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Photochemical cross-linking of histones to DNA in nucleosomes

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ABSTRACT

Ultraviolet (UV)-induced cross-linking was utilized in order to identify histone-DNA interacting regions in the chromatin repeating unit. Fractionated mononucleosomes which contained 185 base pairs of DNA and a full complement of the histones, including histone H1, were irradiated with light of λ>290nm in the presence of a photosensitizer. Equimolar amounts of histones H2A and H2B were found, by two independent labeling experiments, to be cross-linked to the DNA. Based on previous finding that the UV irradiation specifically cross-links residues which are in close proximity, irrespective of the nature of the amino acid side chain or the nucleotide involved, our results indicate that the four core histones are not positioned equivalently with respect to the DNA. This arrangement allows histones H2A and H2B to preferentially cross-link to the DNA.

A water soluble covalent complex of DNA and histones was isolated. This complex was partially resistant to mild nuclease digestion, it exhibited a CD spectrum similar to that of chromatin, and was found to contain histone H1. These results are compatible with a model which suggests that histone H1, though anchored to the linker, is bound to the DNA at additional sites. By doing so it spans the whole length of the nucleosome and clamps together the DNA fold around the histone core.

INTRODUCTION

Evidence which has been accumulated in the last few years has shown that chromatin is composed of repeating units called nucleosomes,[1-3] each containing a defined length of DNA,[4,5] and complexed with histones. Digestion of rat liver nuclei by micrococcal nuclease has shown that the DNA content of the nucleosome is approximately 200 base pairs (bp).[5,6] Further nuclease digestion results in a structure with a DNA content of 140 bp designated "nucleosome core".[6-11] The histone part of the nucleosome core is an octamer of histones H2A, H2B, H3 and H4.[12] It has been suggested that the above histones serve as a core around which the DNA is folded to form nucleosomes and chromatin.[1,13,14] Histone H1, which is also associated with the nucleosome, has been suggested to be bound to the DNA piece which is not present in the "nucleosome core" and serves as a linker between nucleosomes.[10,11,15]

A variety of interactions occur among the components of the nucleosome. These interactions have an important role in forming and stabilizing this nucleoprotein complex. A large body of literature has already dealt with the problem of histone-histone interactions, both in solution and in chromatin.[16] It is apparent that protein-DNA interactions also play an important role in the structure and function of chromatin. Recently it has been shown that histones H3 and H4 are located at the ends of the core DNA.[17] However, the detailed nature of these interactions is yet unknown. In the present study we approach this problem of protein-DNA interactions by using ultraviolet (UV)-induced cross-linking as a probe for identifying histones DNA interacting regions within the nucleosome.

Protein nucleic acid cross-links have been shown by numerous studies to be significant products of UV irradiation of prokaryotic and eukaryotic organisms. Such cross-links occur also in vitro upon irradiation of native nucleoprotein complexes and have been shown to be major products in irradiated chromatin.[18] Several attempts have been made recently to study protein-nucleic acid interactions by the photochemical cross-linking approach.[19] The validity of this approach to the study of protein-nucleic acid interactions, requires that covalent bonds would be formed specifically between interacting regions in the native nucleoprotein structure. The compliance with this requirement has recently been demonstrated by employing a protein-nucleotide complex of known structure: Sperling and Havron[20] photochemically cross-linked the complex of RNase and a pyrimidine nucleotide inhibitor of the enzyme, and identified the amino acid residues which were involved in the cross-linking.[21] It was thus shown that the photochemical cross-linking occurred solely at the enzyme's binding site for the pyrimidine inhibitor. Similarly, it was shown by Yue and Schimmel that the covalent attachment of ATP to Ile-tRNA synthetase occurred to a single peptide.[22]

In this publication we describe experiments in which we have induced covalent cross-links between histones and DNA in factionated rat liver mononucleosomes. The mononucleosomes contained 185 bp of DNA and a full complement of the histones, including histone H1. Thus, the photochemical cross-linking of these mononucleosomes enables the study of histone-DNA interactions both within the "nucleosome core" as well as interactions between histone H1 and DNA on the outside of the "nucleosome core".

EXPERIMENTAL

Preparation of Nucleosomes. Rat liver nuclei were prepared as described

before.[23] The nuclei were washed once with cold 0.2 M sucrose-TKM [50 mM Tris·HCl (pH 7.4) 25 mM KCl, and 5 mM $MgCl_2$] and then suspended in 0.25 M sucrose, 1 mM Tris·HCl (pH 8.0), and 0.2 mM $CaCl_2$. Digestion of the nuclei (46 - OD_{260}/ml) with micrococcal nuclease (Worthington Biochemical Corporation) was accomplished at an enzyme concentration of 110 U/ml for 2 min at 37°C. The digestion was terminated by chilling on ice and adding 0.1 M EDTA (pH 7.0) to a final concentration of 2 mM. The nuclei were centrifuged at 4000 g for 10 min at 0°C. The precipitated nuclei were suspended in 0.2 mM EDTA (pH 7.0) and lysed by forced entry into a Pasteur pipette. The chromatin particles were then layered on 36 ml, 5-10% sucrose gradients containing 1 mM EDTA (pH 7.0), and centrifuged for 13 hr at 4°C at 27000 rpm in Beckman SW 27 rotor. The gradients were monitored for absorbancy by passing the effluent from the bottom of the tube through a turbulene free cell (Molecular Instruments Co.). The fractions corresponding to mononucleosomes were pooled and resedimented on the same gradients. The pooled mononucleosomes were dialyzed exhaustively against 5 mM sodium cacodylate (pH 7.0), 0.2 mM EDTA, and then against 5 mM sodium cacodylate (pH 7.0).

UV-Irradiation of Nucleosomes. Irradiations were carried out in a Wild Universal unit (Wild Heerbrugg, Switzerland) with a 200-W super pressure mercury lamp (HBO 200 W, Osram, West Germany). The solution of nucleosomes ($O.D_{260}$=5.0, in 5 mM cacodylate buffer containing 5% acetone) in a 3-ml spectrophotometric cell, was placed at a distance of 15 cm from the light source in an ice-water-cooled jacket maintained at 25°C. IR radiation and UV radiation of wavelengths shorter than 290 nm were eliminated by introducing into the light path a Pyrex cell of 2 cm path length filled with distilled water. The light intensity, determined as described earlier,[21] was 2.1×10^{-6} einstein min^{-1} cm^{-2}.

Dissociation of Irradiated Nucleosomes. The solution of irradiated mononucleosomes was made 1 mM in EDTA (pH 7.0), 0.1% in SDS, and 1 M in NaCl. It was extracted twice with an equal volume of water-saturated phenol, or alternatively, with chloroform:isoamylalcohol (24:1). The upper phase was dialyzed extensively against water at 4°C, freeze dried, and redissolved in a small volume of water. This "DNA fraction" was stored at -20°C until use. The interphase precipitate of the chloroform extraction was taken up in water dialyzed extensively against water at 4°C and freeze dried. This "protein fraction" was refrigerated until use. Non-irradiated mononucleosomes were dissociated as above into their protein and DNA fractions. These were used as controls in subsequent experiments. The protein and DNA contents of each

fraction were determined by the methods of Lowry et al.,[24] and Burton,[25] respectively.

3'-End Group Labeling of Protein Fractions. (a) DNase I digestion. The protein fraction of irradiated mononucleosomes, as well as a control sample of that of non-irradiated mononucleosomes, was dissolved in 0.3 ml of 40 mM potassium cacodylate, 4 mM Tris·HCl, 10 mM $MgCl_2$ (pH 6.8),and dialyzed against two changes of the same buffer. Dithiothreitol (DTT) was added to a final concentration of 1 mM followed by DNase I (10 U; Serva, 2000 U/mg). The mixture was incubated at 37°C for 10 min and then for 3 min in a boiling-water bath. Solid NaCl was added to a final concentration of 1 M followed by 50 µg of whole histones mixture and the solution was extracted with an equal volume of chloroform:isomylalcohol (24:1). The interphase protein layer was taken up in 1 M NaCl and reextracted with chloroform:isoamylalcohol. The organic and aqueous layers were removed and the protein precipitate was dried under a stream of nitrogen. (b) Terminal Transferase Reaction.[26,27] The dried protein precipitate was dissolved in 0.1 ml of 100 mM potassium cacodylate, 10 mM Tris·HCl, 10 mM $MgCl_2$,and 0.1 mM DTT (pH 6.8). [α-^{32}P]CTP (50 pmol, Radiochemical Centre, Amersham, specific activity 20 Ci/mol) was added followed by 3'-terminal deoxynucleotidyl transferase (4 U, a gift from Dr. H. Kössel) and the mixture was incubated for 5 hr at 37°C. Unreacted CTP and oligonucleotides were removed by passing the reaction mixture through a column (1 ml) of the acetate form of Dowex-1 and elution with 0.1 M acetic acid. The breakthrough fraction was freeze-dried and then analyzed on a 13% SDS-polyacrylamide gel.

Iodination of Protein Fractions. The protein fractions of irradiated and non-irradiated nucleosomes were iodinated with radioactive iodine.[28] The reaction mixture (50 µl) contained histones and cross-linked products (1 mg/ml), ca. 0.1 mM) in 10 mM Tris·HCl (pH 7.5), 1% SDS, 5 mM carrier NaI, 0.5 µCi ^{125}I (Radiochemical Centre Amersham), and 0.8 mM chloroamine-T. The reaction was allowed to proceed at room temperature for 15 min and then stopped by the addition of sodium metabisulfite to a final concentration of 2.4 mM. The reaction mixture was subjected to polyacrylamide gel electrophoresis. The band containing the marker dye was discarded and the remining gel was autoradiographed (wet in a Saran wrap) for 5-10 min.

Gel Electrophoresis. Proteins were analyzed by electrophoresis in SDS-13% polyacrylamide slab gels, according to Laemmli.[29] DNA fragments were analyzed by electrophoresis in 1.7% polyacrylamide, 0.5% agarose composite slab gels,[30] calibrated with Hind II+III fragments of 776 SV40 DNA (kindly given to us by Dr. T. Vogel).

CsCl Density Gradient Equilibrium Centrifugation. Samples in 5 mM cacodylate buffer (pH 7.0) were mixed with CsCl solutions to obtain a final average density 1.638 g cm^{-3} and centrifuged in a Beckman SW 50 rotor at 35000 rpm for 72 hr , at 20°C.

Circular Dichroism Measurements. CD spectra were recorded by a Cary Model 60 circular dichrometer using 1cm path length cells at 20°C. DNA concentrations were determined by the method of Burton.[25]

Immuno-Electron Microscopy Using Anti-H1 Antibodies. Lyophilized anti-H1 antibodies[23] were kindly given to us by Dr. M. Bustin. Samples for immuno-electron microscopy were prepared as described before,[31] by incubating the DNA fractions with anti-H1 antibodies (1/10 dilution) on grids which had been previously incubated with BSA. The grids were stained with 1% uranyl acetate in double distilled water. Excess liquid was removed and the grids were air dried. A Philips EM300 electron microscope operating at 80KV was used.

RESULTS

Preparation of Nucleosomes. Nucleosomes were prepared by in situ micrococcal nuclease digestion of rat liver nuclei and fractionation on sucrose gradient.[32,33] The fractionated nucleosomes were composed of multiples of 185 bp of DNA, as determined by gel electrophoresis using Hind II+III fragments of 776 SV40 DNA as markers. The nucleosomes contained a full complement of the histones including histone H1. The protein to DNA ratio in the mononucleosomes was 1.3 and did not vary significantly along the gradient.

Photochemical Cross-Linking of Nucleosomes. Fractionated mononucleosomes were irradiated with light of λ>290 nm in the presence of acetone, which serves as a photosensitizer, as described in the Experimental section. It should be pointed out that 5% acetone does not affect the cleavage pattern of nuclei by micrococcal nuclease, nor does it affect the shape of mononucleosomes as observed by the electron microscope (Sperling and Arbit, unpublished data). The disappearance of histones from their normal electrophoretic migration positions on SDS-polyacrylamide gels, and the appearance of new bands were followed as a function of the irradiation time. Figures 1 and 2 show that histone H1 was the first to disappear. Histones H2A and H2B disappeared at a slower rate, whereas histones H3 and H4 disappeared at the slowest rate. New bands appeared upon irradiation. A prominent new band coincided with the faster moving band of the histone H1 doublet, and had an apparent molecular weight of 29000, as calibrated with histone H3 multimeters.[34] This band is designated as the "dimer" band. Iodination experiments, which will be described below, indicate

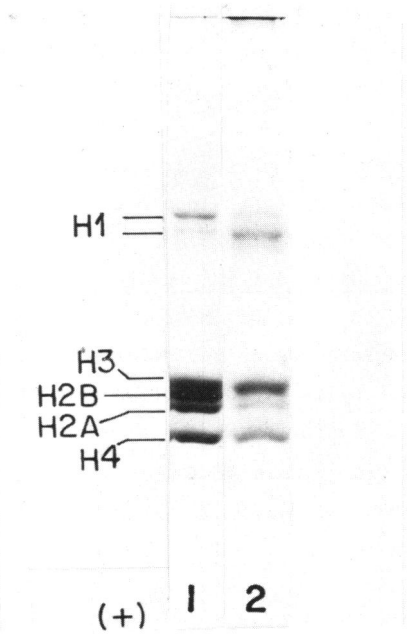

Figure 1. SDS-polyacrylamide gels of the protein component of mononucleosomes (1), and of mononucleosomes irradiated for 5 min

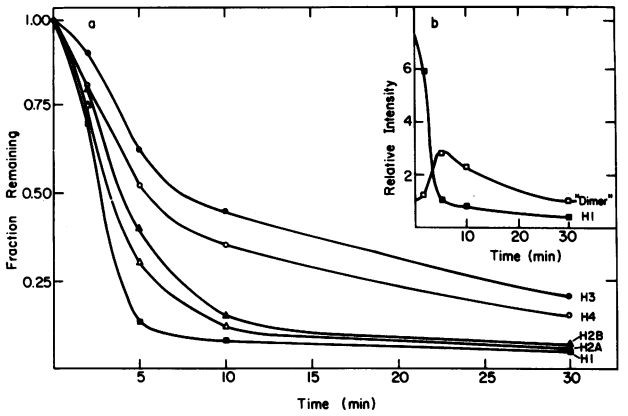

Figure 2. Time course of disappearance of histone bands upon irradiation. Mononucleosomes were irradiated as described in the Experimental section. Aliquots were withdrawn at various time intervals, and analyzed on SDS-polyacrylamide gels. The plots were constructed from densitometer traces of the gels. (a) Histone H1 (slower moving band) (■); histone H2A (Δ); histone H2B (▲); histone H4 (O); histone H3 (●). (b) A comparison between the relative intensities of the slower moving band (■),and the faster moving band (□) of histone H1 doublet. The latter band contains the cross-linked photoproduct designated "dimer".

that the "dimer" band represents a newly formed photoproduct and not enrichment of the faster moving band of histone H1. Figure 2b shows that with further irradiation the intensity of the "dimer" band decreases with the concomitant appearance of high molecular weight products concentrated at the top of the gels. We have thus concentrated our efforts on identifying the initial histone-DNA cross-linking products by studying mononucleosomes which were irradiated for 5 min. During this irradiation period the "dimer" band was maximal and there was full recovery of the disappearing histones on the gel (including the material appearing at the top of the gel, which probably contained histone-DNA cross-links).

Characterization of Photochemically Cross-Linked Mononucleosomes. Mononucleosomes composed of 185 bp of DNA and a full complement of the histones including histone H1 were irradiated for 5 min and resedimented on a sucrose gradient. Figure 3 shows that this irradiated mononucleosomes fraction sedimented as a symmetrical peak. For comparison, non-irradiated mononucleosomes were also resedimented. Each of the above peak fractions was pooled and analyzed for its protein and DNA content. The length of the DNA of the irradiated and non-irradiated mononucleosomes was similar and they were both composed of 185 bp. The SDS-polyacrylamide gel shown in Figure 3 (insert 'b') indicates that the resedimented irradiated mononucleosomes had the same characteristic gel pattern shown by the irradiated mononucleosomes before fractionation (Figure 1 panel 2). This indicates that the new photoproducts were still associated with the mononucleosomes. All further studies were done with the refractionated mononucleosomes.

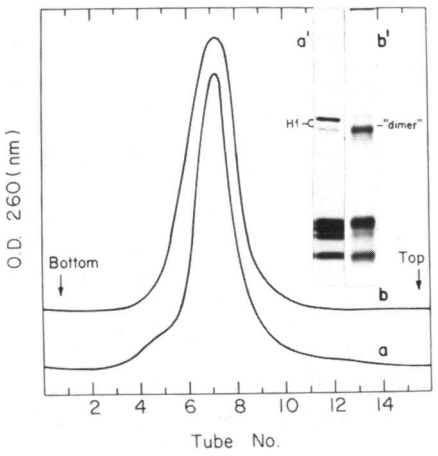

Figure 3. Resedimentation on sucrose density gradients of non-irradiated (a) and of 5-min irradiated (b) mononucleosomes. The peak fractions of each gradient were pooled, dialyzed against 0.2 mM EDTA, and analyzed on SDS-polyacrylamide gels: (a') protein pattern of peak a; (b') protein pattern of peak b.

To further characterize the histone-DNA cross-linking products, the irradiated mononucleosomes were dissociated into DNA and protein fractions. The DNA fraction was obtained by phenol extraction, whereas the protein fraction was obtained by chloroform: isoamyl alcohol extraction. Cross-linked products were looked for in both fractions using the respective fractions of non-irradiated mononucleosomes as controls.

Characterization of the DNA Fraction of Irradiated Mononucleosomes. The DNA components of irradiated and non-irradiated mononucleosomes were both shown by polyacrylamide gel electrophoresis to have the mobility of 185 bp DNA. However, CsCl density gradient centrifugation of irradiated and non-irradiated mononucleosomes, and of their respective DNA fraction, indicated that the DNA peak of the irradiated fraction had a lower maximal density (see Table I). Table I also indicates that the DNA fraction of irradiated mononucleosomes contained about 15% protein (by weight) whereas the non-irradiated

Table I. Properties of DNA Fractions Extracted from Irradiated and Non-Irradiated Mononucleosomes.

	DNA extracted from irradiated mononucleosomes	DNA extracted from non-irradiated mononucleosomes
DNA length (bp)	185	185
Protein content (%)	15	0
Micrococcal nuclease digestion	-	+
θ_{275} (deg cm^2 dmol^{-1})	3500	8000
ρ_{max} (g cm^{-3})[a]	1.70	1.72

[a] Determined by CsCl density gradient centrifugation with micrococcus lysodeicticus DNA (ρ=1.73 g cm^{-3}) as marker.

DNA fraction was free of protein. These results indicate that covalent protein-DNA cross-links occurred due to irradiation and some of the proteins which were cross-linked to the DNA were extracted into the DNA fraction.

Figure 4 shows the gel analysis of a mild micrococcal nuclease digestion of the irradiated and non-irradiated DNA fractions. It can be seen that under the digestion conditions we used, the non-irradiated DNA fraction was completely digested whereas the irradiated DNA fraction was somewhat resistant to the nuclease digestion. These results indicate that the photochemically cross-linked DNA fraction of the irradiated mononucleosomes differs from the

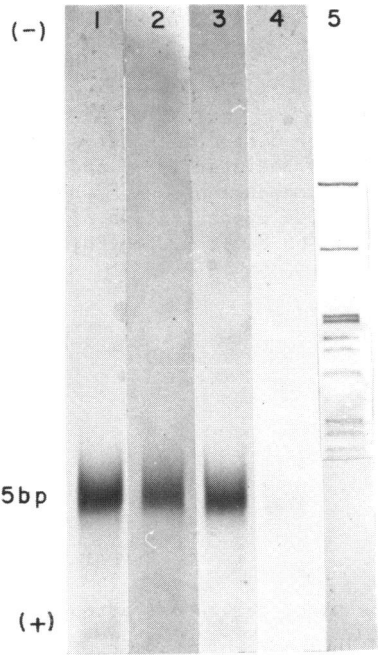

Figure 4. Mild micrococcal nuclease digestion of the DNA fractions of irradiated and non-irradiated mononucleosomes. Samples of the DNA fractions were adjusted to 0.5 mg/ml in 1 mM Tris·HCl (pH 9.0), 10 mM $CaCl_2$, and treated with micrococcal nuclease (6 U/ml) for 30 min at 37°C. The reaction was terminated by cooling on ice and addition of EDTA to a final concentration of 10 mM. Aliquots were electrophoresed on agarose-polyacrylamide composite gel. (1) DNA fraction of irradiated mononucleosomes; (2) DNA fraction of irradiated mononucleosomes digested with micrococcal nuclease; (3) DNA fraction of non-irradiated mononucleosomes; (4) DNA fraction of non-irradiated mononucleosomes digested with micrococcal nuclease; (5) Hind II+III fragments of SV40 DNA.[58]

non-irradiated DNA fraction in its susceptibility to nuclease digestion.

The circular dichroism spectra of the DNA fraction of irradiated and non-irradiated mononucleosomes are shown in Figure 5. The DNA fraction of non-irradiated mononucleosomes displayed the expected spectrum of naked DNA with θ_{275}=8000 deg cm^2 $dmol^{-1}$. However, the DNA fraction of irradiated mononucleosomes had a spectrum similar to that of chromatin[35] with a maximal ellipticity of 3500 deg cm^2 $dmol^{-1}$ at 275 nm. This observation suggests that the DNA fraction of irradiated mononucleosomes retained some aspects of the DNA configuration of intact mononucleosomes.

In order to identify the protein, or proteins, which became cross-linked to DNA, we extensively digested the DNA fractions with micrococcal nuclease and analyzed them by SDS-polyacrylamide gel electrophoresis. Figure 6 shows that whereas no protein band appeared in the DNA fraction of non-irradiated mononucleosomes, either digested with micrococcal nuclease or not, a new band (doublet) appeared in the digestion pattern of the DNA fraction of irradiated mononucleosomes. This band migrated on the gel as a 60000-dalton protein and is designated P-band. It should be noted that the P-band represents only a fraction of the proteins which were cross-linked to the DNA, since a major fraction of proteins, presumably cross-linked to DNA, is still present at the

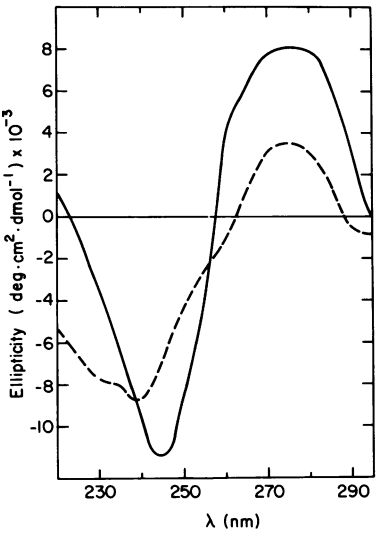

Figure 5. Circular dichroism spectra of the DNA fractions of irradiated (---) and non-irradiated (——) mononucleosomes. The spectra were recorded at DNA concentration of 50 µg/ml in 1 mM Tris·HCl (pH 8.0), 0.2 mM EDTA, and 50 mM NaCl.

top of the gel. An electron micrograph of the DNA fraction extracted from irradiated mononucleosomes is shown in Figure 7b. Part of this fraction has a flat circular appearance with a diameter of about 90Å, and in many of the particles a central hole, 40-50Å in diameter, is observed. For comparison, electron micrographs of mononucleosomes (Figure 7a) and of the DNA fraction of non-irradiated mononucleosomes (Figure 7d) are shown. Incubation of the DNA fraction of irradiated mononucleosomes with specific antibodies elicited against histone H1 resulted, as Figure 7c indicates, in the formation of spheres with increased diameter up to 250Å. Incubation of the non-irradiated DNA fraction with anti-H1 serum resulted in no change in the appearance of the DNA fraction under the electron microscope. We have previously shown[31,33] that specific interaction of histone antibodies with nucleosomes results in a substantial increase in their diameter, as observed by the electron microscope, and therefore can serve as an indicator for specific interactions. Our results thus indicate that histone H1 is present in the cross-linked DNA fraction whereas it is absent from the non-irradiated fraction. It is not clear at this stage whether and how the P-band is related to histone H1. It is possible that the P-band is a UV-induced cross-linked product of histone H1. However, since non-histone chromosomal proteins are included in our nucleosomes the possibility that the P-band is related to one of them should also be considered. These points are currently being investigated.

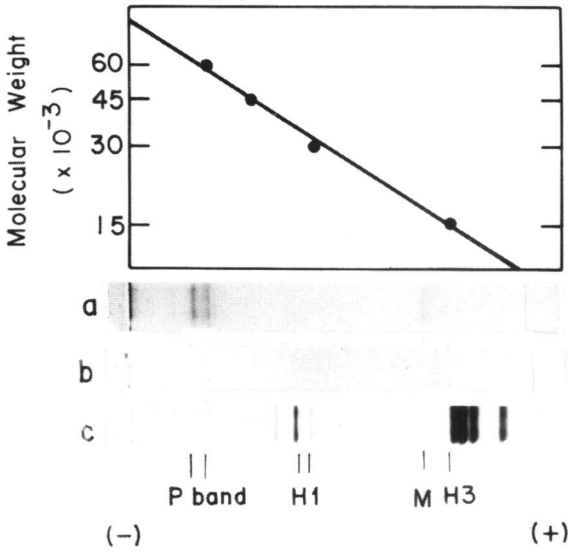

Figure 6. Analysis of proteins present in the DNA fraction of irradiated mononucleosomes. DNA fractions at 1 mg/ml in 10 mM sodium borate (pH 8.5), 10 mM $CaCl_2$ were treated with micrococcal nuclease (100 U/ml), for 30 min at 37°C, and analyzed on SDS-polyacrylamide gel. (a) Digestion products of the DNA fraction of irradiated mononucleosomes; (b) Digested DNA fraction of non-irradiated mononucleosomes; (c) Proteins extracted from rat liver mononucleosomes. The gel was calibrated with the monomer, dimer, trimer and tetramer of histone $H3^{34}$ as shown by the semi-log plot. M - denotes the migration position of micrococcal nuclease.

Characterization of the Photoproducts in the Protein Fraction of Irradiated Mononucleosomes. The protein fraction of irradiated mononucleosomes displayed the same gel electrophoretic pattern as that of the undissociated irradiated mononucleosomes. A prominent band with a mobility of a 29000-

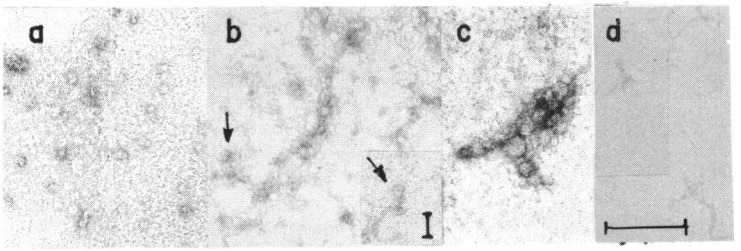

Figure 7. Electron micrograph of the DNA fraction of irradiated mononucleosomes reacted with antiserum to histone H1. (a) Rat liver mononucleosomes; (b) DNA fraction of irradiated mononucleosomes, insert (1) x2 magnified image. The arrows point at circular particles; (c) DNA fraction of irradiated mononucleosomes reacted on the grid with antiserum to histone H1; (d) DNA fraction of non-irradiated mononucleosomes. Bar indicates 1000Å.

dalton protein, the "dimer" band, appeared on the gel, as well as a high molecular weight band which appeared at the top of the gel (see Figure 1). It should be noted that only small amounts of histones H2A and H2B can be seen on that gel. Determinations of the protein and DNA contents of this fraction have shown that it contained about 10% (by weight) of DNA, whereas no DNA was found in the protein fraction of the non-irradiated mononucleosomes. We therefore conclude that the protein fraction contained cross-linked DNA.

In order to identify the histones which became covalently linked to DNA we carried out the following labeling experiment. The protein fraction of cross-linked mononucleosomes was digested with DNase I to yield histones which were cross-linked to short oligodeoxynucleotides with free 3'-OH groups. These were labeled with ^{32}P by the addition of [α-^{32}P] CTP using 3'-terminal deoxynucleotidyl transferase, under reaction conditions designed to add one residue of CTP to a short primer.[26,27] The labeled proteins were separated and analysed by SDS-polyacrylamide gel electrophoresis using a mixture of the five histones as carriers (see Experimental section).The densitometer traces of the autoradiographed dried gel and of the staining pattern of the five histones mixture, which were added to the labeled histones as carriers,are shown in Figure 8. Most of the radioactivity appears as a doublet peak which comigrates with histones H2A and H2B. A minor radioactive band corresponds to the "dimer" band which comigrates with H2A·H2B dimer[34] and with the faster moving band of histone H1. A similar labeling experiment conducted after mild digestion with DNase I revealed the presence of a labeled high molecular weight band at the

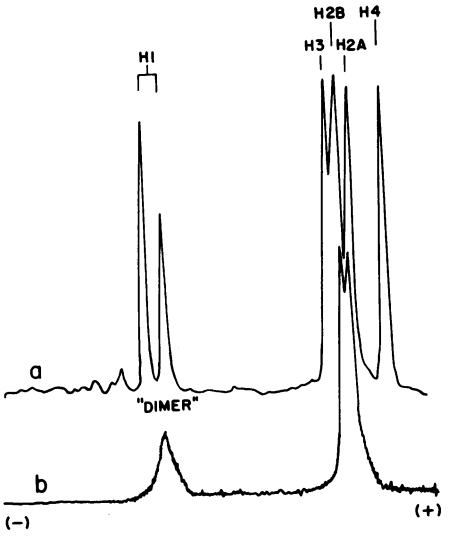

Figure 8. 3'-terminal labeling of DNA fragments cross-linked with histones. The protein fraction of irradiated mononucleosomes was digested with DNase I, treated with 3'-terminal deoxynucleotydil transferase in the presence of [α-^{32}P] CTP, and analyzed on SDS-polyacrylamide gel using total histones mixture as carriers. (a) Densitometer trace of the stained gel; (b) The densitometer trace of the autoradiographed stained gel.

top of the gel. This includes, presumably, precursors of the CTP-labeled H2A, H2B, and "dimer" bands. It should be noted that in a control experiment, employing the protein fraction of non-irradiated mononucleosomes, no radioactivity was incorporated into the histones by the trasferase reaction. These results show that histones H2A and H2B were covalently linked to DNA by the UV irradiation. Furthermore, they indicate the existence of a protein fraction which contained DNA and comigrated with H2A·H2B dimer.

In order to identify the proteins cross-linked to DNA in the "dimer" band we iodinated the protein fraction with ^{125}I (see Experimental section) and analysed it by SDS-polyacrylamide gel electrophoresis, alongside the non-irradiated protein fraction. The autoradiographed gel is presented in Figure 9 which indicates that the four histones,H3, H2B, H2A and H4,were iodinated to a considerable extent; whereas histone H1,which has only one tyrosine,was poorly iodinated. Comparison of the iodinated products of the irradiated and non-irradiated protein fractions further supports the formation of a new "dimer" product due to the irradiation, as there is no comparable iodinated "dimer" band in the non-irradiated fraction. The "dimer" band was extracted from the gel, digested with micrococcal nuclease, and analysed by SDS-polyacrylamide gel electrophoresis. The results, presented in Figure 10, show that the digestion of the "dimer" band by the enzyme yielded histones H2A and H2B. Therefore, we conclude that the dimer band contained histones H2A and H2B bound to one another through a piece of DNA. We do not know yet the length of the DNA stretch that connects histones H2A and H2B in the "dimer" band, nor do we know the length of the whole DNA bound initially to the two histones. It is possible that due to irradiation-induced chain breakage[36] a piece of DNA shorter than 185 bp was cross-linked. The "dimer" band, most probably, also

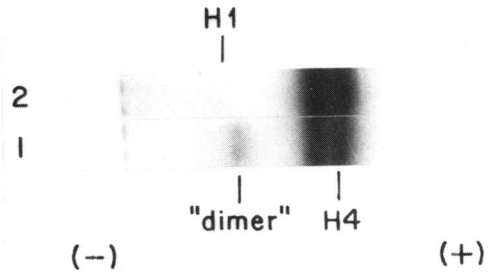

Figure 9. Autoradiograms of electrophoretic gels of iodinated protein fractions. (1) Irradiated, and (2) non-irradiated mononucleosomes.

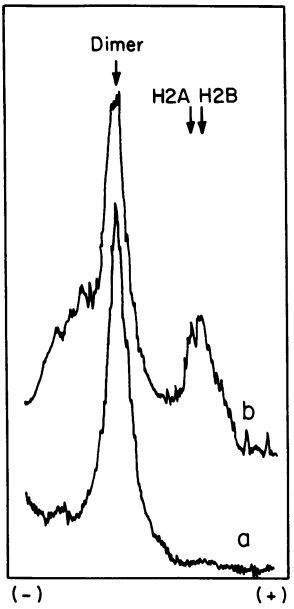

Figure 10. Micrococcal nuclease digestion of the "dimer" band. The iodinated "dimer" band (see Fig. 9 gel 1) was cut out of the remaining gel, crushed and extracted with water (3x300µl). Half of the extract was brought to 10 mM in sodium borate (pH 8.5), 10 mM in $CaCl_2$, and treated with 200 U of micrococcal nuclease for 1 hr at 37°C. The solution was concentrated and analyzed on SDS-polyacrylamide gel. The plots were constructed from the densitometer traces of the autoradiograms of the gels. (a) Undigested "dimer"; (b) micrococcal nuclease digested "dimer".

contains some H2A·H2B protein-protein dimer which has been previously reported by Martinson et al.[37] These points are currently being investigated.

DISCUSSION

The irradiation of nucleosomes with UV light induced the formation of histone-DNA cross-links. In addition, histone-histone cross-links[37,38] also occurred as a result of the UV irradiation. In this study we concentrated our efforts on identifying histones cross-linked to the DNA in the nucleosome.

The validity of conclusions, deduced from photochemical cross-linking experiments, regarding protein-nucleic acid contacts relies on: (a) the ability of both purines and pyrimidines to form UV-induced covalent adducts with a major number of amino acids, and (b) on the formation of covalent bonds only between neighbouring residues in the native nucleoprotein structure. Concerning the first condition, it has been reported that a large variety of amino acids are capable of forming covalent addition products with the pyrimidine moieties of DNA.[39] The involvement of purines in photochemical cross-linking to proteins has been demonstrated in the complex of ATP with histone H4,[38] in the complex of ATP with Ile-tRNA synthetase,[22] and in the photoaffinity labeling of membrane proteins with cGMP.[40] The second condition has already been verified by showing that the UV-induced cross-linking of an RNase-

pyrimidine nucleotide inhibitor exclusively involved amino acid residues at the binding site of the enzyme,[20,21] and by showing that the photochemical cross-linking of ATP to Ile-tRNA synthetase involved a single peptide.[22] These studies have demonstrated that a necessary requirement for the occurrence of a photochemical cross-link is the proximity of the residues in the native nucleoprotein structure. The photochemical cross-linking thus occurs between neighbouring residues - irrespective of the nature of the amino acid side chains or the heterocyclic bases involved.

Upon UV irradiation of fractionated mononucleosomes histone H1 was the first to disappear from its usual migration position on SDS-polyacrylamide gels. Histones H2A and H2B disappeared at a slower rate, whereas histones H3 and H4 disappeared at the slowest rate. These results indicate that the first proteins involved in the UV-induced cross-linking were histones H1 and histones H2A and H2B. The possibility that the disappearance of these histones was due to their UV-induced cleavage can be excluded since full recovery of proteins was obtained under the reported irradiation conditions. Furthermore, the UV irradiation induced the formation of photoproducts which could be detected by gel electrophoresis. The most prominent photoproducts of a 5-min irradiation appeared as a band corresponding to a protein of 29000 dalton, which we designate the "dimer" band, and a band at the top of the gel. In order to identify the UV-induced histone-DNA cross-links we have fractionated the irradiated mononucleosomes into a DNA and a protein fraction and looked for the cross-linked products in both fractions.

The DNA fraction of irradiated mononucleosomes contained 15% protein whereas the non-irradiated fraction did not contain any. The irradiated DNA fraction was more resistent to micrococcal nuclease digestion than the respective non-irradiated fraction, and it also had a CD spectrum similar to that of chromatin.[35] These two latter observations suggest that a DNA fraction from which the majority of the histones had been removed, retained some aspects of the DNA conformation in intact nucleosomes, most probably due to cross-linking to proteins. In order to identify the cross-linked proteins in this fraction we digested it extensively with micrococcal nuclease and obtained a band (P-band) which migrated as a 60000-dalton protein on electrophoretic gels, as well as a high molecular weight protein-DNA product which appeared at the top of the gel. Immuno-electron microscopy of this fraction with anti-H1 serum showed that it contained histone H1, whereas the non-irradiated fraction contained neither histone H1 nor the P-band protein. It is not clear yet how the P-band is related to histone H1 and this point is currently being investigated.

It has been suggested that histone H1 is located on the outside of the nucleosome,[14] and that it is bound at the DNA linker.[10,11,41] Our results are consistent with the notion that histone H1 spans the whole length of the nucleosome and binds to the DNA at additional sites. By doing so it clamps the folds of the DNA once it has been coiled around the nucleosome core. The photochemical cross-linking of H1 to DNA freezes this fold. Therefore, removal of the core histones after cross-linking still leaves the structure partially intact. This suggestion is substantiated by the following observations: (a) binding studies of ATP to histones (J. Sperling, M. Bratu and R. Sperling, unpublished data) show that whereas the core histones have 3-5 specific ATP binding sites per histone molecule, histone H1 binds 12 ATP molecules; (b) immuno-electron microscopy of nucleosomes with anti-H1 serum (D. Goldblatt, M. Bustin and R. Sperling, unpublished data) also indicates that histone H1 spans the whole length of the nucleosome; and (c) it has been shown that histone H1 binds preferentially to supercoiled DNA.[42,43]

The identification of the histones cross-linked to DNA in the protein fraction was achieved by two independent labeling techniques. The first procedure was designed to label short oligonucleotides which remained covalently bound to histones after treatment with DNase I. This experiment revealed that histones H2A and H2B, in nearly equal amounts, and the "dimer" band contained covalently bound DNA. In the second experiment the "dimer" band was iodinated with ^{125}I and subjected to nuclease digestion which yielded histones H2A and H2B. We therefore conclude that in the protein fraction only histones H2A and H2B, in nearly equal amounts, were cross-linked to the DNA. In addition, the "dimer" band contained histones H2A and H2B bound to one another through a short piece of DNA. The UV-induced disappearance of histones H3 and H4, as well as part of histones H2A and H2B, from their normal migration position on polyacrylamide gels, can be accounted for by histone-histone cross-linking with aggregate formation.[37,38] In addition, it should be noticed that histone H4, though being capable of forming covalent adducts with ATP,[38] has not been found to cross-link to DNA under the short irradiation period used in our studies.

It has been suggested that the nucleosome is composed of an octamer of histones[1,12] wrapped by the DNA from the outside,[1,13,14] with about 1 3/4 turns of DNA per nucleosome.[44] It has further been proposed that the histone periodicities determine the fold of the DNA in chromatin.[45] The fold of the DNA in chromatin is presumably responsible for its digestion pattern by DNase I which produces a set of DNA fragments differing in size by 10 bp.[46] A quanti-

tative analysis of the occurrence of these cutting points[47] indicates that high frequency cuts occur at points which are 30-40 or 80-90 bases apart. The low frequency cutting points in the DNA have been suggested to be protected by neighbouring histones. Our photochemical cross-linking studies indicate that within the nucleosome core histones H2A and H2B are in close proximity to the DNA. It is possible, therefore that part of the most infrequent cutting sites of DNase I are at the contact points of the DNA with histones H2A and H2B.

We have pointed out above that the photochemical cross-linking occurs only between neighbouring residues in the native structure - irrespective of their type. This means that a necessary condition for the occurrence of cross-links is the proximity of the residues involved. Reconstitution[48-52] and salt effect[53,54] studies have indicated that histones H3 and H4 have a crucial role in the folding of the DNA, and that they are tightly bound to the DNA. Thus, the absence of cross-links between DNA and histone H3 and H4 may seem surprising. Our results, however, showing that histones H2A and H2B cross-link preferentially to the DNA, are not contradictory to these findings. Although histone H3 and H4 have a crucial role in folding the DNA into nucleosome-like structures, it is possible that after the DNA has been folded into its nucleosome conformation, binding sites for H2A and H2B are created in which H2A and H2B are in close proximity to the DNA. Thus, a possible explanation of the preferential cross-linking of histone H2A and H2B to the DNA is that they are in closer proximity to the DNA than histones H3 and H4.

On the other hand, though proximity is a necessary requirement for the occurrence of cross-linking, additional factors may have to be considered in order to explain the preferential cross-linking of H2A and H2B. Such a factor is the steric requirement for an appropriate orientation of the residues at the contact points prior to their cross-linking. It is difficult to assess the importance of this steric factor without the knowledge of the detailed chemical mechanism of the cross-linking reaction. However, by analogy to studies with model compounds,[55] in which the chemical structure of photo-adducts between nucleic acid constituents and a variety of ligands (e.g. alcohols or amines) has been determined, it can be assumed that the potential points of attachment on the nucleic acid constituents are at the C-8 of purines, the 5,6-double bond of pyrimidines, and the C-5 methyl group of thymine. It has also been shown that structural features of nucleic acids control the reactivity of the various moieties in these reactions.[56] For example, the reactivity of pyrimidines in dinucleoside monophosphates and trinucleoside diphosphates has been shown to be related to their involvement in intramolecular

stacking interactions and to be favoured by unstacking. Thus, the preferential cross-linking of H2A and H2B indicates that the conformation of the DNA in the region where these histones are bound favours their cross-linking. This may be explained by assuming more unstacking of the DNA bases at the binding regions of H2A and H2B relative to those of H3 and H4, or on the basis of disruption of interbase hydrogen bonds and thereby increasing "breathing"[57] at these particular regions. In any event our results show, that within the nucleosome core the four core histones are not equivalently positioned with respect to the DNA. This histones-DNA arrangement leads to the preferential cross-linking of histones H2A and H2B to the DNA.

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