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Histone-DNA interactions within chromatin. Isolation of histones from DNA-histone adducts induced in nuclei by UV light

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ABSTRACT

We have developed a method by which to isolate histones that have been crosslinked to DNA following irradiation of calf thymus nuclei by UV light. The procedure involves separation of protein-DNA adducts from uncrosslinked protein by Sepharose 4B chromatography under dissociating conditions. Histones which are crosslinked to DNA are released by chemical hydrolysis of the DNA and identified by SDS gel electrophoresis. The results indicate that, of the histones, H1 and H3 become crosslinked to the DNA most readily under our irradiation conditions.

INTRODUCTION

Many general features of chromatin structure are now well established (reviewed in ref. [1]). The basic structural subunit, termed the nucleosome, consists of two each of the so-called core histones, H2A, H2B, H3 and H4, tightly bound with about 140 base pairs of DNA. An additional histone, H1, is associated more loosely with chromatin.

Information concerning specific interaction sites between histones and DNA is lacking. Irradiation by ultraviolet light induces protein-nucleic acid crosslinks (2) and is especially appropriate for deriving structural information because it is a zero-length crosslinker. Thus it can "freeze" certain sites of noncovalent histone-DNA interaction in chromatin into covalent histone-DNA adducts which can more easily be characterized. Some examples in which photochemical crosslinking of protein and nucleic acid has been used to derive structural information involve ribosomal proteins and ribosomal DNA (3), gene 5 protein and M13 single-stranded DNA (4), and RNase A and its pyrimidine nucleotide inhibitor, pUp (5).

Here we describe a procedure for isolating histones which have been photochemically crosslinked to DNA in calf thymus nuclei. We find that of the histones, H1 and H3 become crosslinked to DNA most readily.

METHODS

Isolation of Nuclei

Calf thymus was frozen within 15 min of slaughter. 20 g were homogenized in 750 ml saline-phosphate-EDTA buffer (45 mM NaCl, 20 mM sodium phosphate, 24 mM EDTA, pH 8.0) in a Waring blender. Phenylmethylsulfonyl fluoride (PMSF, 100 mM in dimethylsulfoxide) was added to 1 mM final concentration during homogenization to inhibit proteolysis, and a small volume of n-octanol was added to prevent foaming. The homogenate was filtered successively through several layers of Chix wipers and Miracloth (both produced by Chicopee Mills, Inc.), and the nuclei were pelleted at 2000 g for 10 min. The nuclei were washed twice in the same buffer before use.

Photolysis

Ultraviolet irradiation was conducted in a cold room using a 450-watt Hanovia medium-pressure mercury lamp (Ace Glass, Vineland, N. J.) surrounded by a Vycor filter sleeve and a quartz water jacket. The energy output of the lamp between 230 nm and 290 nm is about 27 watts, approximately 70% of which passes the Vycor filter. The Vycor is essentially opaque below 200 nm. The entire lamp and cooling jacket assembly was lowered into an outer Kimax reaction vessel which had inlet and outlet ports at the top. 50 ml of a concentrated suspension of nuclei in saline-phosphate-EDTA buffer were stirred in the reaction vessel for about 45 min under a stream of nitrogen. Additional thoroughly purged buffer was then added anaerobically until the vessel was filled (about 1.1-1.2 liters). The nuclei suspension, at an A_{260} of about 2.5 (determined following lysis in 2 M NaCl, 5 M urea), was irradiated for 30 min under nitrogen with stirring.

Isolation of Crosslinked Histone-DNA Adducts

Following irradiation, the nuclei were pelleted, resuspended in saline-phosphate-EDTA (to which PMSF was added to 1 mM), and then lysed by adding solid NaCl to 2 M. In order to reduce viscosity the chromatin was sonicated for 90 seconds at an output control setting of six on a Branson W-350 Sonifier. Thirty second bursts of sonication were alternated with cooling periods all in ice. The sonicated chromatin was dialyzed into 1 M $NaClO_4$, 5 M urea, 0.2% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM Tris, 0.02% Na azide, pH 7.5. After adding PMSF to 1 mM and β-mercaptoethanol to 5% (v/v), the sample was centrifuged at 16,000 g for 15 min at 25°C, and the supernatant was loaded on a 2.3 cm x 95 cm column of Sepharose CL-4B (Pharmacia) equilibrated with the salt-urea-SDS buffer. This column was operated at 30°C to insure continued solubility of the SDS protein complexes. The average flow rate was about

30 ml/hr. Fractions which contained DNA-histone adducts and no free histone were pooled, dialyzed against 0.1% SDS, 1 mM EDTA, pH 7, and concentrated using a Millipore Immersible Separator. Following addition of PMSF and β-mercaptoethanol, the sample was centrifuged at 16,000 g as before. In order to further purify the sample under different denaturing conditions prior to characterization, the supernatant was loaded on a second Sepharose CL-4B column of identical dimensions equilibrated with a buffer of 2% SDS, 1 mM EDTA, 10 mM Tris, 0.02% Na azide, pH 8.0, at room temperature. Desired fractions were again pooled, dialyzed against 0.1% SDS, 1 mM EDTA, and concentrated.

Hydrolysis of DNA

DNA was hydrolyzed by adding 2 volumes of formic acid (98%) and solid diphenylamine to 2% (w/v) (6), and the solution was incubated at 37°C for 18-20 hours. The hydrolysis mixture was extracted with ether to remove diphenylamine and most of the formic acid, dialyzed against distilled water and lyophilized.

Polyacrylamide Gel Electrophoresis

Histones were analyzed on 0.8 mm thick SDS slab gels using the recipe of Laemmli (7), except that the resolving gel contained 18% acrylamide and 0.24% bis-acrylamide, and the stacking gel was 6% acrylamide and 0.03% bis-acrylamide. Staining with Coomassie Blue and destaining with acetic acid were as previously described (8).

RESULTS

Nuclei were isolated from calf thymus, suspended in saline-phosphate-EDTA buffer, and irradiated with ultraviolet light. Figure 1 shows the histone pattern obtained when nuclei were lysed in 2% SDS and loaded directly on an SDS gel, before and after 30 min irradiation. It is evident that certain histone-histone crosslinked species are generated such as the H2A-H2B dimer (9), an H2B-H4 dimer and an H2A-H2B-H4 trimer (manuscript in preparation). Figure 1 also demonstrates that most of the histone remains free of DNA following this UV exposure since the total intensity of histone bands is comparable before and after irradiation. Although histones can be quantitatively driven into very high molecular weight material (presumably histone-DNA adducts) given sufficient irradiation, we have kept well below such UV doses in order to minimize overall photolytic damage.

Histone-DNA adducts were separated from bulk histone by Sepharose 4B molecular exclusion chromatography. We first utilized a Sepharose column

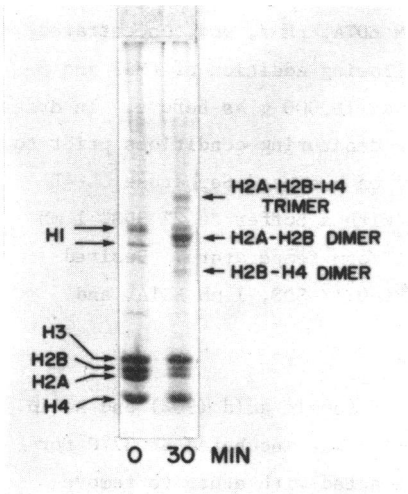

Figure 1. Histone patterns on an SDS gel before and after irradiation (30 min). Equivalent volumes of nuclei were withdrawn from the irradiation vessel, pelleted, and lysed in 2% SDS, 0.125 M Tris (pH 6.8), 10% glycerol, 5% β-mercaptoethanol. The gel samples were then heated at 100°C for a couple of minutes and loaded on an 18% SDS gel.

equilibrated with a buffer containing 1 M $NaClO_4$, 5 M urea, and 0.2% SDS. A salt-urea combination was chosen because it is known to dissociate histones from DNA. However, we found that the common combination of 2 M NaCl and 5 M urea was not completely effective. Therefore $NaClO_4$, a chaotrope, was used instead of NaCl, and SDS was added to the limit of its solubility in this buffer. $NaClO_4$ is known to dissociate histones from chromatin at lower concentrations than NaCl (10). The efficiency with which free histone was removed from DNA by the salt-urea-SDS Sepharose column is demonstrated in Figure 2. The column fractions were assayed by absorbance (Figure 2A) and by SDS gel electrophoretic analysis (Figure 2B). It is evident that most of the DNA and DNA-protein adduct fractions (i.e., fractions 14-26) are well-resolved from those fractions which contain free histone (i.e., fractions 30-36). We have no explanation for the biphasic nature of the elution profile for the irradiated sample (Figure 2A) between fractions 10 and 30. However, this phenomenon is reproducibly observed. Fractions containing DNA and no detectable uncrosslinked histone were pooled and concentrated, and then run over a second Sepharose column containing 2% SDS as the dissociating agent in order to remove any remaining uncrosslinked histone. Faint bands on SDS gels loaded with fractions from this second column indicated that some free histone was eluted in later fractions (results not shown). However, this free histone may be the result of partial reversal of the histone-DNA crosslinks (see Discussion).

The DNA fractions from the 2% SDS column were pooled and concentrated, and the histones crosslinked to DNA were liberated by chemical hydrolysis of

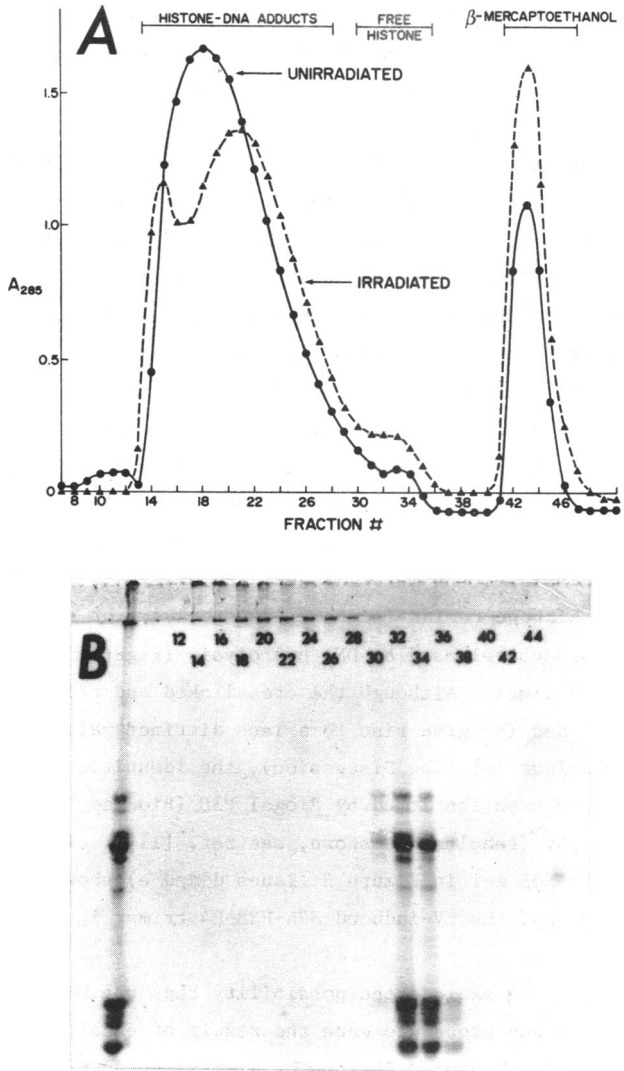

Figure 2. Spectrophotometric elution profile (A) and electrophoretic analysis (B) of fractions from the $NaClO_4$-urea-SDS Sepharose column. In (A) the absorbance of column fractions (approx. 8 ml each) at 285 nm was recorded. The elution profiles for both irradiated (----) and unirradiated samples (——) are shown. In (B) 7 µl from alternate fractions were loaded directly on an 18% SDS gel. Only the results from an irradiated sample are shown. Note the high molecular weight histone-DNA adducts at the top of the gel in the early fractions. In the far left lane was loaded 3 µl of the irradiated sample taken before fractionation on the Sepharose column.

the DNA and analyzed by SDS gel electrophoresis. The results are shown in Figure 3A along with those from an unirradiated sample of nuclei treated by the same Sepharose column procedure. The unirradiated sample is free of histone both before and after hydrolysis of the DNA (Figure 3, lanes a and c). In contrast, DNA hydrolysis gives rise to definite histone bands for the irradiated sample (lanes b and d). Although there are some faint histone bands present before hydrolysis (lane b), these bands are much more intense following hydrolysis (lane d) for an equivalent amount of material loaded on the gel. The small amount of material present before hydrolysis (lane b) probably represents reversal of histone-DNA crosslinks (see Discussion). The faint Coomassie Blue staining at the top of lane (a) for the unirradiated sample presumably is due to free DNA, since it is removed by the hydrolysis procedure (lane c). Notice however that the crosslinked sample gives rise to more intense staining at the top of the gel before hydrolysis than does the uncrosslinked sample (compare lanes b and a). Moreover, following hydrolysis of the crosslinked sample, the material near the origin is largely replaced by protein in the histone region (compare lanes b and d or e). Most prominent among the histones released by DNA hydrolysis (Figure 3, lane d) are H3, H1, and the H2A-H2B dimer. Although the crosslinked and released histones shown in lanes (d) and (e) give rise to a less distinct pattern than the marker histones in lane (f) (see Discussion), the identities of the crosslinked histones have been confirmed by Biogel P30 (Bio-Rad Laboratories) column chromatography (results not shown, see ref. [11]). In addition to these histones, the SDS gel in Figure 3 (lanes d and e) shows faint bands corresponding to H4 and the UV-induced H2A-H2B-H4 trimer in the crosslinked sample.

It was necessary to exclude the possibility that the DNA-associated histones purified by our procedure were the result of enhanced binding of histones to UV-induced denatured regions of a small proportion of the chromatin DNA (12-14). Therefore chromatin in a phosphate buffer containing 0.4 mM Na^+ was heat-denatured at 95°C for 15 min (12), and stripped of histone by the Sepharose column procedure in a parallel manner with an irradiated nuclei sample. The results following the step of chemical hydrolysis of the column-purified DNA are shown in Figure 3B. It is apparent that even in heat-denatured chromatin, no histones remain attached to DNA following the dissociating conditions employed in our Sepharose columns. The pattern of histones obtained from the irradiated nuclei of Figure 3B (lane y) is similar to that shown in Figure 3A (lanes d and e). However, there is no triplet of

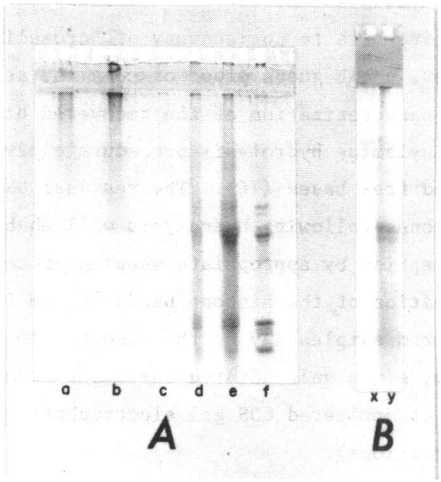

Figure 3-A. Release of histones by chemical hydrolysis of purified histone-DNA adducts. All samples were dissolved in SDS loading buffer following lyophilization, heated, and loaded on an 18% SDS gel (see legend to Figure 1). Equivalent amounts of unirradiated column-purified DNA before and after hydrolysis were loaded in lanes (a) and (c), respectively. Equivalent amounts of irradiated sample before and after hydrolysis of DNA were loaded in lanes (b) and (d), respectively. Lane (e) is the same sample as lane (d), except that four times as much was loaded on the gel. Lane (f) is a sample of free histones from UV-irradiated nuclei (from Figure 1).

B. Comparison of histones released following chemical hydrolysis of column-purified DNA from heat-denatured chromatin (x) and a UV-irradiated sample (y). Samples were prepared as described above.

bands in the H1 region of lane (y) in Figure 3 due to the absence of the H2A-H2B dimer that was present in lanes (d) and (e). We think that this particular sample of nuclei received a lower dose of UV light than previous samples. We currently are investigating the effect of irradiation dose on the histone-DNA crosslinking patterns.

DISCUSSION

Strniste and Rall have provided evidence that covalent chromosomal protein-DNA adducts are formed by ultraviolet irradiation (15). However, they did not identify the proteins involved, and the possibility of strengthened non-covalent binding of protein to UV-denatured DNA was not excluded. We have developed a procedure to identify which histones are crosslinked to DNA in order to investigate histone-DNA binding sites in chromatin. UV-induced histone-DNA adducts are freed of non-covalently bound protein by column chromatography in the presence of salt, urea, and SDS and the histones are

then released by chemical hydrolysis of the DNA. Unirradiated nuclei and heat denatured chromatin give rise to no recovery of "crosslinked" histones by this procedure. However, unambiguous proof of covalent attachment will come only after detailed characterization of the recovered histones.

The formic acid/diphenylamine hydrolysis procedure cleaves at purines to yield pyrimidine tracts and free bases (16). The residual base or pyrimidine tract attached to the histones following hydrolysis will enable ready identification of the attached peptide by appropriate mapping procedures. The trailing and loss of definition of the histone bands on the SDS gel following hydrolysis of the crosslinked samples may be the result primarily of residual attached pyrimidine tracts, since unirradiated chromatin treated with formic acid/diphenylamine yields an unaltered SDS gel electrophoretic histone pattern (unpublished observations).

Several aspects of our results suggest that a certain amount of reversal of crosslinking occurs with time under our conditions. Similar low level reversal appears to occur in several types of histone-histone crosslinked products as well (unpublished observations). The first suggestion of reversal of the histone-DNA crosslinks described here comes from the appearance of free histone in the low molecular weight region of the chromatogram upon rechromatography of the histone-DNA adduct material on the second (2% SDS) Sepharose column (not shown, see Results). In contrast, no additional histones are released from DNA of unirradiated chromatin during this step. Second, the histones which are released are not a random sample but are the same as those recovered in the final hydrolyzed preparations. Third, the existence of continued reversal is further suggested by the appearance of these same free histones at low levels in SDS gels after the purified high molecular weight adduct fractions from the 2% SDS Sepharose step (see above) have been pooled and concentrated (Figure 3A, lane b). Finally, the conspicuous deficiency of H4 in the products of both reversal and hydrolysis argues strongly that reversal rather than mere release of non-covalently attached histone is occurring. In contrast to H4, H3 is a major component among the products of both reversal and hydrolysis. Since the arginine-rich histones (H3 and H4) are known to be dissociated from both native and denatured chromatin coordinately by salt-urea extraction regimes (13), we feel that all free histone which appears in the later stages of the procedure represents reversed crosslinks. This consideration suggests that in future work only the initial salt-urea-SDS chromatography step need be applied to purify histone-DNA adducts. Indeed, a single-step procedure would have the added

advantage of allowing less time for reversal to occur. We are presently studying the nature of reversal by monitoring its occurrence as a function of time under various conditions. This information will give some indication of the chemical nature of the reversible crosslink as well as of the extent of possible chemical heterogeneity among the histone-DNA linkages. It will also provide a guide to the best procedures to use in isolating adduct material for chemical characterization.

We do not know why certain histones are crosslinked more easily to DNA than others. Studies on UV-induced crosslinking of amino acids to nucleic acid bases have demonstrated a range in reactivity in the formation of different heteroadducts (17, 18). In addition, the orientation of the amino acid with respect to the base must be important in the generation of crosslinks. Therefore, since we cannot assume that all sites of intimate association will give rise to crosslinks, the relative rates and extents of crosslinkage of histones to DNA probably do not bear a straightforward relationship to the extents of histone interaction with the DNA. The reverse correlation, however, that a site of crosslinkage represents a site of intimate association, presumably does hold, and characterization of the adducts should give detailed information on the nature of many histone-DNA binding sites within chromatin.

We hope to utilize the UV-induced crosslinking of histones and DNA in future studies of chromatin structure, especially with regard to the investigation of histone-DNA contact sites which may be broken or formed upon induction of conformational transitions in chromatin. Such conformational transition studies may provide insight into the mode of interaction of chromosomal proteins and DNA during the replication and expression of the genetic material.

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