-phase HPLC. The profiles were generated by monitoring at 220 nm so that all peptides released would be detected; the peptides in the major peaks were identified by amino acid analysis. Both chromatograms are quite similar, showing that only Glu-Ser-Tyr and Lys-Glu-Ser-Tyr are quantitatively released from the cross-linked peptide. This clearly demonstrates that for both dimers, tyrosine-37 is not involved to any extent in the cross-link. The cross-link seems to inhibit cleavages at either tyrosine-40 or -42, making it impossible with this method to determine the involvement of either one in the cross-link. The amount of the Lys-Glu-Ser-Tyr peptide varied as a function of the area

Table II: Carboxypeptidase[a] Digests of Cross-Linked Tryptic Peptides

% released[b]		
solution dimer[c]	nuclei dimer[d]	residue released[e]
60	45	glycine-28 (H2A)
66	47	tyrosine-42 (H2B)

[a] Carboxypeptidases A and B for 24 h at 37 °C. [b] Release of penultimate residues as a percentage of the C-terminal residue released for each chain. [c] Cross-linked tryptic peptide isolated from H2A–H2B dimer formed from purified proteins. [d] Cross-linked tryptic peptide isolated from H2A–H2B dimer formed in isolated nuclei. [e] Identified by amino acid analysis.

Table III: Edman Degradation[a] of the Cross-Linked Region[b] of H2A–H2B Dimer

residues released from H2B chain			residues released from H2A chain		
amino acid analysis (nmol)[c]	HPLC of PTH-AA (nmol)[d]	residue	residue	HPLC of PTH-AA (nmol)	amino acid analysis (nmol)
8.7	16	Glu	Ala	16	11.6
3.2[c,e]		Ser	Gly	20	9.3
9.3	16	Tyr	Leu	13	7.8
2.3[c,e]		Ser	Gln	20	7.2
2.3	8	Val	Phe	0	0
0	0	Tyr	Pro	0	0

[a] Beckman automated 890C sequencer. [b] A total of 60 nmol of the cross-linked tryptic fragment isolated from H2A–H2B dimer formed in solution. [c] Following acid hydrolysis (5.7 N HCl) at 150 °C for 15 h, except for serine, which was read as alanine upon acid hydrolysis (5.7 N HCl) in 0.1% stannous chloride for 4 h. [d] Peaks monitored at 254 nm. [e] PTH-serine is destroyed under these conditions.

of the HPLC peak (Figure 4B) used as starting material for the chymotryptic cleavage and was not significantly different for either dimer.

Carboxypeptidases A and B were used to determine if tyrosine-42 could be released from the cross-linked tryptic peptides (residues 35–43 of H2B and 21–29 of H2A) of either dimer. Table II shows that lysine-43 and tyrosine-42 of H2B are released upon carboxypeptidase treatment along with glycine and arginine from the H2A fragment. Variations in digestion time from 15 min to 24 h did not have any effect on the percentage or amount released from either residue. The results are again similar for both dimer peptides, although the peptide from the nuclei dimer was always slightly lower in the percentage released for both glycine and tyrosine.

Sequence Analysis. Automated Edman sequence analysis was performed on a tryptic fragment of dimer formed from purified H2A and H2B. To avoid NH_2-terminal heterogeneity, we trypsinized the dimer without prior modification. Purification of the cross-linked peptide was achieved by using reverse-phase HPLC on both preparative μBondapak C_{18} and analytical Vydac C_{18} columns. The sequence data presented in Table III indicate that each NH_2 terminus of the cross-linked peptide was homogeneous, with the yields of the two chains being nearly identical. The second step shows glycine by both methods, with serine being detected as alanine following hydrolysis in stannous chloride. The third step shows equal release of both tyrosine and leucine by both methods, confirming that within the limits of the experiment none of tyrosine-37 is involved in the cross-link. Step 4 shows a large release of glutamine, with serine being detected by hydrolysis in acid containing stannous chloride. Step 5 produced lower amounts of valine, and no phenylalanine was detected. Since phenylalanine should be detected at this step [the cross-linked peptide contains phenylalanine (Table I)], we conclude that

the cross-link inhibits the formation or release of the phenyl isothiocyanate derivative. In step 6, no proline, tyrosine, or other residues were detected above background levels, as was true for all subsequent steps.

Discussion

We are interested in the internal structural features that represent the basic architecture of the nucleosome. The ability of UV irradiation to produce cross-linked residues within the histone core without perturbing the structural integrity of the particle enables us to probe the interaction between H2A and H2B. This technique allows us to compare the H2A/H2B interaction in very different environments and also gives the precise juxtaposition of residues from neighboring polypeptide chains, since no "linker arm" is introduced upon cross-linking.

Previous studies have demonstrated that histones 2A and 2B become cross-linked following UV irradiation of whole cells, chromatin, or nucleohistone complexes (Martinson et al., 1976). The data presented in this paper clearly demonstrate that the H2A–H2B cross-link produced in solution is identical with that formed in isolated nuclei. This indicates that the binding of the H2A/H2B complex by the core histones, DNA, or other chromosomal proteins does not alter the interaction of H2A and H2B at this region of the binding site. These results demonstrate that the acid-extracted histones are capable of associating in a manner that resembles the undenatured state of the histone complex.

All the various methods of sequence analysis presented confirm that, within the error of the methods, the UV cross-link involves proline-26 of H2A and tyrosine-40 of H2B. The tryptic fragment (0.7 nmol) was also analyzed on the Applied Biosystem 470A gas-phase protein sequencer (Hewick et al., 1981), and the results were similar to those from the Beckman 890C system through the first four steps (Table III). Phenylalanine was detected at 20% of the initial yield at step 5. Step 6 showed proline at a yield of 10% compared to the initial yield, while tyrosine was not detected. The tryptic maleylated fragment of the cross-linked region from the dimer formed in solution and in nuclei was analyzed with the Beckman 890C sequencer. Although the NH_2-terminal heterogeneity resulted in complex data, quantitative release of tyrosine-42 was observed. These sequence analyses substantiate the data presented in this paper and indicate that the loss of yield following step 5 in the analysis of tryptic cross-linked fragment (Table III) is due to washing out of the remaining peptides.

Since there are two other proximal tyrosines (residues 37 and 42) in H2B, it is unclear why only residue 40 forms a cross-link with H2A, but some possible reasons include (a) the absence of appropriately positioned H2A residues in the vicinity of these tyrosines, (b) other residues in H2B itself being more favorably positioned to form intramolecular covalent linkages with these activated tyrosines [this could explain the low analysis for tyrosine in several studies (Table I)], (c) tyrosine–tyrosine interactions that result in tyrosine-40 being more photoreactive than the other tyrosines, etc. These three H2B tyrosines, as well as all the H2A tyrosines, are involved in the H2A/H2B interaction (Butler & Olins, 1982), demonstrating the precise conformational requirements that must be fulfilled for cross-link formation. Therefore, the production of identical cross-links in the H2A/H2B complex in solution or in nuclei reflects a conservation of the quaternary structure of that region.

Complete conversion of H2A and H2B into the heterodimer is observed when a mixture of the isolated histones is irradiated. This indicates that minor variants present in calf thymus H2A and H2B (Franklin & Zweidler, 1977) also associate in solution (Hatch et al., 1983) in a manner that will produce covalent dimers upon UV irradiation. Mammalian H2A has many sequence variants, but the sequence of tryptic peptide 21–29 is strictly conserved among all variants (West & Bonner, 1980) and species tested (Bohm et al., 1980). This region is one of the most highly conserved portions of H2A, and we have demonstrated that this region is directly involved in binding to H2B in chromatin. This suggests that strict conformational constraints are invoked at this H2A/H2B binding site that may be critical to the physiological function of the histones.

UV cross-linking of H2A and H2B under increasingly dilute conditions (data not shown) indicated that the binding constant for the H2A/H2B complex, which allows for cross-linking, is as strong as the association constant for H2A and H2B (Isenberg, 1979). The cross-link could not be produced in the absence of salt, which is probably due to the reported inability of H2A and H2B to fold properly under such conditions (D'Anna & Isenberg, 1972, 1974). Salt concentrations as high as 4 M NaCl did not reduce the rate of cross-link formation. These observations are consistent with the characteristics of the H2A/H2B complex, indicating that the UV-induced cross-link between H2A and H2B is only produced from the properly associated H2A/H2B complex.

The production of the proline–tyrosine cross-link does not prove that there is an interaction between these two residues in the H2A/H2B complex, only that such an association results in proline-26 of H2A and tyrosine-40 of H2B being placed in close proximity (within the length of a carbon–carbon bond, 0.15 nm). The specific interaction may more likely involve phenylalanine-25 of H2A in a ring-stacking phenomena with the tyrosyl-rich region of H2B. Proton magnetic resonance has shown the possible involvement of these aromatic residues in the H2A/H2B interaction (Moss et al., 1976) and that this interaction includes at least residues 25–95 of H2A and 37–114 of H2B. Therefore, the UV cross-link is produced at the NH_2-terminal region of the H2A/H2B binding site.

Registry No. Pro, 147-85-3; Tyr, 60-18-4.

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Accessibility of Tyrosyl Residues Altered by Formation of the Histone 2A/2B Complex†

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ABSTRACT: The availability of tyrosyl residues to surface iodination was analyzed for histone 2A (H2A), histone 2B (H2B), and the H2A/H2B complex. When H2A is free in solution (200 mM NaCl, pH 7.4) tyrosine-39 and one or both tyrosines-50 and -57 were readily iodinated. Tyrosines-83 and -121 of H2B were iodinated, both when the histone was free in solution and when it was associated with H2A, while tyrosines-37, -40, and -42 of H2B were not iodinated under either condition. When H2A and H2B were associated or covalently cross-linked, all tyrosyl residues of H2A were unavailable for iodination. We also found that the iodination of nondenatured H2A and H2B did not inhibit formation of the H2A/H2B complex. These results indicate that the amino-terminal regions of the hydrophobic portions of H2A and H2B undergo significant conformational changes upon formation of the H2A/H2B complex. These conformational shifts occur in the same region of the H2A/H2B complex that contains a contact site between H2A and H2B in the nucleosome, thus indicating an involvement of this region in chromatin assembly.

The fundamental subunit repeat of chromatin is the nucleosome [for reviews, see Felsenfeld (1978) and McGhee & Felsenfeld (1980)], composed of double-stranded, superhelical DNA wrapped around an octameric protein core. These units appear as beads on a string when viewed by electron microscopy (Olins & Olins, 1974; Oudet et al., 1975) and are capable of interacting with one another to produce highly condensed arrays (Finch & Klug, 1976; Renz et al., 1977).

The nucleosome is hypothesized to assemble by the formation of a tetrameric structure of histones 3 and 4, which interacts with the DNA (Roark et al., 1974; Daban & Cantor, 1982). The structure is completed by the addition of one H2A–H2B dimer above the plane of the DNA and one dimer below the plane (Camerini-Otero et al., 1976; Bina-Stein & Simpson, 1977; Worcel et al., 1978; Wilhelm et al., 1978; Ruiz-Carrillo et al., 1979). This H2A–H2B dimer is capable of exchanging with exogenous H2A and H2B without disrupting the nucleosome structure (Louters & Chalkley, 1984).

The hydrophobic regions of the core histones, which are involved in the formation of the H2A–H2B dimer and the

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