Volume 16 Number 10 1988 Nucleic Acids Research

Protein–DNA crosslinking in reconstituted nucleohistone, nuclei and whole cells by picosecond UV laser irradiation

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Received December 11, 1987; Revised and Accepted April 14, 1988

ABSTRACT

A picosecond UV laser was used to cross-link proteins to DNA in nuclei, whole cells and reconstituted nucleohistone. Irradiation of the nucleohistone resulted in crosslinking 15-20% of bound histones to DNA in a very short time (one or several picosecond pulses), the efficiency of crosslinking to single stranded DNA being higher than to double stranded DNA. All histones as well as high mobility group 1 proteins were identified in the covalently linked protein-DNA complexes upon irradiation of isolated nuclei and whole cells. A method is suggested for isolation of crosslinked material from cells and nuclei in amounts sufficient for further analysis. Experiments with reconstituted nucleohistones showed that upon irradiation at a constant dose the efficiency of crosslinking depended on the intensity of the light, thus suggesting a two-quantum process is involved in the reaction.

INTRODUCTION

Protein-nucleic acids complexes are a crucial point in understanding the events involved in gene expression and its regulation. A direct and powerful approach in studying these complexes is protein-DNA crosslinking. A widely used method is the photochemical crosslinking by ultraviolet irradiation,first achieved with light from a low pressure Hg lamp(1-6). In these studies,however,the prolonged irradiation required to obtain reasonable level of crosslinking created conditions for redistribution of proteins and artifactual crosslinking of UV damaged molecules. These drawbacks can be overcome using UV laser which, in a single pulse or in a very short time of irradiation, is capable of yielding a sufficient amount of crosslinked nucleic acid-protein complexes for biological studies(7,8). On the other hand, the efficiency of crosslinking proteins to RNA (per photon absorbed) was reported to be 20-100

times higher upon irradiation with high intensity source (nanosecond or picosecond UV laser) than with low intensity one (UV lamp, see ref.9,10). Moreover, transition from conventional to laser light sources was reported to result in formation of additional crosslinks which are unable to arise at all via lower excited states(9).

The aim of this work was to investigate the generation of protein-DNA crosslinks in both model system (reconstituted histone-DNA complexes) and in isolated nuclei and whole cells by using a picosecond UV laser. Under our conditions of irradiation, 15-20% of histones in the reconstituted nucleohistone were covalently linked to DNA,the reaction being more efficient with single stranded than with double stranded DNA. The increase of the intensity of irradiation in the range 50-1000 megawatts.cm^{-2} at a constant dose resulted in 20-30 times increase in the yield of the covalent links. Irradiation of isolated nuclei and whole cells resulted in analysable number of crosslinks between different chromosomal proteins and DNA. A method is suggested for separation of covalently crosslinked complexes from free DNA and proteins.

MATERIALS AND METHODS

Reconstitution of ^{14}C-labeled histones with DNA.

Radioactively labeled histones were isolated from Ehrlich ascites tumor (EAT) cells. The cells were inoculated intraperitoneally into Agnes Blum white mice. On day 7th after transplantation the ascites fluid was collected, centrifuged at low speed and the pelleted cells were suspended in Eagle·s MEM containing 10% calf serum, heparin(0.5 units/ml) and ^{14}C-protein hydrolysate(final concentration 3μCi/ml). The suspension was incubated at 37°C for 36 hours under gentle shaking.

Nuclei from EAT cells were prepared as described elsewhere (11) Histones were extracted from nuclei with 0.2M H_2SO_4 and precipitated with acetone at -20°C .The specific radioactivity was 400 cpm/ug total histone.

Hen erythrocyte DNA was isolated by pronase treatment of nuclei and subsequent chromatography on hydroxyapatite as

described previously (12). Single stranded M13 phage DNA was prepared according to the protocol in (13).

Reconstitution experiments were carried out at histone:DNA ratio (w/w) 1:2.5. Histones and DNA were mixed in 3M NaCl, 50mM Tris-HCl, pH 7.5, 0.1mM phenylmethylsulfonylfluoride (PMSF), followed by sequential dialysis against decreasing concentrations of NaCl and finally against 2mM Tris-HCl, pH 7.5, 0.25mM EDTA, 5mM NaCl. After centrifugation to remove the aggregated material (5 min, 10 000 x g) the reconstituted nucleoprotein was loaded on 5-25% linear sucrose gradient, containing 5mM NaCl, 5mM Tris-HCl, pH 7.5, 0.25mM EDTA, and run for 8 hours at 37 000 rpm (SW rotor 41) to separate the unbound histones. Fractions containing the nucleoprotein complex were collected and dialysed against 2mM Tris-HCl,pH 7.5, 3mM NaCl, 0.25mM EDTA.

Separation of covalently crosslinked histone-DNA complexes.

The covalently bound ^{14}C-histone-DNA complexes in the reconstituted nucleohistone were separated from non-crosslinked histones by centrifugation through preformed CsCl gradient (four layers with ρ = 1.76, 1.57, 1.54 and 1.32) in SW 41 Beckman rotor at 15°C for 40 hours at 35 000 rpm. Each fraction of the gradient was adjusted to 5xSSC and loaded on nitrocellulose filters. The filters were washed with 0.2xSSC, dried and the radioactivity counted in a liquid scintillation counter.

The covalently bound histone-DNA complexes from irradiated nuclei or whole cells were isolated by digestion of nuclei with micrococcal nuclease (5 units/A_{260} unit, 15 min,37°C) in the presence of 10mM Tris-HCl, pH 7.5, 1mM $CaCl_2$, and after adding 0.12M phosphate buffer, pH 6.8, 2M NaCl, 5M urea to stop the reaction, the material was loaded on a hydroxyapatite column. The column was washed with 5 volumes of the same buffer, then with 0.12M phosphate buffer only. Free DNA and the crosslinked nucleoprotein complex were eluted with 0.48M phosphate buffer, pH 6.8 and run on a preformed CsCl gradient as described for the reconstituted histone-DNA complexes. Selected fractions from the gradient were pooled, dialysed against 5xSSC and aliquots of them loaded on nitrocellulose filters for identification of proteins by dot-immunoassay.

DNA was analyzed by electrophoresis in 1% agarose gels. The integrity of proteins was checked on polyacrylamide-SDS gels(14)

Dot-immunoassay for identification of proteins crosslinked to DNA.

Crosslinked material (about 0.5μg DNA) was loaded on nitrocellulose filters, washed twice with PBS containing 0.05% Triton X-100 (PBS-T) to remove the unbound antigen, followed by two washings with PBS only. The filters were successively incubated with BSA (1% in PBS-T) for 1 hour at 37°C and overnight at 4°C with the antibodies in PBS-T, containing 1% BSA. The filters were extensively washed (3 times with PBS, 0.4% triton X-100 and 3 times with PBS only) and incubated 4 hours at 37°C with peroxidase-conjugated goat antirabbit IgG (Sigma) in PBS containing 1% BSA. After extensive washings, first with PBS-T and then with PBS, the dots were developed using 0.3% 4-chloro-1-naphthol,0.03% H_2O_2 in 50mM Tris-HCl,pH 7.5, 150mM NaCl.

Preparation of antibodies.

Antibodies to H1 and H5 were prepared as described elsewhere(15). Antibodies to H2A, H2B and H4 were raised by immunizing rabbits with histone-RNA complexes (3:1,w/w). Briefly, 200μg of the complex in complete adjuvant was injected at multiple intradermal sites. The same procedure was repeated on the 7th and 14th day with incomplete adjuvant. An intravenous injection of the same quantity of antigen was made a week later. Sera were taken on 7th, 14th and 21st day after an intravenous booster. The antibodies were immunospecifically purified from IgG by affinity chromatography with antigen conjugated to CNBr-Sepharose (15).

Monoclonal antibody to H3 was a kind gift from Dr E. Jockers-Wretou, Institute Pasteur, Athens, Greece. Antisera against high mobility group protein 1 (HMG1) were the gift of Dr L. Marekov. The specificity of all antibodies was tested by enzyme-linked immunosorbent assay.

Lasers and irradiation techniques.

As a source of powerful UV radiation, a home-made passively mode-locked picosecond Nd:YAG laser was used (16). The wavelength conversion was performed by quadrupling the main

frequency by means of angle-matched KDP crystals. The parameters of the laser radiation at 266 nm were as follows: pulse duration 30ps (in a gaussian pulse shape assumption), pulse energy 4mJ, diameter of the beam 0.5cm, repetition rate 0.5Hz. The intensity of the radiation was controlled by focussing and defocussing fused silica lenses. The energy of the radiation was measured with pyroelectrical detectors calibrated with Model Rj 7200 energy meter (Laser precision corp.). The electric signal from the pyrodetectors was transmitted to an Apple II microcomputer for further processing and handling.

Irradiation of air saturated solutions was carried out in rectangular fused silica cuvettes. During the irradiation the solution was continuously stirred. The optical density of solutions was usually kept in the range of $1 < A_{260} < 5$ (optically thick samples). Optically thin samples ($0.1 < A_{260} < 0.5$) were used in some cases only.

RESULTS

Reconstituted ^{14}C-histone-DNA complexes were chosen as a model system for studying laser-induced covalent crosslinks between DNA and proteins because histones bind to DNA at low salt and dissociate completely upon raising the ionic strength up to 3M monovalent ions. This allows the efficiency of crosslinking to be fairly well controlled by centrifugation of irradiated material in CsCl:banding of free DNA, the crosslinked histone-DNA complex and non-crosslinked histones can be determined by following the distribution of optical density and radioactivity through the gradient. The sedimentation profiles built on radioactivity measurements (^{14}C-histones) and optical readings (DNA) of both crosslinked material and the control non-irradiated samples are compared in Fig.1. In the latter case all of the radioactivity was found on the top of the gradient: no detectable amounts of histones remained associated with DNA during centrifugation. The radioactivity of the irradiated samples, however, banded in two peaks: one on the top of the gradient (peak I, free ^{14}C-histone molecules) and one close to the bottom (peak II). The distribution of the optical density along the gradient showed one peak with non-irradiated samples.

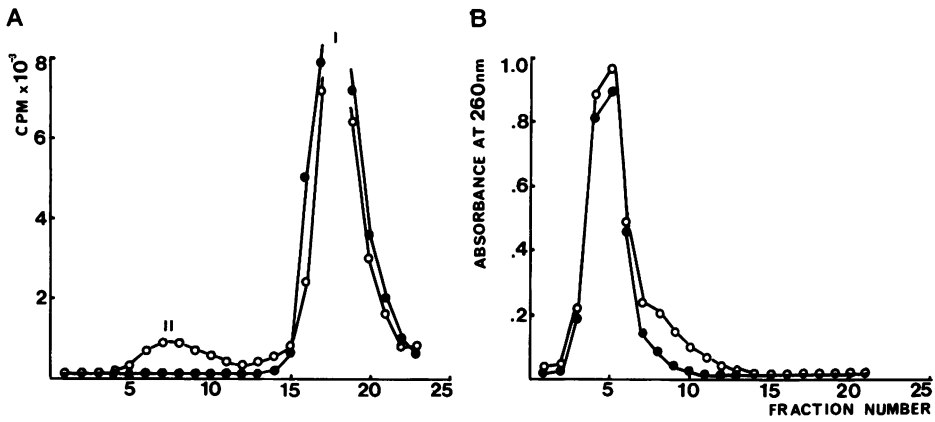

Fig.1. CsCl sedimentation profiles of irradiated (o——o) and non-irradiated control(●——●) ^{14}C-histone-DNA complexes: A, radioactivity measurement; B, optical density reading at 260 nm.

The same peak was observed with the irradiated nucleohistone, part of it forming a clear-cut shoulder on the light side of the peak. The position of the shoulder coincided with peak II in the radioactivity profile. Their appearance we attribute to the covalently linked ^{14}C-histone-DNA complexes.

The efficiency of the crosslinking was investigated relative to the amount of absorbed photons, the intensity of the UV light and the secondary structure of DNA. The increase of the dose absorbed resulted in an increase of the relative amount of crosslinked complexes (Fig.2). The initial slope of the curve showing crosslinking of histones to single stranded DNA is about an order of magnitude greater than that of double stranded DNA, the saturation being achieved at a relative crosslinking of about 24% and 16%, respectively (relative crosslinking is defined as the amount of crosslinked histones relative to their total amount). Thus, the efficiency of crosslinking with single stranded DNA at the level of saturation is 1.5 times higher than with double stranded DNA and, what is more important, only 1-2 absorbed photons per nucleotide are needed to obtain a significant crosslinking (10-15%) with single stranded DNA versus a value of 0.7-1% for double stranded DNA.

The efficiency of crosslinking depended on the intensity of the incident UV light. As Fig.3 shows, at a constant dose of

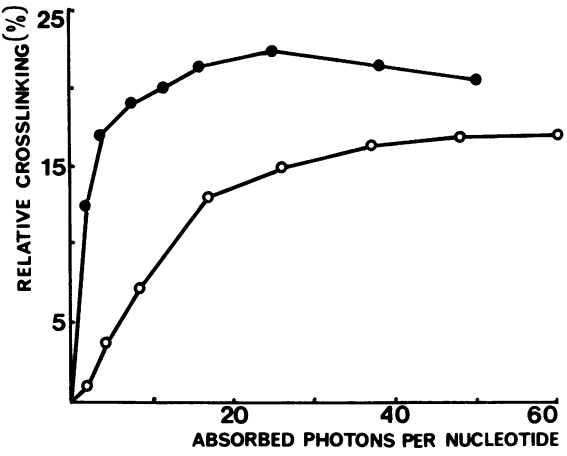

Fig.2. Relative crosslinking vs absorbed dose for complexes of histones with single stranded M13 DNA (●——●) and with double stranded DNA (o——o).

irradiation the number of crosslinks increased with the intensity of radiation. This finding suggests that a biphotonic process is involved in the reaction (17). Again, as in the dose-dependent experiments, the initial slope of the curve, reflecting the crosslinking of histones with single stranded DNA is about 10 times higher than that for double stranded DNA; the plateau levels were achieved with 50 MW.cm^{-2} and 600 MW.cm^{-2}, respectively.

Besides the generation of covalent bonds between proteins and DNA, the high power laser pulse might damage their structure also. Electrophoretic analysis of irradiated histone complement showed neither the appearance of new bands, nor loss of protein into the background of the gel under the conditions of irradiation used (not shown).

Protein-protein crosslinks were not observed. Damage to DNA was assessed by electrophoresis in agarose gels. Fig. 4 shows that the molecular mass of DNA isolated from irradiated nuclei is comparable with this of the control non-irradiated DNA; however, prior treatment of irradiated DNA with single strand specific S1 nuclease revealed single-strand breaks in DNA.

A mechanism supposed to be involved in the crosslinking is the generation of free radicals (18,19). Experiments with

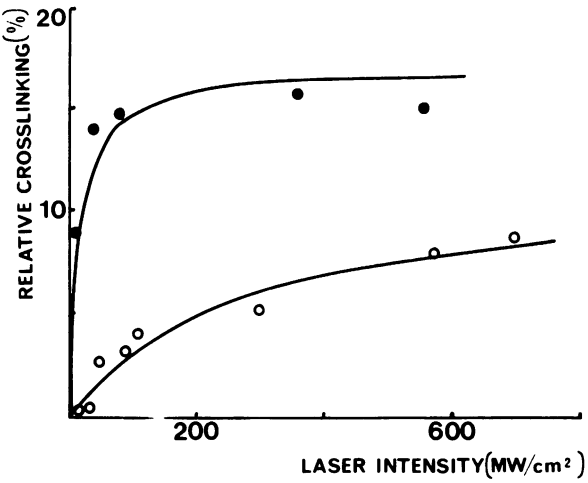

Fig. 3. Relative crosslinking vs laser intensity for complexes of histones with single stranded M13 DNA irradiated at 3 absorbed photons per nucleotide (●——●) and with double stranded DNA at 10 abs. photons/nucleotide (o——o). The experimental data were computer fitted to dependencies reflecting two-quantum processes (for more details see ref. 17, p.16).

quenching long lived free radicals by using scavengers such as 2-mercaptoethanol have shown reduced efficiency of crosslinking of some proteins to DNA (7,8) and RNA(9). We have found that 5mM dithiothreitol reduces crosslinking of the reconstituted nucleohistone by about 50-60%.

The UV laser irradiation of isolated nuclei and whole cells induced an amount of crosslinkage sufficient for subsequent purification of the complexes and identification of some of the covalently bound proteins by immunochemical reactions. Fig. 5 shows a typical sedimentation profile in CsCl of DNA isolated from irradiated nuclei and passed through hydroxyapatite column (the size of DNA thus obtained is presented in Fig. 4). A well defined shoulder appeared on the light side of the peak of free DNA (see the profile of DNA from non-irradiated control nuclei, which has been processed in the same way). The appearance of the shoulder obviously reflected the banding of covalently linked protein-DNA complexes (note that the position of the shoulder

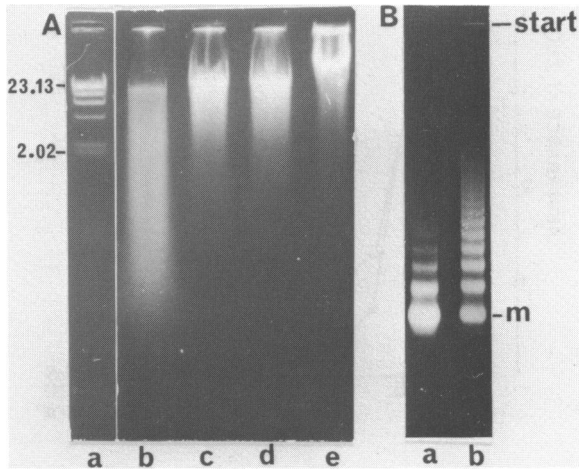

Fig.4. A. Electrophoresis in 1% agarose gels of DNA isolated from control (e) and irradiated (c) hen erythrocyte nuclei by treatment with pronase and extraction with phenol-chloroform. Slots d and b represent S1 nuclease digested DNA (1 enz. unit/ug DNA, 10 min., 37°C) from control and irradiated DNA, respectively. Slot a is Hind III digest of λDNA; the numbers refer to the size of the marker DNA bands in base pairs x 10^{-3}. B.Agarose gel electrphoresis of DNA isolated from irradiated hen erythrocyte nuclei by treatment with micrococcal nuclease and subsequent chromatography on hydroxyapatite (a). Slot b is digest of hen erythrocyte nuclei with micrococcal nuclease, used as a reference; m marks the position of mononucleosome DNA.

coincided with that of peak II of the irradiated reconstituted nucleoprotein). This was proved by following the distribution of histone H5 through the peak region of the gradient using dot-immunoassay (Fig.5 ,inset): binding of anti-H5 antibody was registered only to the material of the shoulder, a maximum reaction being observed with the peak fraction of the shoulder. A second run in CsCl of pooled shoulder fractions showed that the quantity of trapped free DNA in them did not exceed 10-15% (not shown).

The covalently bound proteins were identified immuno-chemically. Using antibodies to core histones, histones H1 and H5 and high mobility group proteins 1 we were able to show that all these proteins were present in the complexes (Fig. 6).

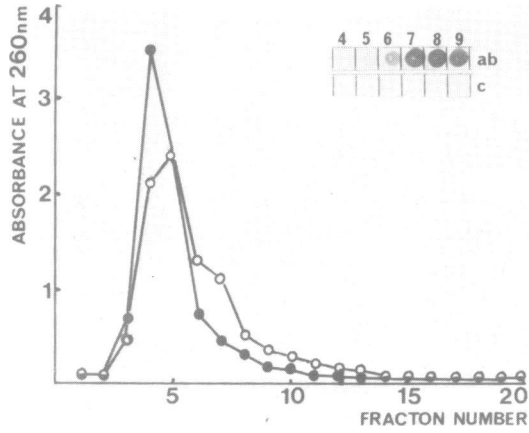

Fig.5. CsCl sedimentation profiles built on the optical density reading at 260 nm of control non-irradiated (●——●) and irradiated (o——o) hen erythrocyte nuclei. Irradiation at 266 nm; E=15 absorbed photons per nucleotide; I=0.8 GW.cm^{-2}. Inset: aliquots from fractions 4-9 containing equal amounts of DNA were analyzed for the presence of histone H5 by immunodot assay with specific antibody (ab); c, control dot with non-immune IgG.

DISCUSSION

The main goal of the present work was to develop an efficient method for covalent crosslinking of proteins to DNA which is fast enough to "freeze" in vivo existing interactions. The data reported by Harrison et al.(7) for crosslinking E. Coli RNA polymerase to DNA using UV laser stimulated us to apply this powerful method for crosslinking proteins to DNA not only in model systems (7,8) but in nuclei and whole cells. To this end

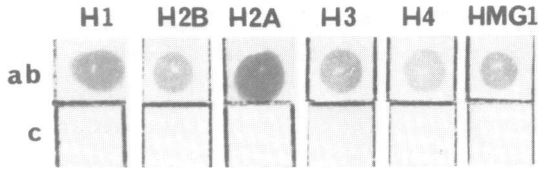

Fig. 6. A dot immunoassay for the presence of histone H1, core histones and high mobility group 1 proteins in crosslinked protein-DNA complexes, obtained upon irradiation of Ehrlich ascites tumor cells with UV laser. Irradiation was performed under the conditions described in Fig. 5; ab, antibodies against the respective proteins; c, control dot with non-immune IgG.

we used picosecond laser technique supplying an amount of photons sufficient to generate protein-DNA adducts in the cell.

The first application of a picosecond UV laser for cross-linking proteins to DNA required knowledge of the parameters of irradiation in order to calibrate the method and to select conditions for inducing crosslinks in vivo. For this reason the experiments were first carried out with a model system: reconstituted nucleohistone. These experiments provided some insights into the photochemistry of the laser-induced covalent bonds between proteins and DNA.

The efficiency of crosslinking histones to single and double stranded DNA showed a non-linear dependence of the intensity of the incident UV light at a constant dose of irradiation. Such a dependence suggests a two-quantum process is involved. This finding is not surprising having in mind that several laser-induced photochemical reactions were qualified as two-quantum processes (17). It was recently shown that crosslinking of some proteins to RNA by nanosecond (9) and picosecond (10) laser is also a two-quantum reaction.

In the pioneering work of Harrison et al.(7) and later in the paper of Hockensmith et al. (8) the covalent crosslinking of proteins to DNA is explained as a monophotonic process. It is difficult to account for the contradiction between these data and our findings as well as those of Budowsky et al. (9) and Arbieva et al.(10). The results of Harrison et al.(7) and especially their interpretation seem not convincing: the experimental points reflecting the dependence of the efficiency of crosslinks on the intensity of the UV light are situated in a way that one cannot accept unequivocally as a linear dependence (see Fig.4, Harrison et al). As for the data of Hockensmith et al.(8) one explanation of the lack of dependence of the crosslinking efficiency on the intensity might be that these authors have worked in the region of saturation of the crosslinking. Indeed, saturation of photocrosslinking proteins to single stranded DNA we observed at a relatively low intensity (about 50 $MW.cm^{-2}$). Having in mind that Hockensmith et al.(8) worked also with single stranded fragments and that the saturation of the high lying levels of thymidine (the excitation

of which we consider as essential for the formation of crosslinks) in the case of nanosecond laser is achieved at about one order of magnitude lower intensity (20) than upon picosecond excitation, such a suggestion seems quite reasonable.

The mechanisms involved in inducing protein-nucleic acid adducts are not known. Harrison et al.(7) assume that a monophotonic process operates in the reaction, which proceeds via intermediates (radicals). The nature of the radicals was not considered, neither a mechanism for their formation was suggested. Hockensmith et al.(8) found that at excitation wavelengths higher than 245 nm photocrosslinking occurred mainly through thymidine. Recently, Budowsky et al.(9) using nanosecond laser irradiation showed that crosslinking of RNA with ribosomal proteins was a biphotonic process. They assumed that crosslinkage proceeds via higher excited states either directly or via short lived intermediates of unknown nature.Indications for generation of cation radicals as a result of laser-induced photolysis of thymidine and DNA, first reported by Oraevsky and Nikogosyan (21), were recently confirmed (16,22). With this in mind and considering the data we have obtained, the possible events occurring upon picosecond laser irradiation might be outlined as follows. Two-quantum excitation of thymidine singlet state results in a very rapid (subpicosecond time scale) generation of thymidine cation radicals (Thd^{+}) and hydroxyl radicals ($OH^{\cdot}$). Thd+ are due to a direct photoionization of thymidine; $OH^{\cdot}$ result from sensitized ionization and dissociation of water molecules (21). We suppose that crosslinks are formed via these two primary radicals, the contribution of Thd+ being at least 40-50% (the amount of crosslinks, found to resist the effect of the scavenger); the remaining 50-60% of the adducts are probably formed via $OH^{\cdot}$ (23) and quenchable derivatives of pyrimidine cation radicals.

Induction of crosslinks directly via highly excited singlet or triplet states seems unlikely because of the very short lifetime of these states (less than 10-12 psec). Provided that crosslinking requires favourable mutual disposition of the two reactants at a moment, such a short time is obviously insufficient: otherwise, the efficiency of the picosecond laser-induced

crosslinking should be less than even that of conventional UV sources. For these reasons we assume that the characteristic time for crosslinking is determined by the lifetime of the transient radical species. Until a detailed radical-mediated mechanism of crosslinking is suggested, this lifetime cannot be precisely determined but if one considers the data of pulse radiolysis studies of primary pyrimidine radicals (24), it does not exceed the microsecond time range.

This paper describes the first application of a powerful picosecond UV laser for generation protein-DNA crosslinks in isolated nuclei and whole cells. Irradiation caused covalent bonds sufficient to isolate the crosslinked material and to identify immunochemically the bound proteins. All histones as well as HMG1 proteins were found covalently linked to DNA. Crosslinking is accomplished in a very short time (single or several picosecond pulses) so that structural rearrangements are unlikely to occur. The efficiency of crosslinking to single stranded DNA is about an order of magnitude higher than to double stranded DNA.

The dose of irradiation as well as the intensity of the UV light we used does not result in a quantum yield for damage to proteins. However, the laser irradiation induced single strand breaks in DNA. Strand scissions in DNA, observed also upon UV lamp irradiation (22) was well characterized by other authors (7,17).

The method suggested for crosslinking proteins to DNA in isolated nuclei and whole cells by using picosecond UV laser is reproducible, generates crosslinks in a very short time and in relatively high amounts. This method, in combination with immunoaffinity chromatography and hybridization techniques might be a valuable tool in studying the localization of specific proteins on DNA, e.g. the distribution of RNA- and DNA polymerases during transcription and replication.

Acknowledgements. We are very grateful to Dr. von Hippel for sending us a preprint of a paper on laser-induced protein-DNA crosslinking. This work was partially supported by Bulgarian Commetee for Science (grant 212).

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