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Photocleavage of DNA and photofootprinting of *E.coli* RNA polymerase bound to promoter DNA by azido-9-acridinylamines

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ABSTRACT

The long-wavelength ultraviolet (λ ~ 420 nm) radiation induced reaction between 6-azido-2-methoxy-9-acridinylamines and supercoiled plasmid DNA results in single strand scissions and formation of covalent adducts (ratio ~ 1:10). By treating azidoacridine-photomodified DNA with piperidine at 90°C, additional strand scissions are observed in a complex sequence dependent manner with an overall preference for T ≥ G > C >> A. The resulting DNA fragments migrate as 5'-phosphates in polyacrylamide gels.

Photofootprinting of the binding site of RNA-polymerase on promoter DNA is demonstrated with an azido-9-acridinylamino-octamethylene-9-aminoacridine. Similar experiments using 9-amino-6-azido-2-methoxyacridine indicate that this reagent recognizes changes in the DNA conformation induced by RNA polymerase binding, in relation to open complex formation.

INTRODUCTION

Footprinting of protein binding sites on DNA is a powerful and versatile technique which can be used both *in vitro* and *in vivo* to study protein-DNA interactions. Originally DNaseI and alkylating agents such as dimethyl sulphate were employed as footprinting reagents [1-3]. Recently, organic transition metal complexes with DNA affinity have been introduced as chemical DNA cleavage and footprinting reagents [4-7]. DNA modification by short wavelength ultraviolet light has also shown potentials in photofootprinting [8-9].

The use of light has three major advantages over enzymatic or chemical footprinting: i) the reaction is controlled by an external agent (light) which ii) can be administered very accurately and within very short time intervals (< 10^{-6} sec by flash irradiation) and iii) the reactions can usually take place at low temperature (0°C or lower if desired).

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Although ultraviolet radiation ($\lambda \sim 254$ nm) by itself can be used in photofootprinting, improvements could be made by using long wavelength ultraviolet radiation ($\lambda > 300$ nm) which is not absorbed by most cellular components. Since DNA does not absorb at such wavelengths, external agents have to be employed. These could either be triplet sensitizers (e.g., acetone or acetophenone), singlet oxygen generators (e.g., methylene blue or acridine orange) or compounds that photoreact covalently with DNA.

In the latter case photoreactive intercalators come into mind. The photoreactions between psoralens and DNA are well described [10]. However, the detection of psoralen DNA adducts requires tedious enzymatic procedures [11,12]. Azidoethidium derivatives have been shown to photobind to DNA [13] but phenanthridinium derivatives are complicated to prepare [e.g., 14] and procedures for detection of the adducts have not been described. Conversely, azido derivatives of 9-aminoacridine are fairly easy to obtain [15,16] and we have previously used 9-amino-6-azido-2-methoxyacridines as the DNA reactive moiety of protein-DNA photo-crosslinking reagents [17].

As an extension of our efforts to design photochemical probes for the study of protein-DNA interactions [17-22] we now report that azidoacridine-DNA photoadducts can be cleaved with piperidine and we show that 9-amino-6-azido-2-methoxyacridines can be used as DNA-photocleaving and DNA-photofootprinting reagents.

MATERIALS AND METHODS

Chemistry

The IR spectra were recorded on a Perkin Elmer model 157 spectrometer and NMR spectra on a JEOL FX90Q spectrometer. Elemental analyses were carried out in the microanalytical laboratory of this department by Mr. Preben Hansen. Melting points are uncorrected.

9-Amino-6-azido-2-methoxyacridine, HCl (1) was prepared [16] by heating 6-azido-9-chloro-2-methoxyacridine with ammonium carbonate in phenol followed by treatment with hydrogen chloride.

<u>N-(6-Azido-2-methoxy-9-acridinyl)-N'-(9-acridinyl)octane-1,8-diamine, 2 HCl</u> (2). N-Boc-octane-1,8-diamine, HCl (Boc = t-butoxycarbonyl, prepared according to ref. 23, 0.30 g, 1 mmol) was treated with 12 M NaOH (5 ml) and extracted with diethyl ether (3 x 25 ml). The extract was dried and evaporated to dryness. The residue was heated to 80°C in phenol (2 g), 6-azido-9-chloro-2-methoxy-acridine [16] (0.30 g, 1 mmol) was added and the mixture was heated to 100°C for 5 h. The Boc protected acridinyl compound was isolated as light brown crystals after washing with diethyl ether (3 x 25 ml) and it was deprotected by treatment with hydrogen chloride in acetic acid (2M, 10 ml) for 1 h at room temperature. Evaporation yielded N-(6-azido-2-methoxy-9-acridinyl)octane-1,8-diamine, 2 HCl, which was heated to 80°C in phenol (2 g). 9-Phenoxyacridine (0.20 g, 0.75 mmol) was added, and the mixture was heated to 100-110°C for 1 h. Washing with diethyl ether (3 x 25 ml) yielded the title compound (2, 0.35 g, 55%) which was purified by recrystallization from diethyl ether-ethanol; mp > 180°C (decomp.) Anal: Found (calculated for $C_{35}H_{37}Cl_2N_7O$, $3H_2O$) C: 60.4 (60.6)%, H: 5.7 (6.2)%, N: 14.7 (14.7)%, Cl: 9.9 (9.8)%). IR (KBr): 2130 cm^{-1} (N_3). The ^{1}H-NMR spectrum was in agreement with the structure.

<u>Photobinding of reagent 2 to calf thymus DNA</u>.
A mixture of calf thymus DNA (750 µg/ml) and reagent 2 (25 µg/ml) in TE was irradiated at 365 nm (Philips TL 20 w/09 fluorescent light tube (PUVA-lamp), 22 $J \cdot m^{-2} \cdot s^{-1}$). Samples were withdrawn at the times indicated (in Fig. 1) and 1 vol of dimethyl sulfoxide was added. The DNA was precipitated with 2 vol of ethanol, 0.2 M NaAc. The DNA was redissolved in 50% dimethyl sulfoxide, again precipitated and finally taken up in 5 N NaOH. After heating for 60 min at 90°C, the fluorescence (λ_{ex} = 390 nm, λ_{em} = 470 nm) was measured and the amount of bound acridine calculated from the amount of acridone formed (21).

The spectral distribution of the common PUVA lamp used in this experiment is not optimal for photolysis of the azidoacridines since it only partly overlaps the long wavelength (400-450 nm) absorption of these. However, due to the high quantum yields for photolysis of the azidoacridines it is quite

adequate. For optimal photolysis rates we used the Philips TL 40 W/03 tube.

Plasmid relaxation assay

pUC19 DNA was transformed into E.coli DH5α and isolated by the alkaline extraction procedure [24]. In a typical experiment 0.3 μg DNA in 10 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was mixed with 1 μl of the reagent dissolved in DMSO, and irradiated in Eppendorf tubes from above at room temperature using a Philips TL 40W/03 fluorescent light tube at a distance of 15 cm (λ ~ 420 nm, 20 $J \cdot m^{-2} \cdot sec^{-1}$). The DNA was analyzed by electrophoresis in 1% agarose in 0.5 x TBE buffer (45 mM Tris-borate, 0.5 mM EDTA, pH 8.3).

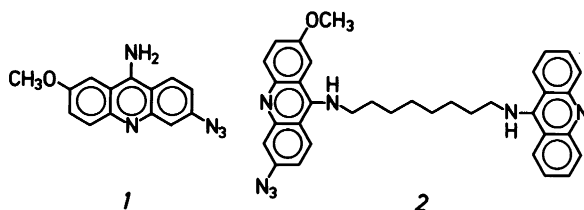
Photocleavage of ^{32}P-end labeled DNA fragments. The 232 bp EcoRI-Pvu II fragment of the plasmid pUC19 was used to study DNA cleavage at the nucleotide level. The fragment was 3'-end-labeled at the EcoRI site and purified using standard techniques. In a typical cleavage reaction 0.1-0.2 pmol of end-labeled fragment was mixed with 0.5 μg calf thymus DNA (Sigma) in 100 μL TE. After addition of 5 μl of reagent (diluted with H_2O from a stock solution (10 mg/ml) in dimethyl sulfoxide) the samples were irradiated from above with 420 nm light for 30 min using the Philips TL 40W/03 lamp. The DNA was recovered by EtOH precipitation and treated with 1 M piperidine at 90°C for 20 min followed by precipitation with 10 vol. of 1-butanol. The DNA pellet was washed with 70% EtOH and lyophilized. The samples were subsequently fractionated on 8% PAG/50% urea sequencing gels and the cleavage products visualized by autoradiography.

Footprinting of E.coli RNA polymerase bound to the deoP1 promoter. For these experiments a 231 bp BamHI-BglII fragment (from the plasmid pGD11) containing the region from -101 bp to +104 bp around the transcription initiation site of the deoP1 promoter was used [26,27]. The fragment was ^{32}P-endlabeled at either end and isolated using standard procedures. The E. coli RNA polymerase (a gift from Dr. Kaj Frank Jensen) was purified from E.coli K12 [27]. 0.1-0.2 pmol of 3'-endlabeled DNA fragment and 10 pmol of RNA polymerase were mixed in 100 μl buffer (40 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 2.5 μg/ml calf thymus

DNA, 2.5% glycerol). After incubation at $37^{o}C$ for 10 min the samples were mixed with 5 µl reagent (50 µg/ml of 1 or 15 µg/ml of 2 in H_2O) and irradiated as described above (or alternatively treated with 1 ng of DNase I for 10 min at room temperature). Following ethanol precipitation the samples were treated with 1 M piperidine at $90^{o}C$ for 30 min, precipitated with n-butanol, dried and taken up in 90% formamide loading buffer. The samples were analyzed on 6% polyacrylamide, 50% urea gels in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.3) followed by autoradiography.

Calculation of photonicking efficiency and photoadduct to nicking ratio for reagent 2

A reagent to base pair ratio (Fig. 2, lane 6) of 0.05 results in 90% relaxation which, assuming a Poisson distribution of the scissions, corresponds to 2.2 scissions per 2.7 kbp or 8×10^{-4} scissions/bp (i.e. 0.0016 nicks/reagent molecule). It is furthermore observed, lanes 6 and 8 (± irradiation) of Figure 2, that the gel migration of the supercoiled pUC19 is retarded as a result of the photoreaction. We ascribe this retardation to the formation of covalent adducts which do not result in strand scission, but which due to the intercalation of the second acridine group unwind the DNA. We have previously found that some oligoacridines stay bound to DNA during electrophoresis [25]. This is, however, not the case with reagent 2 (or reagent 1) as evident from the result of Fig. 2, lane 8. Upon comparison with these results (ref 25, Fig. 6) we estimate that the retardation resulting from photoreaction of pUC19 DNA with reagent 2 at r = 0.05 (Fig. 2, lane 6) corresponds to an intercalation density of 0.008 acridines per bp. Assuming that only the non-photo-reactive acridine moiety of reagent 2 causes DNA unwinding (an assumption supported by the observation that no similar gel retardation of supercoiled pUC19 DNA was seen upon photoreaction with reagent 1) with an unwinding angle of 34° : 2 = 17° (cf. ref. 25, Fig. 6), we estimate that 0.008 adducts per bp are formed by photoreaction of 2 with pUC19 DNA at r = 0.05. These results therefore indicate that photoreaction of reagent 2 with DNA results in one single strand scission for every ten photoadducts formed. The estimate of 0.008 adducts/bp corresponds to a yield of 0.008 : 0.05 ~15%.



Scheme 1 Chemical structures of the azidoacridine photoprobes.

RESULTS

In our attempt to use azidoacridines for DNA-photofootprinting we have synthesized 2-methoxy-6-azido-9-aminoacridine [16] (Scheme 1, reagent 1) and a diacridine, N-(2-methoxy-6-azido-9-acridinyl)-N'-(9-acridinyl)octane-1,8-diamine (Scheme 1, reagent 2), in which the second acridine moiety was introduced in order to enhance the DNA-affinity of the reagent by intercalation.

Due to the extreme sensitivity of reagents 1 & 2 to ambient light we have not undertaken any elaborate study of their reversible interaction with DNA. However, binding to DNA is evident since, the presence of DNA induces a 9 nm bathochromic shift and a 27% hypochromicity of the 433 nm absorption band of the

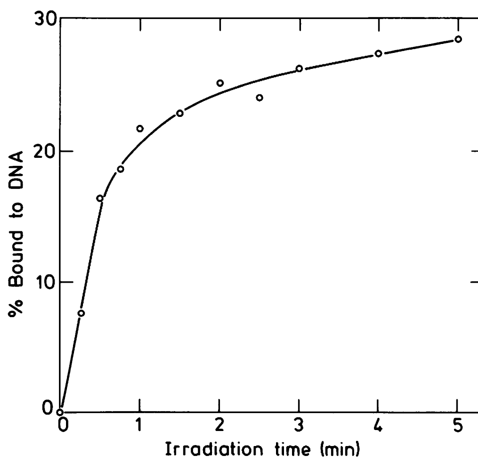


Figure 1 Photobinding of reagent 2 to calf thymus DNA.

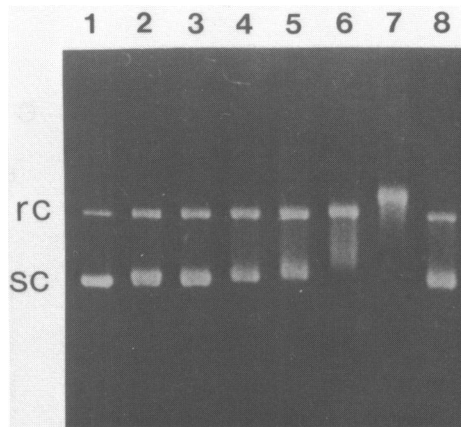
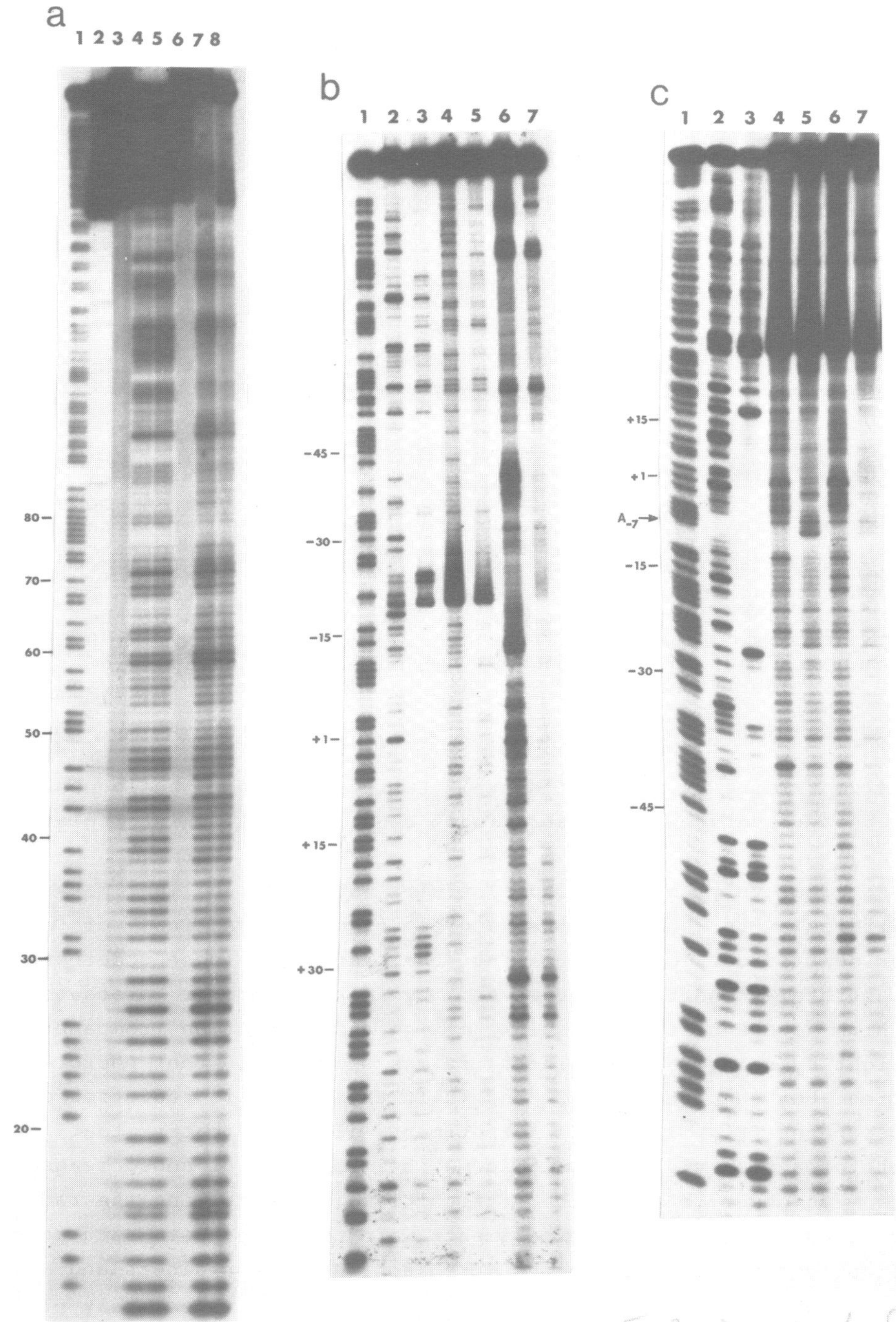


Figure 2 Photonicking of supercoiled circular pUC19 DNA.
0.3 μg pUC19 DNA in 10 μl TE was mixed with the desired amount of reagent 2 in 1 μl dimethyl sulfoxide. The samples were irradiated for 15 min at 420 nm and analyzed by electrophoresis in 1% agarose (0.5 x TBE buffer). The gel was stained with ethidium bromide and photographed. Quantification was performed by densitometric scanning of the photographic reproduction of the gel. The following amounts of reagent were used: Lanes 1-8: 0,5 ng, 7.5 ng, 10 ng, 15 ng, 20 ng, 40 ng and 20 ng. The sample of lane 8 was not irradiated. Reagent 1 showed similar cleavage efficiency.

azidoacridine 1 (data not shown). Furthermore, the analogous 2-methoxy-6-chloro-9-aminoacridines bind to DNA by intercalation [29].

The very high photoreactivity with visible light makes it difficult to control the degree of photoreaction of the azidoacridines by controlling the radiation dose under usual laboratory conditions. However, by taking proper precautions (high concentrations, quick mixing and protection from ambient light by using red containers) it was shown that the photoreaction between reagent 2 and calf thymus DNA is radiation dose dependent (Fig. 1). This experiment also indicates that the photoreaction yield (adduct formation) is ~ 30% of added reagent.

In order to avoid the difficulties in controlling the radiation dose all further experiments were performed with saturating light doses. Stock solutions were kept in red containers and all dilutions and mixings were performed quickly, immediately prior


a
1 2 3 4 5 6 7 8
80
70
60
50
40
30
20
b
1 2 3 4 5 6 7
-45
-30
-15
+1
+15
+30
c
1 2 3 4 5 6 7
+15
+1
A_{-7}
-15
-30
-45

to use, thereby minimizing the exposure of the samples to ambient light until they were ready for irradiation.

The photoreaction between the azidoacridines and double stranded DNA results in single strand DNA scissions detected as photoinduced relaxation of supercoiled pUC19 DNA (Fig. 2). Millimolar concentrations of azide ion did not have any effect on the DNA cleavage by the azidoacridines indicating that singlet oxygen is not involved. Control experiments using either 9-aminoacridine or the unsubstituted diacridine corresponding to reagent 2 [25] showed that these compounds did not photocleave DNA (data not shown). From the results in Fig. 2 (cf. Materials and Methods) a photonicking efficiency of 1.5% of added reagent can be estimated. Furthermore, these results indicate that one photonick is produced by reagent 2 for every ten photoadducts since the yield of photoadducts is estimated as 15%. Within experimental error the latter number is in agreement with that (30%) determined by the fluorescence method (Fig. 1).

Subsequent piperidine treatment of DNA previously photoreacted with reagents 1 or 2 results in a pronounced ($\geq$ 10 fold) increase in the number of DNA strand scissions (Fig. 3a, lanes 3, 4 & 6,7; Fig. 4). These scissions, which presumably occur at the sites of the photoadducts, are sequence dependent without a strict base specificity. Cleavage occurs most frequently at thymines and only rarely at adenines (Fig. 4). Reagents 1 and 2 resemble each other in terms of sequence

Figure 3. Photofootprinting & DNaseI footprinting of the binding of E.coli RNA-polymerase to promotor DNA.

a. Piperidine dependent cleavage of the ^{32}P-3'-endlabeled 232 bp EcoRI-PvuII fragment of pUC 19 by reagent 1 or 2. The incubation mixtures contained no reagent (lane 2), 2 μg/ml of 2 (lanes 3 & 4) 1 μg/ml of 2 (lane 5), 5 μg/ml of 1 (lanes 6 & 7) or 2.5 μg/ml of 1 (lane 8). Samples in lanes 2, 4, 5, 7 & 8 were treated with piperidine whereas the samples in lanes 3 & 6 were not. Lane 1 shows an A + G formic acid sequencing reaction. Distances from the ^{32}P-labeled EcoRI site are indicated.
b & c. Footprinting of E.coli RNA polymerase on a ^{32}P-3'-endlabeled DNA fragment containing the deoP1 promoter. (b) shows results of the non-coding strand while (c) was obtained using labeling of the coding strand. Lane 1: A + G sequencing reaction. Lanes 2 & 3: DNaseI footprint, lanes 4 & 5: photofootprint using reagent 1, lanes 6 & 7: photofootprint using reagent 2. The samples of lanes 3, 5 & 7 contained RNA polymerase whereas those of lanes 2, 4 & 6 were controls without polymerase.

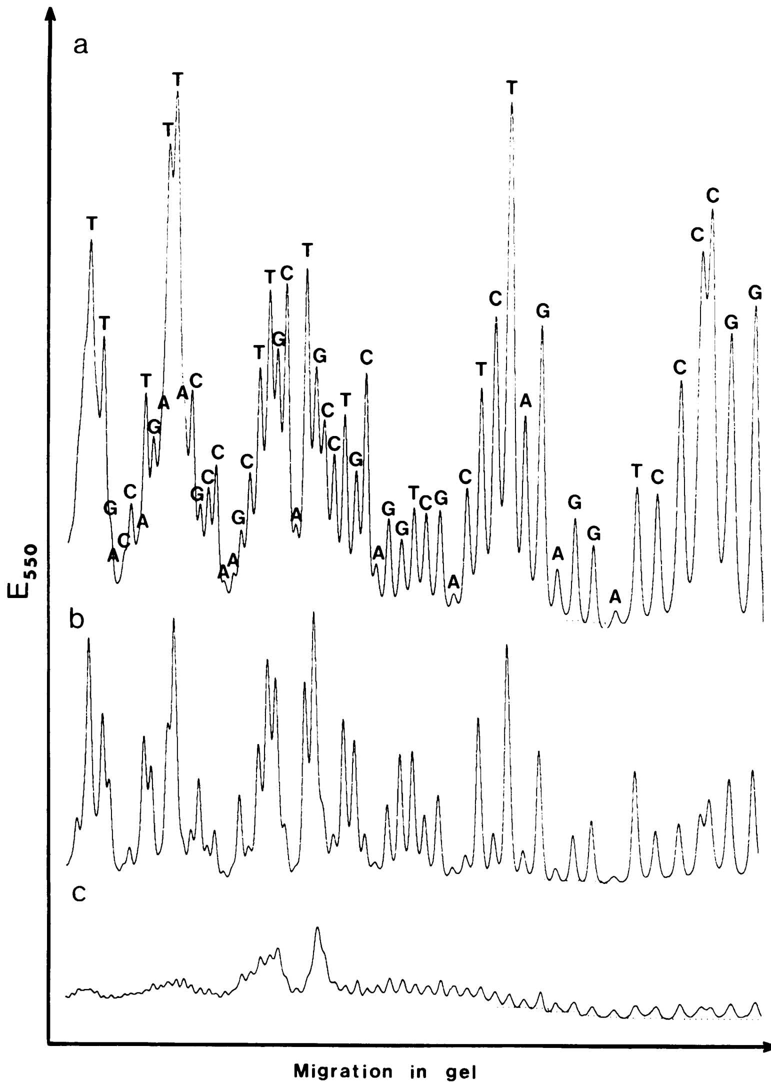


Figure 4 Sequence preference of the DNA photocleavage by reagents 1 and 2

Densitometric scans of the autoradiogram shown in Fig.3a.a: lane 7, reagent 1, b: lane 4, reagent 2, c: lane 3, reagent 2, no piperidine. Quantification of the cleavage frequencies yielded the following results: Reagent 1; T:G:C:A = 1:0.7:0.3:0:1 and reagent 2; T:G:C:A = 1:0.7:0.9:0.3. For comparison a similar quantification of the results of Fig. 3c, lanes 4 & 6 yielded: Reagent 1; T:G:C:A = 1:1:0.4:0.6 & reagent 2; T:G:C:A = 1:1.2:0.6:0.6.

cleavage specificity but do also show pronounced differences (Fig. 3a, lanes 4,7; Fig. 4a,b). These differences can not be described in terms of simple base specificities, and a more detailed interpretation must await a chemical characterization of the azidoacridine-DNA photoproducts.

In order to evaluate the potentials of these compounds as photofootprinting reagents, we analyzed their light-induced reaction with promoter DNA, after binding of E.coli RNA polymerase. The deoP1 promoter chosen for these studies has been characterized as a strong promoter which is independent of CRP-binding [26,30].

Although it was found that Na^+ and especially Mg^{2+} ions cause some inhibition of the photonicking of DNA by reagents 1 or 2 (Table I), photofootprinting experiments exploiting the piperidine catalyzed cleavage could be performed in Na^+ / Mg^{2+} containing buffers (Fig. 3b,c). Binding of the RNA polymerase efficiently protects the DNA from photoreacting with reagent 2 (Fig. 3b,c, lanes 6,7 & Fig. 5) and the protection is analogous to that found for DNaseI digestion (Fig. 3b,c, lanes 2,3 & Fig. 5).

The protection of the DNA from photoreaction with reagent 1 by RNA polymerase is much less pronounced (Fig. 3 b,c lanes 4,5). However, this reagent appears to recognize changes in the DNA structure, some of which are connected to the open complex formation. Two new photoreaction sites are generated within the single stranded DNA loop at A_{-7} and T_{+1} on the coding strand (Fig. 3c, lane 5). Photocleavage at A_{-7} and T_{+1} is temperature dependent

TABLE 1

Buffer conditions	Relative photonicking
TE	100
TE + 20 mM NaCl	60
TE + 50 mM NaCl	35
TE + 2 mM $MgCl_2$	33
TE + 10 mM $MgCl_2$	6

Effect of Na^+ and Mg^{2+} on the photonicking of PUC19 by reagent 1. The experiment was performed analogously to the one described in Fig. 2, and quantification was done by densitometric scanning of photographs of the ethidium stained gel. Reagent 2 gave similar results.

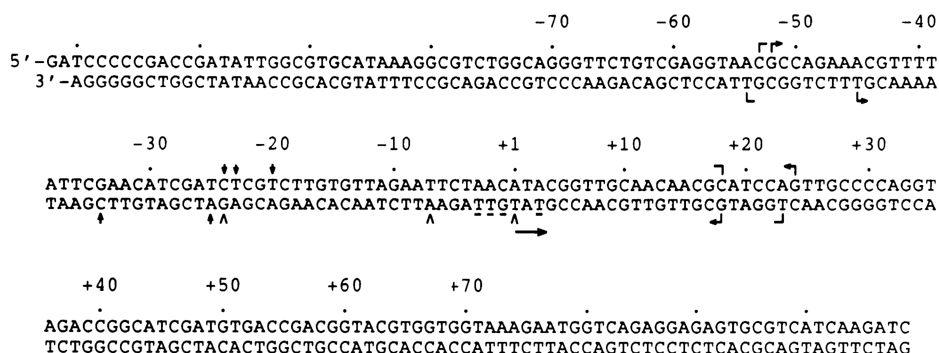


Figure 5 Footprints of RNA polymerase binding to the deoP1 promoter.

Transcription initiation is indicated by (+1) →. DNase I footprint (┌▸ ◂┐) and sensitive sites within the footprint (❙) as well as photofootprint by reagent 2 (┌ ┐) are also shown. Reagent 1 hypersensitive sites are indicated by (v) while sites protected against cleavage by reagent 1 is shown by (----).

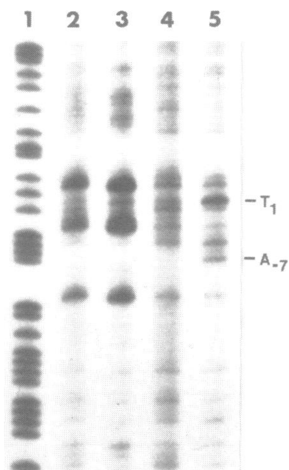


Fig. 6 Temperature dependence of the RNA polymerase induced photocleavage of T_{+1} and A_{-7} by reagent 1.

Lane 1: A+G sequence reaction, lanes 2 , 3: 4°C, lanes 4,5: 30°C. Lanes 2,4: no polymerase, lanes 3,5: in the presence of polymerase. Other experimental conditions were as described in the legend to Fig. 3C, lane 5.

parallel to that of open complex formation (4°C versus 30°C) (Fig. 6). Protection from cleavage by 1 at other positions (most noticeably at positions +2, -3, -11 and -38 on the coding strand (Figs. 3c & 5)) could reflect regions of direct contact between RNA polymerase and DNA.

DISCUSSION

We have found that the photoreaction of azidoacridines 1 & 2 with DNA results in single strand scission, DNA-adduct formation, and the induction of alkaline labile sites in the DNA. We estimate that the yield of adduct formation is 15-30% with approximately one DNA scission for every 10 adducts. We do not know the mechanism involved in adduct formation, but we suggest that they proceed *via* the photogenerated cycloazaheptatetraene intermediate (singlet nitrene rearrangement products) [31-32] of the azidoacridines. These intermediates may react with the bases to form adducts that render the DNA backbone susceptible to hydrolysis. It was observed that the DNA fragments formed by azidoacridine-piperidine cleavage migrate identically to fragments formed by the Maxam-Gilbert sequencing reaction. Thus it is conceivable that 5′-phosphate containing fragments result from the piperidine dependent DNA cleavage. We ascribe the differences in sequence specificity of the two reagents to restrictions on the geometry of the intercalation complex - and thus on the possible photoreactions - imposed by the presence of the linker and the second intercalating acridine ligand in 2. It is unlikely that the sequence specificity is due to the intercalation since we have found that a reagent analogous to 2, where a diazocyclopentadienylcarbonyloxy ligand was substituted for the azidoacridinylamino ligand (ref. 19, compound 3c), photocleaves DNA virtually base and sequence independently [33], showing that the acridine moiety intercalates in DNA with very little sequence preference. We can not rule out that intercalating by the 2-methoxy-6-azido-9-aminoacridine moiety is sequence dependent, although the apparent lack of sequence preference for DNA photonicking (Fig. 4c) argues against this possibility.

The DNA photonicking activity of the azidoacridines could be caused by photoreaction with the deoxyribose units of the DNA

backbone since this reaction appears significantly less base specific than the piperidine dependent DNA cleavage (Fig. 4bc). Previously observed DNA cleavage via deoxyribose reactions most often involves radical species and/or hydrogen abstraction [34-,35] since no good nucleophilic centers are present. It is therefore possible that triplet nitrene photoproducts of 1 and 2 are responsible for their DNA photonicking activity. Hydrogen abstraction is a major pathway for triplet nitrene reaction [36].

The footprinting results (Fig. 5) show that reagent 2 reports an RNA polymerase binding site on the deoP1 promoter which is close to that obtained by DNase I. Minor differences are observed at the bounderies of the RNA polymerase binding site (positions +20 to +25 and -43 to -54) and hypersensitive sites (-18 to -23) are only seen with DNase I. We ascribe these differences to the different mechanism of action of the two probes. DNase I reports hindrance of physical access of the enzyme to the DNA as well as changes in DNA conformation (mainly the width of the grooves) whereas reagent 2 is expected to report inhibition of intercalation, i.e., increase in DNA rigidity and resistance to DNA helix extension and unwinding. Thus it may be concluded that the part of the promoter DNA (-18 to -23) which is accessible to DNase I is kept in a rigid conformation that prevents intercalation.

The mono-azidoacridine 1 does not report any clear footprint of RNA polymerase promoter interaction although some areas of decreased reactivity can be identified (Fig. 5). We take the poor footprinting of reagent 1 as evidence for an intercalation independent DNA photoreaction. It is very interesting, however, that binding of the RNA polymerase to the deo1 promoter greatly enhances two reagent 1 cleavage sites, namely A_{-7} and T_{+1}. These sites are within the single stranded DNA loop formed in the "open complex". Reagent 1 may therefore be used to probe open complex formation, much in analogy to the thermal probe, phenanthroline(CuI) [7,37].

The presently described photofootprinting reagents can be compared to methidium-EDTA(FeII) and phenanthroline(CuI) which are currently being used [4-7, 38]. The azidoacridine reagents have the advantage that they are activated photochemically. They

require neither a transition metal nor a reducing agent and should therefore be applicable for experiments _in vivo_. This aspect is now being pursued. Furthermore, the reactions of _1_ and _2_ are not expected to take place _via_ diffusing species and thus the cleavage sites should precisely reflect the reagent binding sites.

Finally, it should be noted that reagents _1_ and _2_ could be used for time resolved photofootprinting studies using rapid-mixing and flash photolysis [39-40], and for photofootprinting _in situ_ in polyacrylamide gels analogous to use of phenanthroline(CuI) [41].

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