Histone–Histone Interactions within Chromatin. Preliminary Characterization of Presumptive H2B–H2A and H2B–H4 Binding Sites†

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ABSTRACT: Cyanogen bromide peptide analyses of cross-linked histones show that histone 2B possesses separate binding sites for histones 2A and 4. Various polymorphic forms of H2A and H4 in chromatin are bound to H2B through these binding sites. These interactions are thus a fundamental structural feature of chromatin. Additional data strongly suggest that H2B is bound to H2A and H4 simultaneously in vivo. Mouse L cells were treated with either tetranitromethane (TNM) or ultraviolet (uv) light at 280 nm in order to induce the cross-linking of histone 2B in their chromatin to either histone 4 or 2A. The characteristics of these cross-linking agents and the reactions they induce suggest that the cross-links define loci within the actual binding sites between these histones. Characterization of the cyanogen bromide peptide fragments of the H2B–H4 and H2B–H2A dimers showed that the C terminal half of H2B contains the link to H4 (also near *its* C terminus), whereas the N-terminal half contains the link to H2A. Treatment of the cells with both uv light and TNM results in the appearance of a single new trimer which we presume to be H2A–H2B–H4. We conclude from this that H2B interacts simultaneously with H2A and H4 in chromatin through separate binding sites. Further analysis shows that several acetylated subspecies of H4 are represented in the H2B–H4 dimer and that at least two of the polymorphic forms of H2A are present in the H2B–H2A dimer. These data reveal that the H2A–H2B–H4 association is an important underlying structural feature of chromatin.

The fundamental chromatin fiber of higher eucaryotes is now generally believed to be composed of discrete histone–DNA subunits (Elgin and Weintraub, 1975; Van Holde and Isenberg, 1975). Each subunit is thought to possess a core comprised primarily of the C-terminal portions of two each of the histones 2A, 2B, 3, and 4. A length of DNA, comprising about 180 base pairs, is complexed with 25 or so residues of each histone N terminus and collapsed in some way about this core. Association of this fundamental subunit with H1 and an unknown amount of nonhistone protein completes the structure (Elgin and Weintraub, 1975; Van Holde and Isenberg, 1975).

A well-known feature of histones is their unusual resistance to evolutionary change (Elgin and Weintraub, 1975). This phenomenon presumably reflects the need for each histone to interact simultaneously and specifically with a number of different chromatin components—perhaps in a variety of interconvertible conformations. One class of such interactions must be those among the histones themselves. Indeed, solution studies show that each histone species is capable of specific interaction with at least two others (D'Anna and Isenberg, 1974).

In our studies of chromatin structure we have recently shown (Martinson and McCarthy, 1975; Martinson et al., 1976) that highly selective cross-linking can be induced within the chromatin subunits of whole cells by the zero-length cross-linkers tetranitromethane (TNM[1]) and uv light. These agents are able to penetrate hydrophobic clusters and activate tyrosine, so we have used them as probes for histone–histone binding sites. Of particular interest was the finding that TNM induced an H2B–H4 cross-link, while uv cross-linked H2B to H2A. These results strengthened the earlier implication derived from solution studies (D'Anna and Isenberg, 1974) that individual histones possess at least two distinct specific binding sites for other histones. In this report we show, by cyanogen bromide peptide mapping of the cross-linked dimers, that H2A and H4 are indeed linked to different portions of the H2B molecule. Moreover, we present evidence which strongly suggests that H2B is specifically complexed to both H2A and H4 simultaneously in vivo. By pursuing these observations, we seek to increase our understanding of the internal structure of the fundamental chromatin subunit.

Materials and Methods

Mouse LA-9 cells were cultured in Joklik-modified minimum essential medium with 5% calf serum. Chromatin was prepared as previously described (Martinson and McCarthy, 1975, procedure 1) by lysing detergent-prepared nuclei in 50 mM Tris (pH 8). The chromatin was centrifuged and resuspended in 1 mM EDTA (pH 8) for storage at 0 °C until use. Histones were prepared by acid extraction and fractionated on columns of Bio-Gel P-30 as previously described (Martinson and McCarthy, 1975). The acid–urea system of polyacrylamide gel electrophoresis was used (Panyim and Chalkley, 1969). Fifteen percent acrylamide containing urea at 2.8 M was cast as 150 × 110 × 0.8 mm slabs. The gels were generally run at 300 V for 2.5–3 h. Staining with Coomassie blue, destaining, densitometry, and scintillation counting of excised bands were as previously described (Martinson and McCarthy, 1975).

Cells were treated with tetranitromethane (TNM) under anaerobic conditions as follows. Four liters of cells at about 6–8 × 10^5/ml was harvested and resuspended in 30–40 ml of sa-

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[1] Abbreviations Used: uv, ultraviolet; TNM, tetranitromethane; CNBr, cyanogen bromide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

line–phosphate (4 mM KCl, 127 mM NaCl, 15 mM sodium phosphate, pH 7.5). The suspension was stirred gently under a stream of N_2 for 15–30 min at room temperature, after which about 400 ml of additional N_2-purged saline–phosphate was added. The TNM (0.2 ml) was then added and the suspension incubated with stirring for about 2 h at 37 °C. During this period the pH usually decreased from 7.5 to about 7.0. All steps up to this point were carried out under a nitrogen atmosphere. The cells were rapidly harvested and washed free of TNM.

The anaerobic photolysis of cells was carried out following procedures described previously (Martinson et al., 1976). Cells were harvested at a density of about 9×10^5/ml and then resuspended at 3×10^6/ml in phosphate-buffered saline (4 mM KCl, 136 mM NaCl, 10 mM sodium phosphate, pH 7.1). The irradiation was carried out on 100-ml batches of cell suspension in 35-mm diameter Corex cylinders (old formulation) which filter out light of wavelengths less than about 260 nm (Martinson et al., 1976). The exposure time was about 1 h and the sample temperature was maintained at less than 30 °C throughout. No change in pH occurred during photolysis.

Results

Pure preparations of H2A–H2B and H2B–H4 dimers were cleaved at their methionines using cyanogen bromide. The resulting CNBr peptides were then displayed on polyacrylamide slabs by means of gel electrophoresis, and the electrophoretic patterns obtained were interpreted by comparison with reference patterns obtained from CNBr digests of the purified monomers. In the paragraphs below, we describe the preparation of the cross-linked histones and their characterization by CNBr peptide mapping.

Preparation of Pure H2B–H4 Dimer. Earlier when we reported the cross-linking of H2B to H4 by tetranitromethane (TNM), we also pointed out that this primary reaction, though specific, was complicated by an array of nonspecific secondary side reactions (Martinson and McCarthy, 1975). We have since found that the contribution of many of these, presumably oxidative, side reactions can be substantially diminished with a concomitant increase in dimer yield by conducting the cross-linking reaction anaerobically. Accordingly, we now treat cells with TNM under a nitrogen atmosphere in N_2-purged saline–phosphate buffer.

Histones prepared from such cells were fractionated on a column of Sephadex G-200. A typical profile is shown in Figure 1, where it can be seen that this procedure separates the dimer material from the bulk of the histone monomers except for H1. Those fractions containing H2B–H4 dimer were pooled and chromatographed on Bio-Gel P-30, which fractionates histones on the basis of selective affinity as well as size. H4 apparently has a high affinity for the polyacrylamide beads of Bio-Gel P-30. Consequently, the H2B–H4 dimer elutes within the monomer region of the profile well separated from the other high-molecular-weight material which coelutes from Sephadex (see Figure 1, lower panel). Lyophilization of the pooled H2B–H4 dimer fractions yielded a preparation of greater than 90% purity as judged by stained electrophoretograms (see Figure 2, TNM panel).

Preparation of Pure H2A–H2B Dimer. Gel exclusion chromatography on Sephadex G-200 of histones isolated from uv-irradiated cells is illustrated in the upper panel of Figure 1. The H2A–H2B and H2B–H4 dimers behave similarly on this chromatographic support. Rechromatography on Bio-Gel P-30 of the fractions containing H2A–H2B dimer yields relatively pure dimer which elutes in the position shown in the lower panel of Figure 1. The high yield and low background

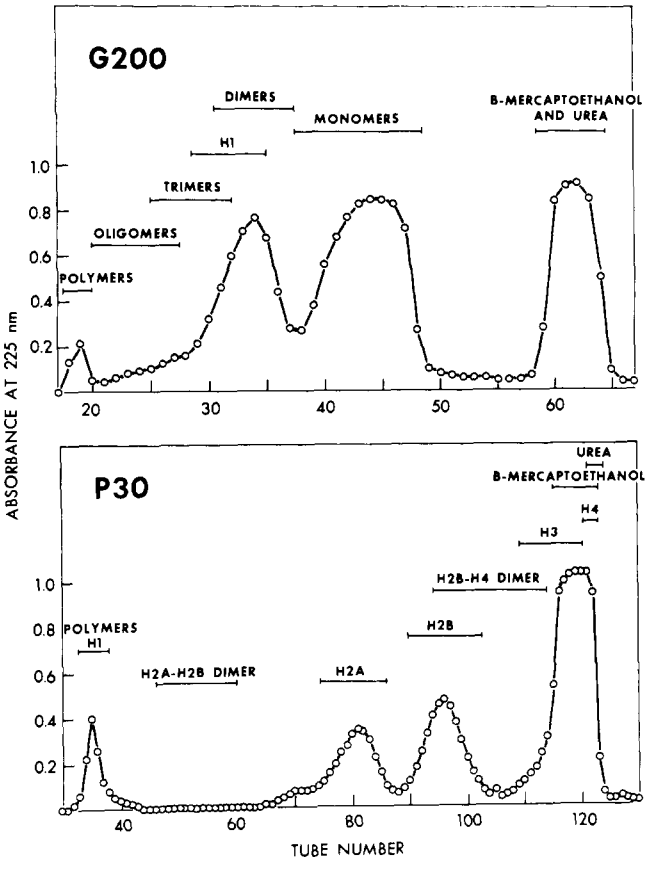

FIGURE 1: Representative elution profiles of whole histone on either Sephadex G-200 or Bio-Gel P-30. G-200: Total histone from 2×10^9 photolyzed cells was dissolved in about 1.3 ml of freshly prepared 5 M urea containing 4% β-mercaptoethanol. The histone solution was loaded on a gel bed 15 mm in diameter and 115 cm long which was connected in series to a second column of similar dimensions. The columns were run at room temperature and eluted with 10 mM HCl–10 mM β-mercaptoethanol at a flow rate of about 7 ml/h. Fractions of 6.5 ml were collected. The elution profile shown is that of the preparation just described. The horizontal bars summarize the data for elution of cross-linked histones from both uv and TNM treated cells. P-30: Total histone from un-cross-linked L cells was loaded on a three-section (3×115 cm) column and eluted as described above, except that the column was run at 4 °C, the flow rate was 5 ml/h, and 4.5 ml fractions were collected. The horizontal bars identify the peaks of monomer histone and also indicate the elution positions of cross-linked histones from uv and TNM treated cells.

characteristic of H2A–H2B dimer formation during photolysis ensure little contamination by extraneous material. When pooled and lyophilized, the H2A–H2B dimer appears to be about 95% pure (see Figure 2, uv panel).

Preparation of Pure Monomers. The chromatography of histone monomers on Bio-Gel P-30 is illustrated in the bottom panel of Figure 1 (see also Candido and Dixon, 1972). The peak fractions for each histone species contained the respective histone monomers in greater than 90% purity (see Figure 2).

Characterization of CNBr-Treated Histone Monomers. Polyacrylamide gel electrophoresis patterns have been determined for the CNBr digests of the individual histone monomers for subsequent use in the interpretation of patterns similarly obtained for the dimers. Under our conditions, partial digestion of histones 2A, 2B, 3 and 4 was obtained yielding a mixture of fully cleaved as well as partially cleaved and/or uncleaved material. As will be discussed in a subsequent section, the resulting patterns of partials are very meaningful and strengthen the overall interpretation of the covalent structures of the

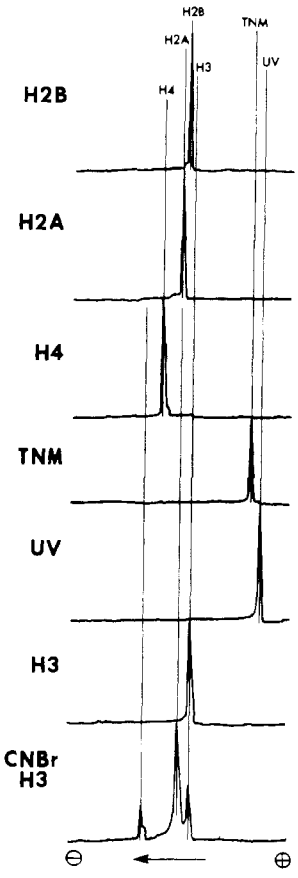

FIGURE 2: Electrophoretic profiles of purified histones and histone dimers. The bottom panel is the electrophoretic profile of H3 treated with CNBr as described in the legend to Figure 3.

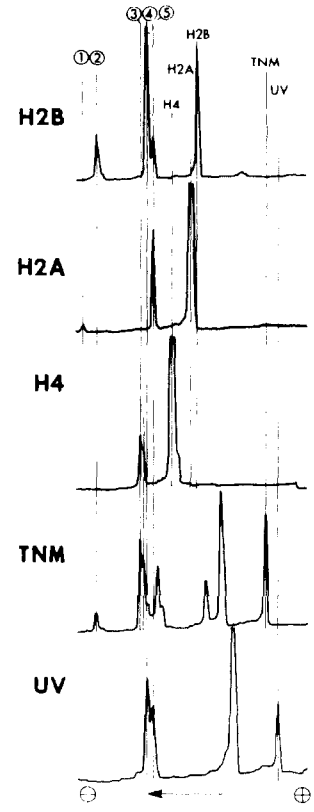

FIGURE 3: Electrophoretic profiles of CNBr-treated histone monomers and dimers (TNM is H2B-H4 dimer; UV is H2A-H2B dimer). About 10 μl of monomer or 20 μl of dimer at concentrations of 4–5 μg/μl was dispensed to separate tubes. CNBr (100 μl) at 20 mg/ml in 0.1 M HCl was then added. The tubes were then sealed with Parafilm and stored in the dark for 15 h at room temperature. Subsequently the contents were lyophilized and taken up in 10 μl of 5 M urea–4% β-mercaptoethanol, of which 1 μl was then taken for electrophoresis. All of the samples were run side by side on a single slab and parallel scans of the samples are shown. In order to fit all peaks into a single scan, it was necessary to begin recording slightly below the origin of electrophoresis. In no case was there any material above background in the region of the gel not scanned.

histone dimers. As a control, H1 was also treated with CNBr. Mammalian H1 is devoid of methionine (Alfageme et al., 1974) and, as expected, no cleavage occurred. The cleavage patterns obtained with the other four histones are discussed below.

Bovine and, we assume also, mouse H2B (125 amino acids) have two closely spaced methionines near the center of the molecule at positions 59 and 62 (Elgin and Weintraub, 1975). Cleavage with CNBr at both residues bisects the molecule into a rapidly migrating N-terminal half (residues 1–59, Figure 3, peak 2) and a more slowly migrating C-terminal half (residues 63–125, Figure 3, peak 4) (Adler et al., 1974) with the loss of residues 60–62. Cleavage only at position 59 would yield a C-terminal half molecule which is three amino acids longer (residues 60–125) than that of fully cleaved H2B. This partial cleavage product presumably accounts for peak 5 of Figure 3. Similarly, cleavage only at position 62 yields an N-terminal half molecule with three extra residues. Accordingly, peak 2, although it appears as a singlet in Figure 3, is usually observed as a doublet similar to peaks 4 and 5. (The relative intensity of peak 2 rapidly diminishes during the storage of the gel in acetic acid with the result that, in Figure 3, the doublet normally at the peak 2 position is reduced in size and only the major member is clearly visible.)

Histone 2A has generally been found to be devoid of methionine (Sautière et al., 1975). However, Alfageme et al. (1974) have reported a minor variant of mouse H2A which does contain a methionine residue and, under our conditions, a small portion of H2A is cleaved by CNBr to yield two fragments (Figure 3). The more slowly migrating of the fragments has the same mobility as the peak 5 fragment of H2B in our gels, but is easily distinguishable from it by the distinctly different color of its stained band. As explained for peak 2 of H2B, excessive destaining accounts for the small size of peak 1 here.

H4 (102 amino acids) has a methionine at position 84 in its sequence (Elgin and Weintraub, 1975). Cleavage at this methionine with CNBr yields a small, rapidly migrating, C-terminal fragment of 18 residues and a considerably larger N-terminal fragment (Pekary et al., 1975). Under our conditions, the large N-terminal fragment of H4 (residues 1–84) migrates on the gel (Figure 3, peak 3) slightly faster than does the H2B C-terminal doublet (Figure 3, peaks 4 and 5) and is itself seen to be a doublet or triplet reflecting the respective acetylated forms of the parent H4 (Wangh et al., 1972). The small C-terminal fragment of H4 is lost under our conditions.

For possible future reference, the pattern for H3 is shown in the bottom panel of Figure 2, although the cleavage pattern of H3 is not relevant to the following discussion on the TNM and uv dimers which contain no H3. H3 (135 residues) has methionines at positions 90 and 120. Presumably, cleavage at position 120 yields the fragment (1–120) which migrates in the same position as H2A, while cleavage at position 90 yields the fragment (1–90) which migrates a little faster than H4 (with the mobility of peak 5, Figure 3). A very rapidly mi-

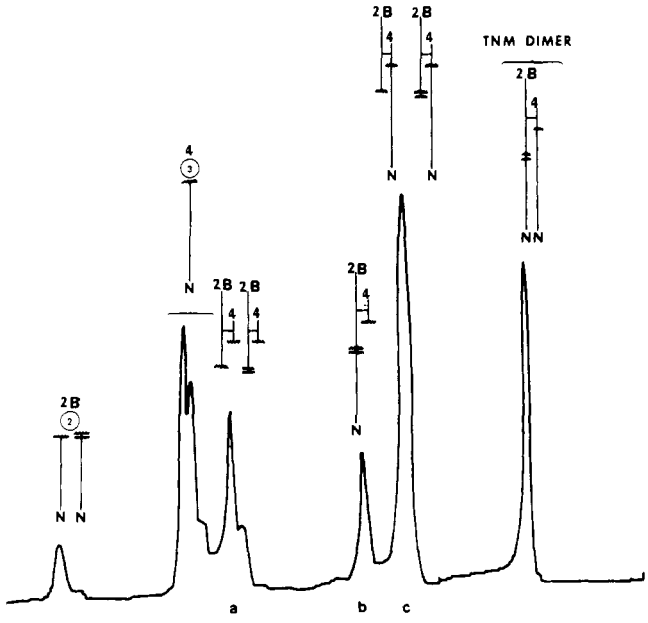

FIGURE 4: Identification of each cleavage product resulting from treatment of H2B-H4 dimer with CNBr. The circled numbers 2 and 3 refer to the same numbered peaks as in Figure 3. The H2B and H4 polypeptide chains are drawn to scale. Their N termini and CNBr cleavage sites are indicated.

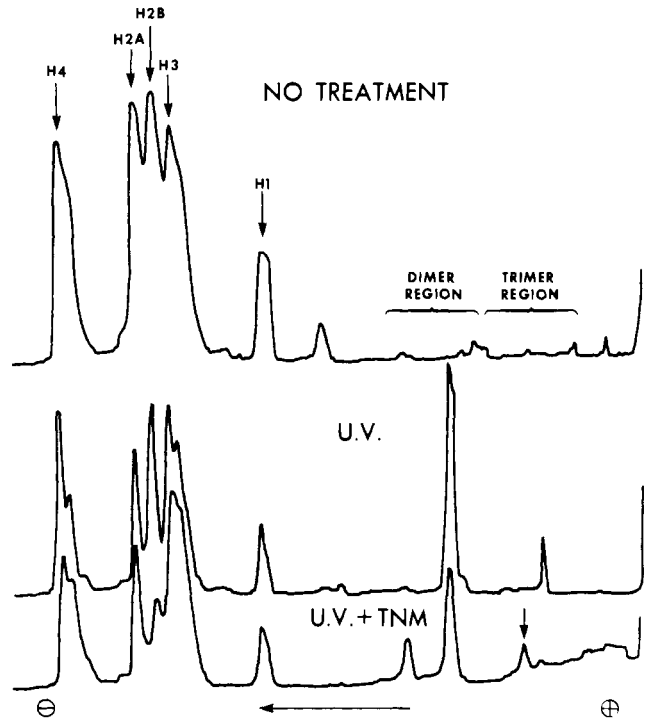

FIGURE 5: Production of putative H2A-H2B-H4 trimer (indicated by arrow) by sequential treatment with uv and TNM.

grating, presumably C-terminal, fragment is also observed if the gel is run and destained for only a short period of time.

Characterization of the CNBr-Treated Histone Dimers. Partial cleavage of the TNM (H2B-H4) and uv (H2A-H2B) dimers with CNBr followed by gel electrophoresis yields the patterns shown in the bottom two panels of Figure 3. The particular peptides which appear in these patterns serve to demonstrate that different halves of the H2B molecule participate in the interactions with H2A and H4. Peak 2, the N-terminal portion of H2B, appears in the TNM dimer pattern but not in that of the uv dimer. Conversely, peaks 4 and 5, which represent the C-terminal end of H2B, appear only in the uv dimer pattern. Clearly, it is the C-terminal region of H2B which is cross-linked to H4 in the TNM dimer, and the N-terminal half which is cross-linked to H2A in the uv dimer. The monomer-pattern fragments which are missing from the dimer patterns must include the cross-linked regions of the dimers. These give rise, in the dimer patterns, to the relatively high-molecular-weight fragments which do not correspond to peaks 1-5 in the monomer cleavage patterns. Thus, in the case of the uv (H2A-H2B) dimer, a new major large peptide appears representing the cleaved N-terminal portion of H2B linked to uncleaved H2A.

The pattern of peptides is somewhat more complex in the case of the TNM (H2B-H4) dimer. This pattern is displayed on a larger scale in Figure 4, together with the assignment of each peak to a peptide present in the monomer patterns or to a composite peptide resulting from the cross-link between the two monomers. The intact TNM product is shown as a dimer induced by a cross-link between a residue in the C-terminal half of H2B and one of the last 18 residues of H4. All possible partial cleavage products can be assigned unambiguously to a peak in the pattern.

Simultaneous Interaction of H2B with H2A and H4 in Chromatin. The involvement of separate regions of H2B in the cross-links to H2A and H4 suggests, but does not prove, that H2B interacts with two other histones through separate domains. Stronger evidence that the binding sites for H2A and H4 do not overlap was obtained by treating cells sequentially with uv and then TNM (Figure 5). After uv treatment, the typical pattern arises (Martinson et al., 1976) in which a prominent dimer (H2A-H2B) and a trimer are seen in the gel electrophoretic profile. Subsequent treatment with TNM generates the usual H2B-H4 dimer together with a new band (marked by an arrow) in the trimer region of the gel, but distinguishable from the uv trimer. We presume this new product to be an H2A-H2B-H4 trimer resulting from linkage of the H2A-H2B dimer to H4 at a separate H2B binding site. This is the most reasonable and certainly the simplest interpretation of these data since treatment with TNM normally produces only a single identifiable product, namely, the H2B-H4 dimer (Martinson and McCarthy, 1975). Attempts are currently underway to positively identify this trimer.

Discussion

Two different histone dimers (H2A-H2B and H2B-H4) both involving H2B were produced within whole cells by zero-length cross-linking procedures and then subjected to CNBr cleavage and peptide mapping. It was found that the N-terminal half of H2B contains the link to H2A, whereas the C-terminal half contains the link to H4. The significance of this observation was borne out by the fact that a trimer, presumably H2A-H2B-H4, was produced by the sequential treatment of cells with uv and then TNM. Thus, H2B apparently has separate binding sites for H2A and H4 and at least a portion of the H2B population in chromatin appears to interact simultaneously with H2A and H4 in vivo. A similar conclusion was arrived at from the preliminary assembly mapping studies which we reported (Martinson and McCarthy, 1975) in which we found that TNM treatment of a reconstituted mixture of H2B, H4, and DNA did not yield the usual H2B-H4 dimer unless H2A were also present.

In our hands CNBr cleavage of the histones did not reach completion but yielded a mixture of fully and partially cleaved as well as uncleaved material. However, the partial cleavage

patterns were reproducible and provided additional confirmatory evidence for the dimer configurations presented above. No attempt has been made to modify the procedure to obtain complete cleavage.

Analysis of the Uv Dimer Cleavage Pattern. CNBr digestion of the H2A-H2B dimer (Figure 3) yields the 4, 5 doublet representing the free C-terminal H2B half molecule as well as a new high-molecular-weight cleavage product which is presumably the H2B N-terminal fragment covalently linked to H2A. However, it can be seen in Figure 3 that the intensity of peak 5 relative to peak 4 is greater in the H2A-H2B dimer pattern than in the H2B monomer pattern. This implies the presence of the H2A as well as the H2B monomer component in peak 5 of the uv dimer pattern and the color of this band in the stained gel (see above) bears this out. The small shoulder on the large cleavage product peak may correspond to the remaining H2A fragment covalently linked to intact H2B. Since only the minor methionine-containing variant of H2A (Alfageme et al., 1974) can be cleaved by CNBr, the fully cleaved cross-linked core of the corresponding variant dimer (i.e., H2A-H2B lacking both the C-terminal half of H2B as well as the peak 5 fragment of the variant H2A) is apparently not produced in sufficient quantities to be detected on the gel. Unfortunately, the position of the variant H2A methionine is not presently known, so we cannot say which half of H2A is involved in the binding interaction.

Analysis of the TNM Dimer Cleavage Pattern. In contrast to the variant H2A, the positions of the methionines in both H2B and H4 are known and a very detailed interpretation of the H2B-H4 dimer cleavage pattern is possible. Indeed, the pattern of partials obtained from cleavage of the TNM dimer (Figures 3 and 4) reveals that all expected partial cleavage products can be accounted for by individual peaks in the pattern. Moreover, only if it is assumed, as shown in Figure 4, that the TNM cross-link occurs in the C-terminal portions of H2B and H4 can the qualitative aspects of yield and mobility for the new peptide fragments be explained. For example, under our conditions most of the H2B is cleaved, whereas most of the H4 is not (Figure 3). Accordingly (see Figure 4) the TNM dimer peak is converted with good yield into peak c since this involves cleavage of the H2B part of the dimer, while in contrast peaks a and b are produced with poor yield since these involve cleavage of the H4 portion of the dimer. The mobilities of peaks a, b, and c also conform to expectation. Thus, from Figures 3 and 4, peaks a and b migrate only slightly more slowly than the H2B C-terminal half (peaks 4 and 5) and the H2B monomer, respectively, as would be expected from the attachment to each of only 18 additional residues derived from H4. Also, as expected on the basis of molecular weight, peak b has a greater mobility than peak c. Finally, although peak c has not migrated far enough to resolve the two similar fragments therein, the resolution is clear in peaks a and 2. Similarly, the nonacetylated and acetylated forms of H4 are not resolved within the dimer peak, but the standard triplet configuration is clearly displayed in peak 3, the N-terminal portion of H4 liberated from the dimer. Thus, at least three different forms of H4 participate in the binding to H2B.

Implications. The presence of multiple forms of H4 in the TNM dimer and at least two forms of H2A in the uv dimer is of particular significance. In light of the high specificity of the cross-linking agents and particularly in view of the great conformational selectivity exhibited by TNM (Martinson and McCarthy, 1975), this shows that the H2B-H4 and the H2B-H2A binding interactions are fundamental structural features of chromatin which are common to both genetic (H2A) and posttranslational (H4) polymorphic forms of these histones. Furthermore, together with the findings that H2B-H2A cross-linking is nearly quantitative (Martinson et al., 1976) and that an H2A-H2B-H4 trimer can apparently be produced in whole cells, these results imply that the H2A-H2B-H4 association is a general feature of chromatin structure. This presumably reflects the existence in chromatin of the tetrameric H2A-H2B-H4-H3 cluster predicted by D'Anna and Isenberg (1974) and later demonstrated in solution by Weintraub et al. (1975).

An interesting question is why TNM and uv have different cross-linking specificities. Both agents induce cross-links via transient phenoxy free radicals which yield bityrosyl bridges in the cross-linked proteins (Williams and Lowe, 1971; Lehrer and Fasman, 1967; Pretorius et al., 1975; Martinson et al., 1976). While TNM may be sterically excluded from the uv site, this cannot be true of the uv with respect to the TNM site. Peptide analyses and sequence studies are currently being undertaken to determine the specific residues involved in the TNM and uv cross-links. These results should shed light on the nature of the actual H2B-H2A and H2B-H4 binding sites and the differences between them.

It should be noted that, while we presume the TNM and uv cross-linked residues to be within the histone-histone binding sites, they certainly do not by themselves constitute the sites. Thus, more information is required. Perhaps the finding of Van Lente et al. (1975) will prove useful. They have shown that formaldehyde treatment of chromatin also cross-links H2B to H2A and H4 with relatively high specificity. However, an important distinction between their result and ours is that formaldehyde presumably cross-links two lysines together which, since both are positively charged, cannot have been associated directly with one another prior to cross-linkage. Nevertheless, formaldehyde contains only one carbon, so the lysines must have been adjacent and may represent regions immediately surrounding the histone-histone binding sites. Thus, sequence analysis of histones cross-linked to each other and DNA by reagents of various sizes and specificities should eventually yield a detailed picture of the three-dimensional structure of the basic chromatin subunit.

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Hydrolytic Action of Aminoacyl-tRNA Synthetases from Baker's Yeast: "Chemical Proofreading" Preventing Acylation of $tRNA^{Ile}$ with Misactivated Valine†

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ABSTRACT: Phe-$tRNA^{Phe}$-C-C-A, Val-$tRNA^{Val}$-C-C-A, and Ile-$tRNA^{Ile}$-C-C-A, which accept their amino acid on the 2′-OH of the 3′-terminal adenosine, are hydrolyzed readily by their aminoacyl-tRNA synthetase. If the 3′-terminal adenosine in these tRNAs is replaced by either 3′-deoxyadenosine or formycin, little if any hydrolysis can be observed. Correspondingly Ser-$tRNA^{Ser}$-C-C-A which accepts serine on the 3′-OH of the 3′-terminal adenosine is hydrolyzed by seryl-tRNA synthetase, whereas Ser-$tRNA^{Ser}$-C-C-2′dA and Ser-$tRNA^{Ser}$-C-C-F are not. Tyr-$tRNA^{Tyr}$-C-C-A and all modified Tyr-$tRNA^{Tyr}$-C-C-N, which can accept tyrosine on either the 2′-OH or the 3′-OH of the 3′-terminal adenosine, are not hydrolyzed by tyrosyl-tRNA synthetase. The data can be rationalized assuming that hydrolysis takes place only if the amino acid is bound to the nonaccepting OH and hence is not positioned at the amino acid binding site upon formation of the complex between aminoacyl-tRNA and aminoacyl-tRNA synthetase. In the formycin-carrying tRNA, the amino acid bound to the nonaccepting OH seems to be inaccessible to the enzymatic groups responsible for hydrolysis. Val-$tRNA^{Ile}$-C-C-3′dA and Ile-$tRNA^{Ile}$-C-C-3′dA cannot be hydrolyzed by isoleucyl-tRNA synthetase. Val-$tRNA^{Ile}$-C-C-A is hydrolyzed by the enzyme five times more rapidly than Ile-$tRNA^{Ile}$-C-C-A. Whereas Ile-$tRNA^{Ile}$-C-C-F is absolutely stable, Val-$tRNA^{Ile}$-C-C-F is hydrolyzed immediately. As shown by the earlier finding that valine misactivated by isoleucyl-tRNA synthetase cannot be permanently transferred to $tRNA^{Ile}$-C-C-A but to $tRNA^{Ile}$-C-C-3′dA, the 3′-OH is essential for preventing transfer of misactivated valine. It thus appears that valine is hydrolyzed off Val-$tRNA^{Ile}$-C-C-N if it is bound to the accepting 2′-OH in the binding site for isoleucine. A hypothesis is offered attempting to explain the experimental observations in mechanistic terms. We consider the hydrolytic action of the aminoacyl-tRNA synthetases as a general mechanism of "chemical proofreading" in the protein biosynthesis.

In protein biosynthesis amino acids are enzymatically activated (eq 1) and transferred to tRNA (eq 2) (Loftfield, 1972; Kisselev and Favorova, 1974; Söll and Schimmel, 1974). In these reactions, the required specificity is dependent entirely on the interaction of aminoacyl-tRNA synthetase with its specific amino acid (aa[1]) and its specific tRNA.

$$\text{ATP} + \text{aa} + \text{enzyme} \rightleftharpoons [\text{E} \cdot \text{aa-AMP}] + \text{PP} \quad (1)$$

$$[\text{E} \cdot \text{aa-AMP}] + \text{tRNA} \rightleftharpoons \text{E} + \text{AMP} + \text{aa-tRNA} \quad (2)$$

In addition to the reactions described in eq 1 and 2, aminoacyl-tRNA synthetases can perform a hydrolytic reaction; aminoacyl-tRNAs are in most cases hydrolyzed on interaction with their enzyme (eq 3). This reaction is known in the literature as AMP/PP independent hydrolysis.

$$\text{aa-tRNA} + \text{enzyme} \rightarrow \text{aa} + \text{tRNA} + \text{enzyme} \quad (3)$$

It has been speculated, whether this hydrolysis might be functional in a control step in order to increase the specificity of the reactions described in eq 1 and 2 (Söll and Schimmel, 1974; Yaniv and Gros, 1969; Lagerkvist et al., 1966; Schreier and Schimmel, 1972; Bonnet and Ebel, 1972; Sourgoutchov et al., 1974), because the specificity of the aminoacyl-tRNA synthetases with respect to amino acids is no one absolute value (Loftfield, 1972). One of the best investigated irregularities is misactivation of valine by isoleucyl-tRNA synthetase from *Escherichia coli* or from baker's yeast according to eq 1, yielding [E^{Ile}·Val-AMP] (Söll and Schimmel, 1974, see section IVD; Bergmann et al., 1961; Loftfield and Eigner, 1965; Baldwin and Berg, 1966; von der Haar and Cramer, 1975). Transfer of this misactivated valine to $tRNA^{Ile}$-C-C-A is prevented by a hydrolytic reaction which leads to the liberation of valine from [E^{Ile}·Val-AMP] on treatment with $tRNA^{Ile}$-C-C-A (eq 4) (Baldwin and Berg, 1966), thus correcting the previous misactivation.

$$[E^{Ile} \cdot \text{Val-AMP}] + tRNA^{Ile}\text{-C-C-A} \rightarrow \text{Val} + \text{AMP} + E^{Ile} + tRNA^{Ile}\text{-C-C-A} \quad (4)$$

Previously we have found a strict specificity for the position

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[1] Abbreviations used: aa, amino acid; E, enzyme; F, formycin (8-aza-9-deazaadenosine); Xxx-$tRNA^{Yyy}$-C-C-N, tRNA specific for amino acid Yyy, fitted with terminal nucleoside N, and aminoacylated with Xxx- at the 2′- or 3′-position as determined by the terminal nucleoside N and the corresponding enzyme; [E^{Xxx}·Yyy-AMP], binary complex of the aminoacyl-tRNA synthetase specific for Xxx and the aminoacyl adenylate of the amino acid Yyy; Tris, tris(hydroxymethyl)aminomethane; uv, ultraviolet; DEAE, diethylaminoethyl; PEI, poly(ethylenimine).