Radiolytic Footprinting. β Rays, γ Photons, and Fast Neutrons Probe DNA–Protein Interactions†

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ABSTRACT: Ionizing radiations induce numerous damages in DNA, especially strand breaks. The hydroxyl radical OH•, produced by the radiolysis of water, is mainly responsible for this effect. The fact that strand breakage occurs at all nucleotides and that bound proteins may locally radioprotect DNA at the binding site lead us to develop a radiolytic footprinting method to study DNA–protein interactions. Three different radiations were used: β rays, γ photons, and fast neutrons. In order to validate this technique, three well-known interaction systems were tested: the *lac* repressor–*lac* operator of *Escherichia coli*, the cyclic AMP receptor protein (CRP) of *E. coli* and its specific site in the *lac* regulation region, and the core nucleosome. Radiolytic footprinting gives results similar to those obtained by more classical probes: DNase I, complexes of orthophenanthroline (OP) and copper, complexes of ethylenediaminetetraacetate ion (EDTA) and iron, and UV light. For the same system (*lac* repressor), irradiation with either γ photons or fast neutrons gives identical results.

The precise determination of the interacting sequence of DNA remains one of the main points in the protein–DNA interaction studies. A score of years ago, Gilbert and Maxam (1973) isolated and sequenced the *lac* operator using a preparative footprinting technique: the total digestion by DNase I of the regions of DNA that are not protected by the *lac* repressor. Some years latter, using the same enzyme, the same biological system, and the sequencing gel electrophoresis technique of Maxam and Gilbert (1977), Galas and Schmitz (1978) realized the first analytical footprint, by partial digestion of the nonprotected DNA. This footprinting method using nucleases has been largely applied to various systems [e.g., to nucleosomes by Lutter (1979) and Prunell (1983)]. Chemical probes, such as DMS (Ogata & Gilbert, 1979; Shanblatt & Revzin, 1987), $[Fe(EDTA)]^{2-}$ [1] (Tullius et al., 1987; Tullius, 1991), or $[Cu(OP)_2]^{2+}$ (Sigman & Spassky, 1989; Sigman & Chen, 1990), have also been developed. A physical probe, UV radiation, was also used in photofootprinting studies (Ogata & Gilbert, 1977; Becker & Wang, 1984).

All these methods give different responses depending on the probe used. DNase I cutting overestimates the length of the DNA region covered by the interacting protein, because of its own size. It also exhibits a relatively high specificity regarding the sequence of bases. Base methylation involves only purine residues, whereas UV photofootprinting deals only with pyrimidine bases. In the case of $[Cu(OP)_2]^{2+}$, which decomposes hydrogen peroxide to oxidize the deoxyribose, the positively charged complex is bound to DNA. The pattern of DNA attack is thus largely modulated by the binding ability of the $[Cu(OP)_2]^{2+}$ complex (Sigman & Chen, 1990). $[Fe(EDTA)]^{2-}$ decomposes H_2O_2 in the presence of ascorbate to generate OH• radicals (Tullius, 1989). The negatively charged complex is not bound to DNA, and the OH• radical can diffuse in the solution. From this property results one of the most precise methods, which provides cutting patterns that are only slightly dependent on the base sequence (Tullius et al., 1987). OH• radicals are also produced by water radiolysis, together with H• and solvated electrons. OH• radicals are considered the most reactive intermediates, mainly responsible for the DNA radiation-induced damages (von Sonntag et al., 1981).

Among the numerous lesions induced by radiolysis in DNA, the strand breaks have been the most extensively studied. The OH• attack of the $C_{4'}$ position of the deoxyribose moiety leads to frank strand breaks. Some other radiolysis-induced damages are revealed by postirradiative treatments, such as heating or exposure to alkali (von Sonntag et al., 1981; Lafleur et al., 1983). A study of the chemical nature of the breaks performed by Henner et al. (1982, 1983) showed that they occur at each nucleotide.

Recently, we have shown that bound proteins radioprotect DNA at the binding site, by blocking of radical attack, and not by scavenging of radicals in the bulk solution (unpublished results). Hayes et al. (1990b) took advantage of this fact in a footprinting study of the λ repressor–operator interaction using γ photons.

The aim of the present work is the validation of the radiolytic footprinting method with other protein–DNA interacting systems, and with other types of radiation. We studied the *lac* repressor–operator of *Escherichia coli*, the CRP and its specific site in the *lac* operon of *E. coli*, and the histone octamer interacting with the 146 bp DNA in the core nucleosome. β rays, γ photons, and fast neutrons were used for irradiation. In all cases, the same radicals are formed in water, but with neutrons, their local concentrations and spatial repartitions

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[1] Abbreviations: CRP, cyclic AMP receptor protein; OP, orthophenanthroline; EDTA, ethylenediaminetetraacetate ion; LET, linear energy transfer.

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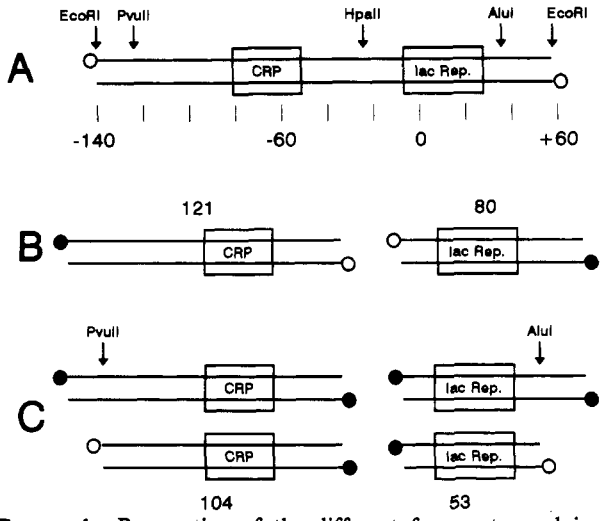


FIGURE 1: Preparation of the different fragments used in the experiments from fragment 203. 5′ ends are marked by open circles, and ^{32}P-labeled 5′ ends are marked by closed circles. (A) Fragment 203 with the restriction sites. Numbering originates at the start of transcription of the *lac* gene. Boxes represent the specific sites of *lac* repressor and CRP protein. (B) Labeling at the *Eco*RI 5′ ends, followed by the *Hpa*II cutting. (C) Cutting by *Hpa*II, followed by the separation of the two fragments. Then they are 5′ labeled and cut by either *Pvu*II or *Alu*I.

are different (Chatterjee & Magee, 1987). Therefore, different results with β rays and γ photons on the one hand and fast neutrons on the another hand might be expected.

MATERIALS AND METHODS

5′-Terminal Labeling of DNA. After alkaline phosphatase (Boehringer) treatment, the 5′-termini were ^{32}P-labeled using T4 polynucleotide kinase (BRL) and [γ-^{32}P]ATP (specific activity >185 TBq; Amersham) (Sambrook et al., 1989).

DNA Fragments. The 203 bp fragment containing the regulation region of the *lac* operon of *E. coli* (Figure 1A) was isolated from *Eco*RI (Biolabs) cutting of a recombinant plasmid derived from pBR322 (Fuller, 1982). The *lac* operator, that is the specific site of *lac* repressor binding, covers the transcription start, and is borne by both fragments 80 and 53. The specific site of the CRP, situated about 70 bp upstream, is borne by fragments 121 and 104.

Fragments 121 and 80 are obtained after *Eco*RI 5′ end labeling (see above) and cutting by *Hpa*II (Boehringer) (Figure 1B). The two fragments were separated on a 7% polyacrylamide gel [acrylamide/bis(acrylamide) ratio of 20/1]. The fragments are recovered from the pieces of gel by incubation overnight at 50 °C in few milliliters of 10 mM Tris, 1 mM EDTA, and 200 mM NaCl, pH 8, buffer. Fragments are purified and concentrated on NACS 37 microcolumns (BRL), ethanol-precipitated, and dialyzed against the binding buffer.

Fragments 104 and 53 are obtained by the following procedure (Figure 1C): *Hpa*II cutting of fragment 203; electrophoretic separation of the two fragments 121 and 80; 5′ end labeling followed by *Pvu*II (BRL) digestion of fragment 121 and *Alu*I (Appligene) digestion of fragment 80. Electrophoretic separation and purification of fragments 104 and 53 are performed as described above.

Proteins. E. coli lac repressor was prepared as already described (Culard et al., 1981) from the overproducing strain BMH 491. Its purity, greater than 98%, was checked by SDS–PAGE electrophoresis. Before use, stock solutions were extensively dialyzed against 0.2 M potassium phosphate, pH 7.25. The final protein concentration was 6.6×10^{-4} M.

E. coli CRP was prepared from an overproducing strain harboring the plasmid pBS crp2 (Cossart & Gicquel-Sanzey, 1982). The purity was of the order of 99%, as judjed by SDS–PAGE electrophoresis. Stock solutions of 2.3×10^{-4} M protein were kept in 10 mM sodium phosphate, 0.4 M NaCl, and 0.2 mM EDTA, pH 6.8.

DNA–lac Repressor Complexes. Complexes of DNA and repressor were prepared by mixing labeled DNA (20 μL at about 10^{-7} M fragment) and repressor in the binding buffer (100 mM potassium phosphate, pH 7.25) to the highest protein/DNA ratio, i.e., 64. Samples with protein/DNA ratios of lower values are obtained by diluting this sample with pure DNA. Since very small volumes of protein are needed, the ionic strength of the final solution does not significantly vary.

DNA–CRP Complexes. They were prepared as DNA–repressor complexes, with some modifications. Since the association constant is lower than for the *lac* repressor, the DNA and protein concentrations were 5-fold increased. This also reduced the relative amount of protein adsorbed on the walls of the vessels. The binding buffer was 1 mM potassium phosphate, 50 mM NaCl, and 10^{-4} M cAMP (Sigma), pH 7.25. The highest protein/DNA ratio was 32. In this case, the concentrations of salts originally present in the protein stock solution were negligible.

Core Nucleosomes and 146 Base Pair DNA Fragments. Core nucleosomes were prepared from chicken blood, according to Libertini and Small (1980). Chromatin was digested using micrococcal nuclease (Pharmacia), leading to 5′-OH termini.

Naked DNA fragments of 146 bp were obtained after proteinase K (Merck) and phenol treatment. Their length measured on sequencing gels after ^{32}P labeling ranged between 144 and 148 bp, with a highly major band at 146 bp.

DNA fragments, either naked or in the core nucleosomes (50-μL samples, both at 0.1 mg/mL DNA), were labeled at the two 5′ termini as described above. The input ^{32}P activity was 6 MBq. The samples were then dialyzed against 50 mM potassium phosphate, pH 7.25, to remove the unreacted labeled ATP. It is during this dialysis that the β radiolysis of the DNA occurs.

Irradiations. Neutrons were obtained by nuclear reaction of 34-MeV protons on a semithick beryllium target (Centre d'Etudes et de Recherches par Irradiation, CNRS, Orléans). As far as microdosimetric parameters are concerned, the dose mean lineal energy in water at the point of interest was 87.7 KeV/μm. The mean dose rate monitored with transmission chambers was 10 Gy/min.

γ photon irradiations were performed with a ^{60}Co teletherapy unit (Centre Hospitalier Régional d'Orléans). The dose mean lineal energy was about 1.9 KeV/μm, and the dose rate was 9 Gy/min.

Samples of 10 μL were irradiated in polypropylene tubes (Eppendorf, 0.4 mL) immersed in an ice bath, 1 cm below the surface.

Radiolysis of the Nucleosomes by β Rays. β rays (electrons) of 1.7 MeV were obtained directly inside the sample by disintegration of ^{32}P borne both by the [γ-^{32}P]ATP used for the labeling and by the labeled DNA molecules. The dose D integrated over the dialysis time T (27 h) has been estimated to 100–300 Gy, using the formula (Kiefer, 1990):

$$D = E_{\text{eff}} \int_0^{\tau} S_0 e^{-t/\tau}\, dt$$

were E_{eff} represents the effective particle energy (generally one-third of the maximum energy for β rays, i.e., 9.6×10^{-14}

J for ^{32}P), S_0 is the activity per mass unit at the beginning of the dialysis (0.1 GBq/kg), and τ is the evaluated time constant of the dialysis (2.5–7.5 h). The dose D is expressed in Grays, the SI unit (abbreviated Gy) which corresponds to 1 absorbed joule per kilogram of matter.

Repressor and CRP Footprinting. After irradiation, the DNA was extracted from the complexes with phenol, ethanol-precipitated, dried, and dissolved in few microliters of 10 mM Tris-HCl/1 mM EDTA, pH 7.6, buffer. Samples can be stored at 4 °C in this buffer for several days, since we observed that Tris and EDTA protect DNA against autoradiolysis by β radiations emitted by ^{32}P.

Before electrophoresis, aliquots containing identical activities were dried and redissolved in 2 μL of loading buffer (98% formamide, 10 mM EDTA, pH 8, 0.025% xylene cyanol, and 0.025% bromophenol blue) and heated for 3 min in boiling water. Naked irradiated DNA underwent the same treatment. Samples were run on 0.4-mm-thick, 40-cm-long, 8% polyacrylamide sequencing gels [acrylamide/bis(acrylamide) ratio of 20/1] for 1–3 h at 35 W. The gel buffer was 89 mM Tris–borate, 2 mM EDTA, and 7 M urea, pH 8.

For the identification of the bands, Maxam–Gilbert sequencing of the fragments (Sambrook et al., 1989) was run on the same gel.

Nucleosome DNA Footprinting. After irradiation, the samples were dried and dissolved in a few microliters of loading buffer (98% formamide, 10 mM EDTA, 7 M urea, pH 8, 0.025% xylene cyanol, and 0.025% bromophenol blue) and heated for 3 min in boiling water. They were run on 0.4-mm-thick, 55-cm-long, 6% polyacrylamide sequencing gels [acrylamide/bis(acrylamide) ratio of 8/1] for 2.5 h at 40 W. The amount of bis(acrylamide) has been increased to reduce the effect of the base composition on the electrophoretic mobility of fragments of the same length (Lutter, 1979). The gel buffer was 89 mM Tris–borate, 2 mM EDTA, and 7 M urea, pH 8.

Analysis of Data. Gels were fixed in a mixture of 10% acetic acid and 10% ethanol, dried onto Whatman 3MM paper, and autoradiographed (3M type R films). Autoradiographs were scanned using a CAMAG densitometer.

The densitograms giving the absorbance of the film (reading at 480 nm) as a function of the migration distance were transformed as follows. We represented the maximum absorbance of each band and the minimum absorbance of each interband as a function of the length of the oligonucleotides. The mobile average over 20 points (10 bands) on each side was subtracted, to suppress the nonuniform background of the film, especially in the vicinity of the intact fragments (top of the gel).

RESULTS

DNA fragments, either naked or complexed with proteins, have been irradiated with ionizing radiations. To obtain correct patterns for the footprints, the number of breaks per fragment should never be larger than 1. Keeping the mean value of breaks per fragment smaller than 0.1 limits to 1% the fraction of fragments bearing more than 1 break. The order of magnitude of doses, D (in Gy), corresponding to this yield for a fragment of L base pairs was determined by extrapolation of the values obtained for a purified supercoiled plasmid (Spotheim-Maurizot et al., 1990): $D = 10^4/L$ for γ rays and $D = 2 \times 10^4/L$ for neutrons.

The doses effectively delivered had to be adjusted, however, for each experiment, since the DNA–protein complex solutions could often contain traces of radioprotectors such as ethanol, dithiothreitol, or mercaptoethanol for instance. Figure 2 shows the autoradiographs of radiolyzed fragments in the absence and in the presence of the proteins.

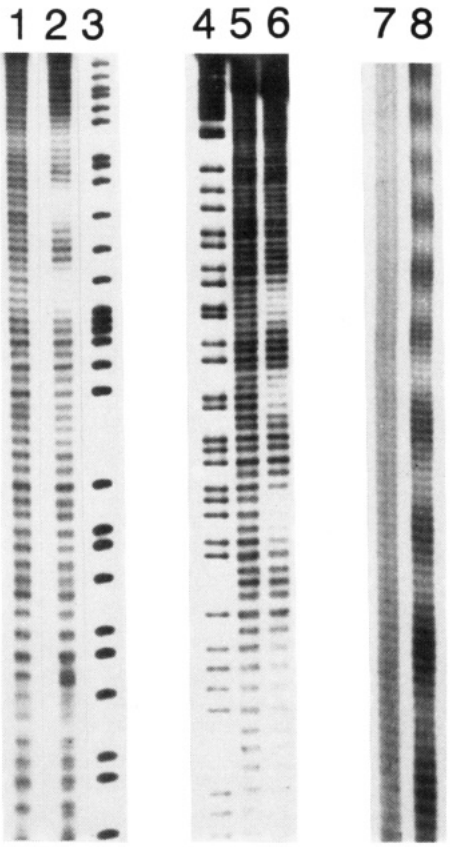


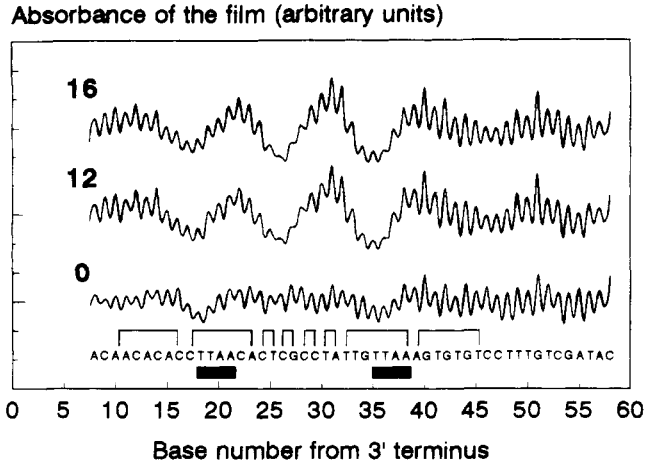
FIGURE 2: Autoradiographs of sequencing gels. Lanes 1–3: fragment 80, fast neutrons irradiation. Lane 1, DNA alone. Lane 2, DNA complexed with *lac* repressor (12 proteins per fragment). Lane 3, Maxam–Gilbert pattern of purines. Lanes 4–6: fragment 104, γ-photons irradiation. Lane 4, Maxam–Gilbert pattern of purines. Lane 5, DNA alone. Lane 6, DNA complexed with CRP (two proteins per fragment). Lanes 7–8: 146 bp fragments from core nucleosomes, β-rays irradiation. Lane 7, DNA alone. Lane 8, DNA complexed with the histone octamer.

Radiolysis of Naked DNA. On the lanes corresponding to naked DNA, we observe a quasi-regular ladder, showing that all sizes of oligonucleotides are obtained. In other words, breakage can occur at each nucleotide site. The intensities of the bands are, however, not identical, showing that the occurrence of breaks depends on the site and may be on the base, but more probably on the sequence. We note that the AATT sequences are always less attacked than the average of the rest of the DNA.

A double banding pattern is easily observed in the bottom of the gels. The upper band of the doublet corresponds to the 3′-phosphate terminus (Henner et al., 1983). Maxam–Gilbert sequencing also gives the 3′-phosphate terminus; the assigned base corresponds to the radiolyzed deoxyribose moiety.

Similar patterns of breakage are obtained for both γ photons and fast neutron irradiated DNA.

Footprinting of the E. coli lac Repressor and CRP. Fragments 80 and 53 both bear the *lac* operator. Fragment 80 is 5′ end labeled on the template strand, and fragment 53 on the opposite strand, that allows footprinting on both strands. We have a similar situation with fragments 104 and 121 that both bear the binding site of the CRP. Retardation gel experiments were performed before irradiation to check that complexes exist and after irradiation to verify that complexes are not destroyed. Figure 2 shows the breakage patterns of



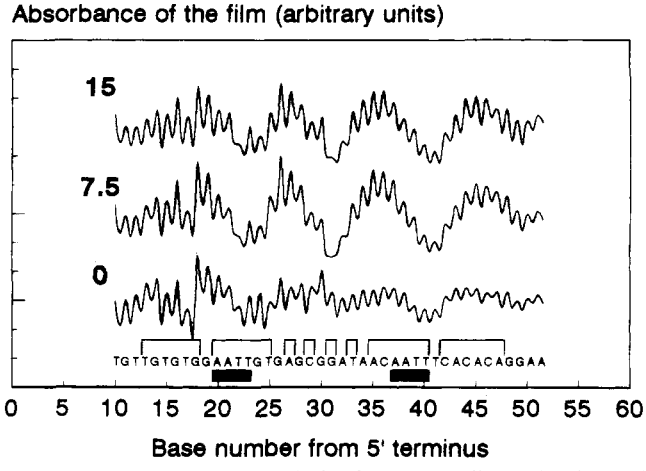
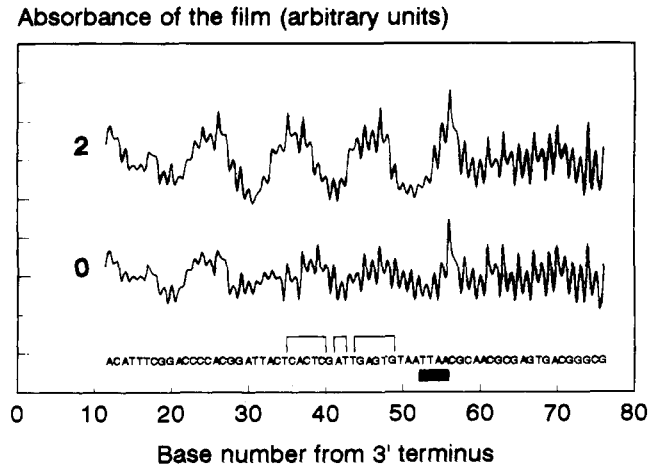


FIGURE 3: Densitometric analysis of the autoradiographs obtained with radiolyzed DNA–*lac* repressor complexes. The numbers represent the ratio of protein per fragment (naked DNA is 0). The mobile average over 10 bands is subtracted from the densitograms. The profiles are shifted along the y axis for the sake of clarity. The sequence and the location of the palindromic site allow the identification of every band. The AATT sequences are underlined. Upper part: fragment 80 (template strand) irradiated with fast neutrons. Lower part: fragment 53 (nontemplate strand) irradiated with γ rays.



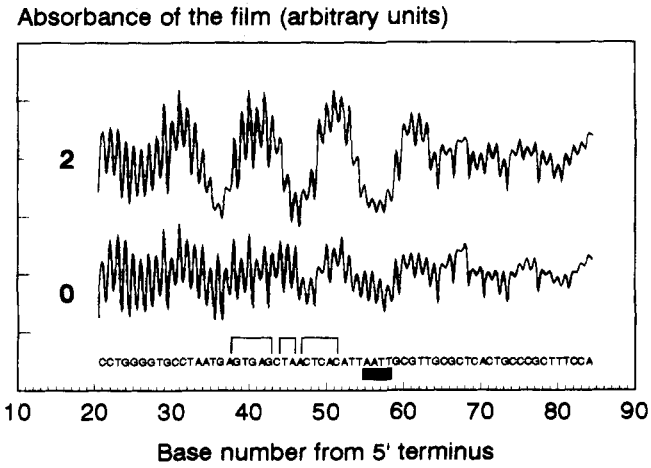


FIGURE 4: Same as for Figure 3, but with DNA–CRP complexes. Upper part: fragment 121 (nontemplate strand). Lower part: fragment 104 (template strand). Both fragments were irradiated with γ rays.

fragment 80 bound to *lac* repressor irradiated with neutrons, and of fragment 104 bound to CRP irradiated with γ photons.

In the presence of protein, we observe that the intensities of the bands were modified in several regions, showing that some sites of radiolytic attack are protected (bleaching of the bands) whereas others are more exposed (intensification of the bands).

For both regulatory proteins, the patterns exhibit three major protected regions that overlap the palindromic binding sites of the proteins (Figures 3 and 4).

After subtraction of the mobile average, the profiles are normalized using parts of the patterns unaffected by these site-specific binding proteins, i.e., as far as possible from the binding site. This treatment allows us to erase the differences in the extent of cutting of naked and complexed fragment, and to overcome the problems due to experimental errors of volume (and consequently of radioactive activity) charged on the gels.

Let us call A_p and A_n the absorbances at the maxima of the bands after this treatment, respectively, in the presence and in the absence of bound protein. The enhancement factor EF could thus be defined as

$$EF = [(A_p + A_0)/(A_n + A_0)] - 1 = (A_p - A_n)/(A_n + A_0)$$

where A_0 is the average absorbance of the film along the electrophoretic track.

Figures 5 and 6 show the enhancement factors calculated for the *lac* repressor and the CRP. Positive values correspond to an enhancement of the radiolytic attack, while negative values reflect a protection of the considered sites. One observes that for both proteins, the cutting patterns for the two opposite strands are symmetrical relative to the center of the palindrome.

Footprinting of the Histones in the Core Nucleosome. The core nucleosome and the corresponding 146 bp naked DNA have not been exposed to a radiation beam, but the irradiation has been performed in situ by the β rays emitted by the ^{32}P isotope used for labeling (see Materials and Methods).

The electrophoretic separation of the resulting fragments (Figure 2) shows that for naked DNA, breakage occurred at all nucleotide sites, with a better uniformity than for the restriction fragments. For the DNA surrounding the core nucleosomes, the electrophoretic pattern shows an alternation of bleached and intensified groups of bands, as shown on the densitogram (Figure 7). A Fourier transform of the data (Figure 7) exhibits only one significant harmonic, showing an

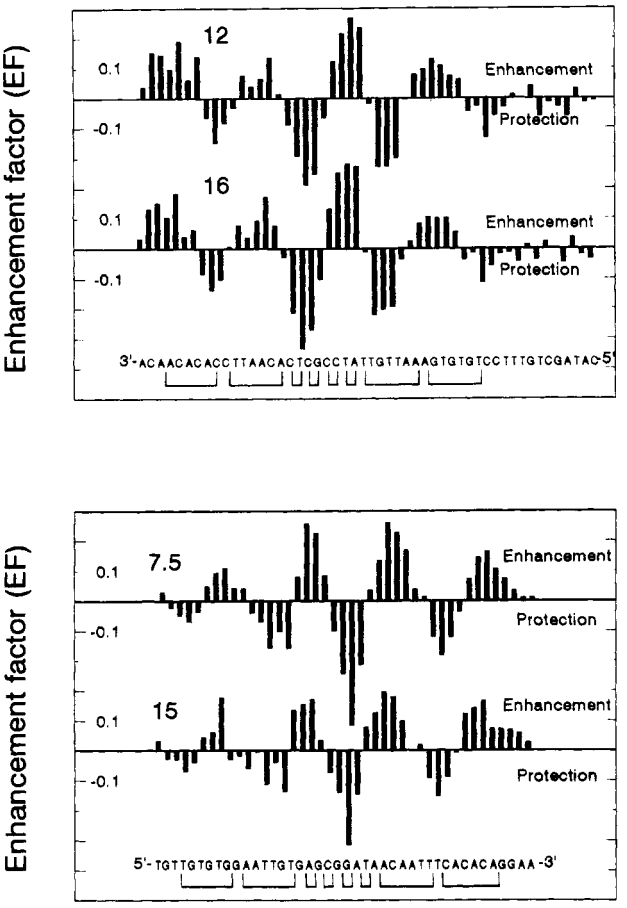


FIGURE 5: Histograms deduced from the data of Figure 3 showing the sites of enhanced or protected radiolytic attack in the DNA–*lac* repressor complexes. EF has been plotted as a function of the site of breakage. The numbers represent the ratio of protein per fragment. Upper part, fragment 80. Lower part, fragment 53.

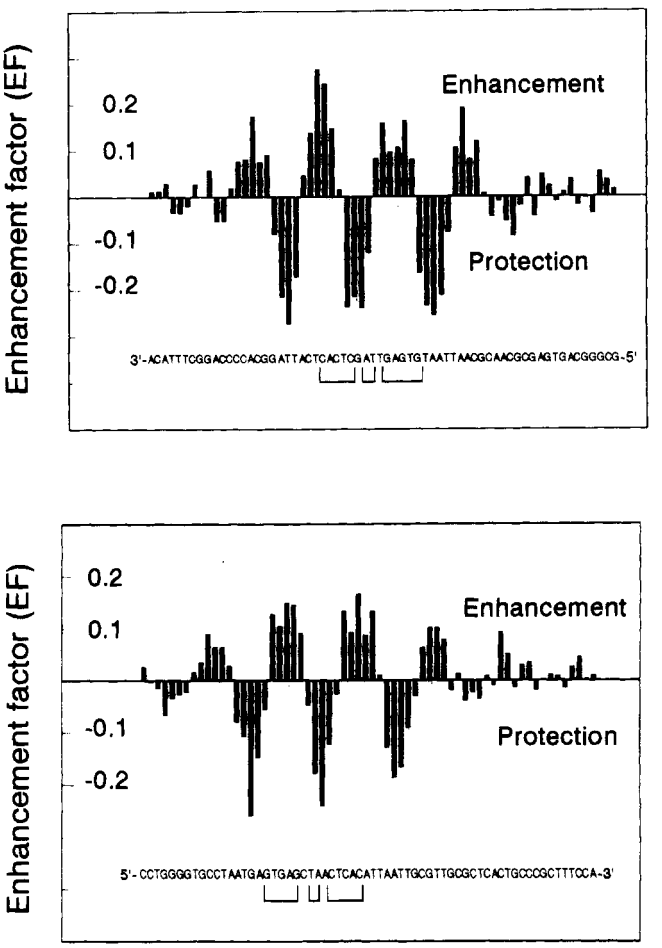


FIGURE 6: Same as for Figure 5, but with DNA–CRP complexes. Upper part: fragment 121. Lower part: fragment 104.

excellent periodicity of the attack of DNA. The period is 10.39 ± 0.16 nucleotide sites.

DISCUSSION

Radiolysis of Naked DNA. Radiation-induced frank strand breaks of DNA result from the cutting of the sugar–phosphate backbone, essentially through the $C_{4'}$ attack of the deoxyribose moieties (von Sonntag et al., 1981). The absence of piperidine treatment in our experiments avoids the revelation of damages such as incomplete sugar destruction or base deletions or modifications, which represent the major part of the alkali-labile bonds (Téoule, 1987). Such alkali-labile bonds would have interfered with the frank strand breaks and complicated the interpretation of the results. Before being layered on sequencing gels, the samples have been heated for a few minutes at 100 °C to denature the DNA, and it may be possible that the observed breaks include some heat-labile bonds (Lafleur et al., 1983).

As already observed by Henner et al. (1982, 1983), we clearly detected doubling of the bands for the radiolyzed 5'-labeled fragments, the upper band corresponding to the 3'-phosphate and the lower one to the 3'-phosphoglycolate termini. We also observed that all deoxyriboses are potential sites of breakage, since no band is lacking whatever fragments were used. Nevertheless, all bands do not have the same intensity, showing that all sites do not have the same sensitivity. This sensitivity seems unrelated in a simple way to the nature of the base, or of its closer neighbors, as already observed in radiolysis of single-stranded oligonucleotides (Duplaa & Téoule, 1985; Téoule & Duplaa, 1988).

We consider that one cannot relate these differences of sensitivity to the nature of the base, if the $C_{4'}$ and not the base itself is the primary target in the frank strand breakage. These discrepancies can more reasonably be related to some locally disturbed conformation of the DNA. For instance, the sequence AATT, that is present 3 times in the fragments (at positions −75, 1, and 18 from the transcription start), is always less sensitive to the radiolysis than the rest of the naked DNA and even than the TTAA sequences (Figures 3 and 4). Using chemically produced OH• radicals as a probe, Burkhoff and Tullius (1988) observed the same "resistance" of the AATT sequences. An explanation comes from the crystallographic data obtained by Fratini et al. (1982) with a B-DNA dodecamer, CGCGAATTCGCG, showing that the minor groove is narrower in the AATT sequence, leading to bending of the DNA. This result acquires all its interest if we keep in mind that the regulatory sequences are AT-rich. Consequently, the promoter regions could be more resistant to radiolysis than the rest of DNA.

The pattern of 146 bp naked DNA from core nucleosome is more uniform than those of restriction fragments. This is due to the superposition of patterns corresponding to fragments of different base composition and sequence.

lac Repressor Footprinting. The first footprinting results concerning the *lac* regulatory region have been obtained by Galas and Schmitz (1978) using DNase I as a probe. Chemical probes, such as dimethyl sulfate (Ogata & Gilbert, 1979) of

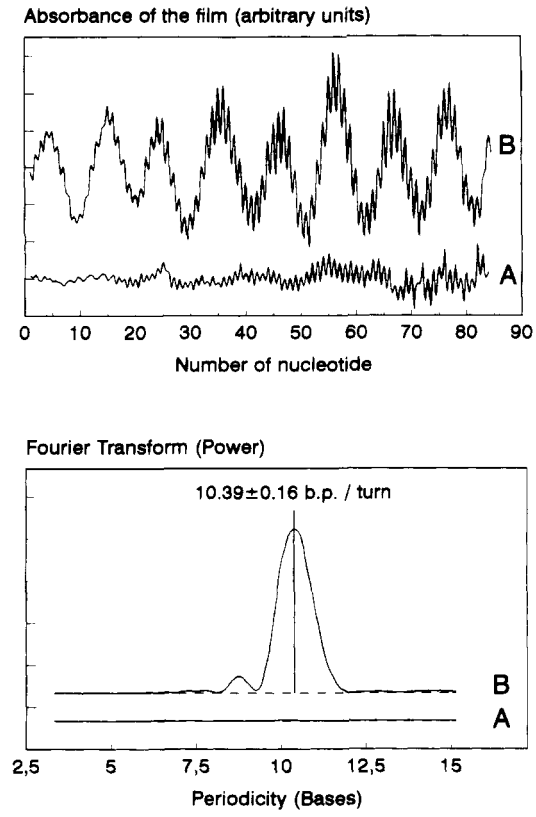


FIGURE 7: Upper part: densitometric analysis of the autoradiograms of the electrophoresis gels. (A) 146 bp naked DNA fragments, extracted from the core nucleosomes. (B) 146 bp DNA fragments in the core nucleosome. The mobile averages over 22 bands have been subtracted, and the profiles are shifted along the y axis. Irradiations with β rays emitted by ^{32}P. Lower part: Fourier transform of the densitometric profiles showing that there is no periodicity in the naked DNA profile (A) and only one major harmonic at 10.39 bp in the DNA surrounding the core nucleosome (B).

$[Cu(OP)_2]^{2+}$ (Sigman & Spassky, 1989), have also been used. As far back as 1977, Ogata and Gilbert (1977), and more recently Wick and Matthews (1991), used breaks photoinduced in bromouracil-substituted DNA to investigate the thymine residues implicated in the interaction. The photofootprinting method of Becker and Wang (1984) was first applied also to the *lac* regulatory region.

Our results allow us to draw the protection pattern shown in Figure 8A. The sites protected by the protein against radiolysis are located on one "side" of a linear B-helix. This protection pattern is very similar to the pattern of *lac* repressor–operator interaction deduced from the other footprinting studies. In Figure 8A, the interaction surface appears slightly left-handed, as already proposed (Charlier et al., 1981), and covers two major grooves, where the helix–turn–helix motifs of the two interacting protomers can penetrate (Weber et al., 1982). The weak protection in the zones exterior to the binding site (the narrow ribbon on Figure 8) may be due to protein hindrance, which considerably reduces the accessibility to $OH^{\bullet}$ radicals.

CRP Footprinting. As for the *lac* repressor, the specific interaction of the CRP with DNA has been extensively studied by footprinting experiments, using DNase I (Schmitz, 1981; Spassky et al., 1984), $[Fe(EDTA)]^{2-}$ (Shanblatt & Revzin, 1987), $[Cu(OP)_2]^{2+}$ (Sigman & Spassky, 1989), and photobreakage of bromouracil-substituted DNA (Simpson, 1980). The 3-Å resolution crystal structure of the specific CRP–cAMP–DNA complex (Schultz et al., 1991) showed that the

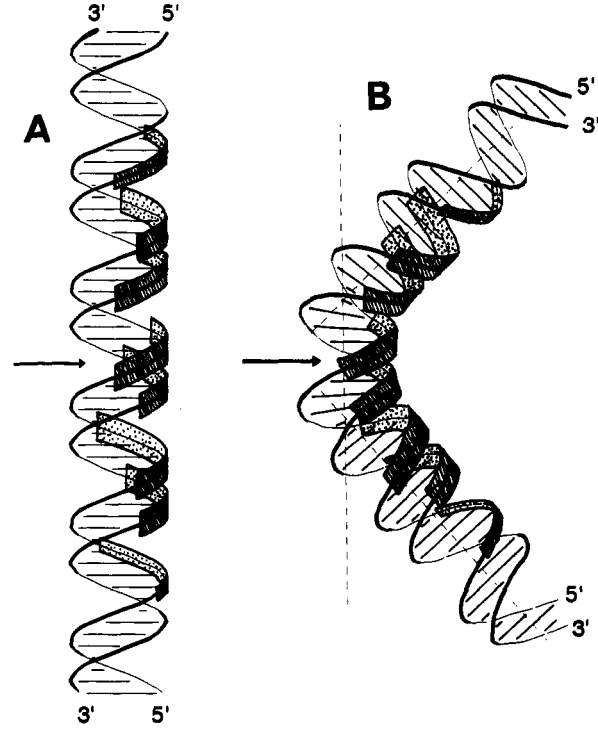


FIGURE 8: Drawing of the sites protected by the proteins on the two strands of a B-form helix of DNA. In the case of the CRP protein, and the helix has been bent, as described by Schultz et al. (1991) from crystallographic data. External zones where the enhancement/protection factors were small have been represented with a narrower ribbon. Arrows indicate the center of the palindromic sites. (A) *lac* repressor. (B) CRP protein.

DNA was bent by 90°, as already suggested several years ago by gel shift experiments (Wu & Crothers, 1984).

In Figure 8B, we present the pattern of protection by CRP against radiolytic cleavage on a DNA bent as described by Schultz et al. (1991). This pattern is consistent with the protection patterns obtained by other footprinting methods. It perfectly agrees with the crystallographic data recently reported by Schultz et al. (1991) concerning the CRP–DNA interaction. The interacting surface is located in the inner part of the kink, and covers, as for the *lac* repressor, two major grooves where both helix–turn–helix motifs of the dimer protein interact. The slightly protected zones external to the binding site (narrow ribbons) could result from a restricted accessibility to the radicals, due to the hindrance of the protein inside the curved DNA.

Core Nucleosome Footprinting. The core nucleosome (histone octamer surrounded by the 146 bp DNA fragment) presents a dyad axis: the two strands of the DNA are thus symmetrical to one another. All fragments have a different base sequence and composition. Therefore, only the distance of the breaks from the 5′ termini is relevant.

The footprinting of histones on the nucleosome DNA was studied using nucleases (Prunell et al., 1979; Lutter, 1979; Prunell, 1983), $[Fe(EDTA)]^{2-}$ (Tullius & Dombrowski, 1985; Tullius, 1989; Hayes et al., 1990a), or photodimerization of pyrimidine bases (Gale et al., 1987). All these works showed a periodic attack of the DNA, corresponding to a helical periodicity of 10.3–10.5 base pairs per turn in the core nucleosome. Our results showing a periodic protection/attack pattern with a period of 10.39 ± 0.16 are in very good agreement with the previously reported data.

Effect of Radiation Quality. On the basis of LET differences between γ photons and neutrons, and their different effects on DNA in vivo or in vitro, we expected different

patterns for fragments irradiated with these radiations. As we observed with fragment 80, either naked or interacting with the *lac* repressor, the effects of γ rays or of fast neutrons are identical. The same bands were either reinforced or bleached, with the same intensity (data not shown). The same agreement between γ photons and fast neutron radiolysis has been observed on fragment 121 naked or interacting with a chromosomal protein (unpublished results). The same doubling of bands show that in both cases, 3′-phosphate and 3′-phosphoglycolate termini are obtained. These results suggest that in the low dose range, the frank strand breaks due either to γ photons or to fast neutrons are induced by a similar chemical process. Nevertheless, significantly higher doses of neutrons than of γ photons have to be used for getting similar intensities of bands, thus similar yields of breaks, as expected from our previous studies with plasmids (Spotheim-Maurizot et al., 1990). A detailed study of the relative proportions of the two termini for the two types of radiation is in progress.

CONCLUSION

By studying several well-known DNA–protein interacting systems, we have validated here the method of footprinting with radiolytically produced OH• radicals proposed by Hayes et al. (1990b). The radiolytic footprinting can thus be reliably used as a method to study unknown DNA–protein interactions. Moreover, we have shown that not only γ photons but also fast neutrons or β rays can be used for radiolytic footprinting.

The immediate advantage of the radiolysis is the absence of chemicals, and consequently the simplicity and the nonperturbation of the biological systems by the reactives. Moreover, in the case of β rays emmited by the radioactive isotope, there is no requirement of an irradiation device. To be effective, i.e., to produce OH• radicals, radiolysis needs only water in the system. Since there is no problem of penetration in cells or in nuclei, this method could be suitable for in vivo experiments.

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