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Identification of Interacting Amino Acids at the Histone 2A–2B Binding Site†

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ABSTRACT: Histones 2A and 2B of calf thymus were cross-linked within intact nuclei by UV irradiation. This procedure induces the formation of covalent cross-links between non-covalently interacting residues in the histones of native chromatin. Tryptic peptide and partial sequence analysis of the cross-linked product has shown that the covalent linkage is between tyrosine-37, -40, or -42 (we have not yet determined which) of H2B and proline-26 of H2A. We conclude that these residues constitute part of the hydrophobic H2A–H2B binding domain within the nucleosomes of native chromatin.

The DNA of eukaryotes is packaged tightly within chromatin together with histones to form compact subunits called nucleosomes (Felsenfeld, 1978). Nucleosomes contain two each of the so-called core histones (2A, 2B, 3, and 4) around which are wrapped 144 base pairs of the core DNA. Some additional DNA (often called spacer) as well as histone 1 is usually associated with the core in a complete nucleosome.

We are engaged in studying the structure of the nucleosome histone core by means of analysis of the products of histone–histone cross-linking. Zero-length cross-links are induced at sites of noncovalent histone–histone association within intact nuclei. This yields cross-linked histone products which contain within their covalent structure information about the noncovalent arrangement of histones in the nucleosome core of native chromatin. Sequence analysis identifies the sites of cross-linking and thus permits precise identification of sites of noncovalent histone–histone associations within the native structure.

UV irradiation of whole cells induces the nearly quantitative conversion of monomer H2A and H2B into an H2A–H2B dimer (Martinson et al., 1976). The nature of the UV cross-linking reaction is such that the cross-link can be presumed to represent the covalent joining of the two histones within their mutual binding sites (Martinson et al., 1976; Martinson & McCarthy, 1976). This expectation arises because UV is known to induce cross-links by activation of tyrosine. Subsequent attack by this tyrosine on a precisely adjacent neighbor then yields a zero-length cross-link. Preliminary CNBr peptide mapping results (Martinson & McCarthy, 1976) have shown that the H2A–H2B link is

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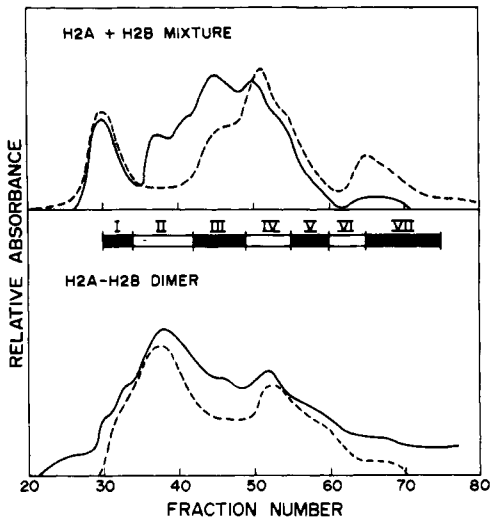

FIGURE 1: Elution profiles of tryptic peptides from a column of Sephadex G-25. Peptides were detected by monitoring at 235 (—) and 280 nm (---). Fractions for both the mixture and the dimer were pooled as indicated by the black and white bar.

somewhere within the NH_2-terminal halves of these histone molecules (see Discussion). Here we show that it is tyrosine-37, -40, or -42 of H2B and proline-26 of H2A that are cross-linked.

Materials and Methods

Preparation of Histones. Calf thymus nuclei were prepared in NaCl–EDTA (Martinson et al., 1979a) and irradiated (Martinson et al., 1979b) in a manner similar to that previously reported (Martinson et al., 1976). Histones were extracted and fractionated to yield H2A, H2B, and H2A–H2B dimer by column chromatography (Martinson et al., 1979b) as described. The dimer was estimated to be greater than 90% pure based on polyacrylamide gel electrophoresis. The H2B (about 90% pure) was slightly contaminated by what appeared to be a proteolytic fragment of H3. The H2A used was taken from an electrophoretically purified stock (Martinson et al., 1979a) and was essentially 100% pure. After purification, all of the histones were acetone-precipitated, dissolved in water, and clarified by centrifugation prior to use in peptide mapping and amino acid analysis.

Tryptic Hydrolysis of Histones. H2A–H2B dimer (20 mg) or a mixture of 10 mg each of monomer H2A and H2B was hydrolyzed with trypsin (0.2 mg, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Worthington) at 37 °C for 5 h in 3.5 mL of 0.1 M NH_4HCO_3, pH 8.0. Diisopropyl fluorophosphate (0.15 mL of a 2.5% solution, v/v, in 2-propanol) was added to the reaction mixture which was then incubated at 37 °C for an additional hour to inactivate the trypsin.

Purification of the Tryptic Peptides. The tryptic peptide mixture was fractionated directly by gel filtration on a column (1.9 × 160 cm) of Sephadex G-25, equilibrated, and eluted with 0.1 M NH_4HCO_3 at a flow rate of 30 mL/h and monitored simultaneously at 235 and 280 nm (Gilson dual-wavelength recording spectrophotometer MD-UV-RP). Fractions of 5 mL were collected, pooled as shown in Figure 1, lyophilized to dryness, redissolved in 10 mL of 30% acetic acid, lyophilized again to dryness, dissolved in 0.5 mL of 98% formic acid, diluted to 10 mL with 30% acetic acid, and concentrated by rotary evaporation to 1.0 mL.

Aliquots of each pool were subjected to descending paper chromatography (Glazer et al., 1976) in 1-butanol–pyridine–glacial acetic acid–H_2O (90:60:18:72) and separately to paper high-voltage electrophoresis at pH 1.9 (Glazer et al., 1976) to determine the number and kinds of peptides present. The papers were first stained with ninhydrin reagent (0.1% ninhydrin in ethanol–collidine, 19:1 by volume) and then were sprayed with Pauly reagent (Glazer et al., 1976) to locate peptides containing tyrosine (and histidine).

Each peptide fraction was subjected to that method of fractionation (Glazer et al., 1976) deemed most suitable (see above), and the purified peptides were eluted from the papers for 40 h with 30% acetic acid. After concentration to 1.0 mL by rotary evaporation, aliquots of each fraction were examined by the chromatography or the electrophoresis system not used for its purification. When necessary, this second system (and sometimes a third system, such as pH 3.6 electrophoresis) was used to purify the peptide further.

Amino Acid Analysis. Each purified peptide (approximately 10–50 nmol) was hydrolyzed with 6 N HCl (containing 1 drop of 5% phenol/1 mL of reagent) at 110 °C for 24 h in evacuated, sealed tubes (Glazer et al., 1976). The HCl was removed over solid NaOH in vacuo in a heated desiccator at 40 °C. The residues were dissolved in 0.1 mL of H_2O, and aliquots of 0.01 mL of each sample were examined by paper electrophoresis at pH 1.9. The remainder of each sample was analyzed with a Beckman 121 amino acid analyzer.

Sequence Studies on Peptide X from the H2A-H2B Dimer. Peptide X is the large cross-linked peptide found only in the dimer tryptic digest. Edman degradation of peptide X (40 nmol) was accomplished as described by Glazer et al. (1976).

Carboxypeptidase A and B digestion (Worthington; 20 μg of each) was carried out on 14 nmol of peptide for 2 h at 40 °C in 0.1 mL of 0.1 M NH_4HCO_3, pH 8.0. The enzymes were treated with diisopropyl fluorophosphate before use to inactivate any contaminating trypsin or chymotrypsin (Glazer et al., 1976). The digestion was ended by the addition of 2 drops of glacial acetic acid, and the digest was lyophilized to dryness for amino acid analysis.

Results

The elution profiles for the gel filtration fractionations of the tryptic peptides from the H2A–H2B dimer and from the mixture of H2A and H2B are shown in Figure 1. It had been anticipated that the cross-link in the dimer would interfere somewhat with tryptic digestion (see below), thereby producing some variation in the elution profiles of the two digests, but the differences were larger than expected. Despite these differences in the profiles, the fractions were pooled identically (see Figure 1), and the same peptides generally were isolated from the same fractions of both digests (see Table I). Several peptides were isolated from each of two adjacent fractions, but usually only the fraction containing the largest amount of a given peptide is indicated in Table I. (In some cases this makes it appear that certain peptides such as 126–127 and 128–129 from H2A were isolated from different fractions of the two digests.) The peptides isolated from each digest account for all of the residues present in both histones 2A and 2B.

We believe the most likely explanation for the differences in the elution profiles of Figure 1 is that steric hindrance by the cross-link in the dimer interferes with tryptic hydrolysis. This would give rise to a number of overlapping peptides which would elute from the column at earlier times in the case of the dimer digest (see Figure 1). It is not unusual that these peptides were not isolated on paper, since they may be expected to streak or remain at the origin. Moreover, each would probably be present in low yield with the result that they would

Table I: Peptides Isolated from a Tryptic Hydrolysate of either the H2A + H2B Mixture or the H2A-H2B Dimer

Sephadex G-25 pool of Figure 1	source of peptide	peptides from H2A + H2B mixture[a]	peptides from H2A-H2B dimer[a]
I	H2A	43-71, 100-118	43-71, 100-118
	H2B	58-72	58-72
II	H2A	89-95	89-95
	H2B	1-11, 47-57, 100-108, 109-116	1-5, 6-12, 47-57, 100-108 peptide X
III	H2A	6-9, 14-15, 21-29, 36-42, 72-75, 82-88, 96-99, 119-125, 126-127, 128-129	6-9, 14-15, 36-42, 72-75, 82-88, 96-99, 119-125
	H2B	13-16, 17-20, 21-23, 25-27, 44-46, 73-79, 87-92, 93-99, 117-120	13-16, 21-24, 25-27, 44-46, 73-79, 87-92, 93-99, 109-116, 117-120
IV	H2A	1-3, 4-5, 10-11, 12-13, 16-17, 18-20, 33-35, 76-77, 78-81	1-3, 4-5, 10-11, 12-13, 16-17, 18-20, 33-35, 76-77, 78-81, 126-127, 128-129
	H2B	12, 24, 28-29, 30-31, 32-34, 35-43, 80-85, 86, 121-125	17-20, 21-24, 28-29, 30-31, 32-34, 80-85, 121-125
V	H2A	30-32	30-32
	H2B		86

[a] Indicated by residue number in sequence. The underlined numbers indicate the residues which comprise peptide X.

Table II: Characterization of the Cross-Linked Peptide X of the H2A-H2B Dimer and of Its Parent Peptides from Monomers

	amino acid analysis				Edman degradation of peptide X						
	peptide 21-29 from H2A	peptide 35-43 from H2B	sum of 21-29 and 35-43	peptide X from dimer	analysis after first step	difference from original	amino acids regen-erated[a]	analysis after second step	difference from step 1	amino acids regen-erated[a]	difference from original
Lys	0.04	1.00(1)	1	1.27(1+)[b]	0.66(1)[c]	−0.61[c]	+	0.64(1)[c]	−0.02		−0.63[c]
Arg	1.00(1)	0	1	1.00(1)	1.00(1)	0		1.00(1)	0		0
Asp	0.06	0.21		0.18	0.29	+0.11		0.26	−0.03		+0.08
Thr	0.04	0.20		0.13	0.30	+0.17		0.23	−0.07		+0.07
Ser[d]	0.10	2.12(2)	2	1.94(2)	1.80(2)	−0.14		1.29(1)	−0.51	*e*	−0.55
Glu	1.12(1)	1.14(1)	2	2.00(2)	1.61(1)	−0.39	+++	1.39(1)	−0.22	+	−0.61
Pro	1.10(1)	0	1	0	0			0			
Gly	2.09(2)	0.23	2	2.23(2)	2.32(2)	+0.09		1.57(1)	−0.75	+++	−0.66
Ala	0.91(1)	0.15	1	0.96(1)	0.47(0)	−0.49	+++	0.33(0)	−0.14		−0.63
Val	0.94(1)	2.07(2)	3	2.80(3)	2.82(3)	+0.02		2.80(3)	−0.02		0
Ile	0.06	0.06		0.26	0.21	−0.05		0.25			−0.01
Leu	1.10(1)	0.21	1	1.03(1)	1.03(1)	0		1.03(1)	0		0
Tyr	0	2.54(3)	3	1.54(2)	1.38(2)[f]	−0.16[f]		1.23(2)[f]	−0.15		−0.31[f]
Phe	1.00(1)	0	1	0.83(1)	0.72(1)	−0.11		0.76(1)	+0.04		−0.07
total	9	9	18	16							
column pool of Figure 1	III	IV		II							
yield (%)	22	10		12							
migration, solvent II[g]	2.43	2.36		2.03							
migration, pH 1.9[h]	0.73	0.60		0.65							

[a] As judged by pH 1.9 electrophoresis of the amino acids which were regenerated by acid hydrolysis of the thiazolinone derivatives removed by the Edman degradation. [b] The dimer peptide appears to have about 25% of its molecules with NH_2-terminal lysine (plus NH_2-terminal alanine) and the remainder with NH_2-terminal glutamic acid (plus NH_2-terminal alanine). This peptide migrates as a streak rather than as a uniform spot on paper which probably accounts for the cross contamination. [c] Lysine usually gives low values after Edman degradation due to derivatization of the ϵ-NH_2 group and incomplete regeneration during acid hydrolysis. However, the drop in lysine also reflects those molecules with NH_2-terminal lysine (see footnote *b*). [d] Values for serine have been corrected by 10% after acid hydrolysis. [e] The anilinothiazolinone derivative of serine does not regenerate to serine upon acid hydrolysis. [f] Tyrosine usually gives low values after the Edman degradation. In both steps 1 and 2 approximately half of the tyrosine was analyzed as a tyrosine derivative which eluted before lysine on the short column and in the nitrotyrosine position on the medium column. [g] Solvent II = 1-butanol-pyridine-glacial acetic acid-H_2O (90:60:18:72). Migration is relative to alanine. [h] pH 1.9: high-voltage paper electrophoresis at this pH. Migration is relative to alanine.

be observable to a significant extent only during the column chromatographic step.

The characterization of the three peptides which are of particular significance to this work is shown in Table II. Peptide 21–29 from H2A and peptide 35–43 from H2B were found in the tryptic digest of the mixture of histones 2A and 2B but not in the tryptic digest of the dimer. Peptide X (for cross-linked) was isolated from the dimer but not from the histone mixture. This peptide contains all of the residues of peptide 21–29 of H2A and peptide 35–43 of H2B except for one proline (peptide 21–29) and one tyrosine (peptide 35–43) (see Figure 2). Therefore, we conclude that the cross-link is between a tyrosine and a proline.

The first step of the Edman degradation of peptide X removed mainly alanine and glutamic acid but also a lesser amount of lysine (Table II). Apparently, some molecules of peptide X have the NH_2-terminal sequence Lys-Glu which would be produced by tryptic hydrolysis of the preceding Arg–Lys bond (see Figure 2). Because of the NH_2-terminal position of the lysine residue and the presence of the adjacent negatively charged glutamic acid residue, the Lys–Glu bond would be hydrolyzed very slowly by trypsin, and even after

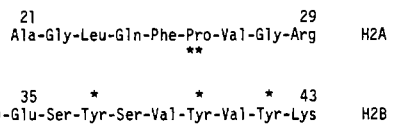

FIGURE 2: The amino acid sequences of H2A and H2B in the vicinity of the cross-link formed by ultraviolet irradiation. It is not known which tyrosine(s) (*) in H2B is involved in the cross-link with the proline (**) residue in H2A. Some molecules of the cross-linked tryptic peptide (X) isolated from the dimer have lysine adjacent to the glutamic acid as indicated by (Lys) (see text).

5 h of tryptic hydrolysis about 25% of the molecules apparently still retain this bond. The removal of alanine and glutamic acid by the Edman degradation was as expected for the proposed cross-linked peptide. The second step of the Edman degradation removed mainly glycine and serine as expected but also a lesser amount of glutamic acid from those molecules with the NH_2-terminal Lys-Glu sequence (Table II). There was insufficient material to carry the Edman degradation further in order to identify directly the cross-linked residues, but the results confirm that the cross-linked peptide itself is correctly identified.

A carboxypeptidase analysis of the COOH termini of peptide X was also carried out. If it is assumed that the carboxypeptidases cannot proceed past a cross-linked tyrosine (there are no data on this point), then one would expect the position of the cross-link to be reflected in the tyrosine to lysine ratio (see Figure 2). A ratio of zero would indicate a cross-link at tyrosine-42, a ratio between 0 and 1 would suggest tyrosine-40, and a ratio of greater than 1 would implicate tyrosine-37. We obtained a ratio for tyrosine/lysine of 0.5, suggesting that the cross-link is at tyrosine-40. However, these experiments were very preliminary in nature, and the involvement of tyrosine-37, or even a mixture of tyrosine-37 and -42, is suggested by other features of the data. This problem currently is being investigated further.

Discussion

We have shown that the covalent link between H2A and H2B induced by UV irradiation of nuclei is from a tyrosine in tryptic peptide 35–43 of H2B to proline-26 of H2A. The tryptic peptide containing the cross-linked residues was identified by comparison of the tryptic digestion products of the cross-linked dimer with those of a mixture of H2A and H2B monomers. Amino acid analysis of the purified, cross-linked peptide was that expected for the sum of the two corresponding monomer peptides except for the lack of a proline and a tyrosine. Since the two monomer peptides contained between them only one proline, it was possible to assign one member of the cross-link directly to proline-26 of H2A.

Peptide 35–43 of H2B contains three tyrosines at positions 37, 40, and 42. In principal, any of these could be involved in the cross-link. Indeed, the cross-linked peptide could be a mixture of isomers. This possibility was suggested by a preliminary analysis of the cross-linked peptide by digestion from the COOH terminus with carboxypeptidases A and B. However, uncertainties regarding the mode of action of these enzymes in the neighborhood of a cross-linked residue has prevented a firm conclusion as yet. An independent indication that the dimer may indeed be a mixture of cross-linked isomers comes from the observation that occasionally (apparently on better than average gels) the H2A–H2B dimer resolves into a barely discernible doublet upon acid–urea electrophoresis. Acid–urea gels have been shown to resolve configurational isomers of dimers (Panyim et al., 1971). If the H2A–H2B dimer is a mixture of configurational isomers, this would suggest either that more than one tyrosine of H2B is in contact with proline-26 of H2A at the binding site or that the binding site can exist in at least two different conformations within chromatin.

Although the tyrosine–proline adduct to which we attribute the cross-link was not itself isolated and characterized, our results cannot be explained by trivial photolytic damage and consequent change in properties of the histone peptides. First of all, peptide assignments were based on a comparison of the tryptic digests and amino acid analysis of dimer and monomers isolated from the same preparation of photolyzed nuclei. Secondly, although H2A and H2B monomers can be almost quantitatively converted to dimer by UV irradiation, we chose an irradiation dose which gave only 40% conversion, thus minimizing UV exposure and random photolytic damage. Finally, our light source was filtered to remove the 254-nm emission. This reduces damage to DNA and minimizes histone–DNA cross-linking (Martinson et al., 1976).

The results reported here are consistent with several independent lines of evidence. Our previous peptide mapping data (Martinson & McCarthy, 1976) showed by CNBr cleavage that the H2A–H2B cross-link involved the NH_2-terminal half of H2B. In view of some recently published results, those same data now also show that it was the NH_2-terminal half of H2A as well that became cross-linked. This can be deduced because H2B became cross-linked not only to the predominant mammalian H2A (which lacks methionine) but also to a methionine-containing variant of H2A (Martinson & McCarthy, 1976). Since it is now known that the methionine of the H2A variant is at position 51 (Franklin & Zweidler, 1977), it can be deduced, by examination of the previous CNBr peptide mapping data (see Martinson & McCarthy, 1976), that the cross-link to H2A must occur within its first 51 residues. We have also observed (unpublished experments) that partial proteolysis of cross-linked chromatin reduces the molecular weight of the dimer but does not excise the cross-link, thus causing the dimer to disappear. This shows that the cross-link is not within the NH_2-terminal "fingers" which are removed by gentle proteolysis (Weintraub & Van Lente, 1974). This is consistent with our present assignment of the cross-link to positions at least 26 and 37 amino acids in from the NH_2 terminus. Finally, the action spectrum of H2A–H2B cross-linking (Martinson et al., 1976) and the known properties of UV cross-links implicated the involvement of at least one tyrosine from the outset. Thus, a collection of data totally independent from those presented here is in complete agreement with the present assignment of the cross-link from tyrosine-37, -40, or -42 of H2B to proline-26 of H2A.

It is noteworthy that these cross-linking assignments involve residues which may border almost directly on the NH_2-terminal fingers of H2A and H2B which have been implicated as being involved primarily in DNA binding (Kato & Iwai, 1977). Thus, the DNA-binding region of H2A extends to about residue 20, while the DNA-binding region of H2B extends to about residue 35 (Kato & Iwai, 1977). Since it is proline-26 and tyrosine-37, -40, or -42 that become cross-linked at the H2A–H2B binding site, this implies that the NH_2-terminal fingers of H2A and H2B must bind adjacent regions of DNA.

It seems likely that the cross-link which we have characterized represents an important and fundamental noncovalent interaction within the H2A–H2B binding site. First of all, irradiation with UV under appropriate conditions gives rise to almost quantitative conversion of monomer H2A and H2B

to the dimer. Moreover, we have found recently (Martinson & True, 1979) that plant (leek) chromatin is cross-linked by UV with the same specificity and yield characteristic of mammalian chromatin to give a dimer which CNBr analysis shows is completely homologous to the mammalian counterpart. In addition, preliminary analysis of plant (pea) H2B peptides (Hayashi et al., 1977) shows that tyrosines-37 and -40 are completely conserved evolutionarily and that tyrosine-42 has sustained merely a conservative change to phenylalanine. Furthermore, tyrosines-37, -40, and -42 are all conserved among calf, trout, sea urchin, and drosophila despite the fact that the intervening amino acids are not (Koostra & Bailey, 1978; Elgin, personal communication). Finally, acid-extracted H2A and H2B bind each other in solution, free of the constraints of the nucleosome and the other histones, to give complexes which are cross-linked by UV with specificity comparable to that of intact nuclei (Martinson et al., 1979a). Taken together, these data suggest that the tyrosine–proline contact which we have identified does not represent merely the fortuitous adjacency of two amino acid residues but rather reflects a substantial thermodynamic involvement in the H2A–H2B binding interaction.

When this work was begun, it was fully anticipated that tyrosine would be at least one of the residues involved in the cross-link induced by UV irradiation (see introduction). A preliminary experiment had indicated that the H2A and H2B peptides, 21–29 and 35–43, were not present in the tryptic digest of the dimer, and it was thus anticipated that the second member of the cross-link might be phenylalanine-25 of H2A (see Figure 2). However, the data leave little doubt that the adjacent proline (residue 26) rather than the phenylalanine is the amino acid which is attacked by the UV-activated tyrosine residue in H2B. This strongly suggests that, in nucleosomes, proline-26 of H2A and one or more tyrosines (37, 40, 42) of H2B are adjacent and precisely positioned for such a cross-link to form. It is possible that a tyrosine of H2B and phenylalanine-25 of H2A are involved in some type of interaction (such as ring–ring stacking) which firmly positions the adjacent proline for attack by the activated tyrosine. The spatial relationship of the residues is apparently more important than the type of residue in determining which is attacked to form cross-links of this type. Because of this, the determination of the cross-linked structure can contribute much to our understanding of precise histone–histone interactions in chromatin.

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