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PHOTOSENSITIZATION OF SV 40 DNA MEDIATED BY PROMAZINE DERIVATIVES AND 4′-HYDROXYMETHYL-4,5′,8-TRIMETHYLPSORALEN

INHIBITION OF THE *IN VITRO* TRANSCRIPTION

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Abstract—*In vitro* transcription by *E. coli* RNA polymerase was carried out on SV40 DNA photoreacted with various promazine derivatives. Inhibition of the template activity was recorded with increasing irradiation times in the presence of promazine derivatives. Promazine covalent adducts on guanine did not terminate RNA synthesis and seemed to be bypassed by the enzyme. HMT (4′-hydroxymethyl-4,5′,8-trimethylpsoralen) photoreaction with DNA was carried out under two conditions: (i) irradiation with $\lambda > 395$ nm favouring monoadduction on pyrimidine residues and (ii) irradiation at 360 nm inducing a maximum of interstrand diadducts. Both adducts were able to terminate RNA synthesis on the phototreated SV40 DNA and using the *O*-methyl-nucleotide sequencing procedure, the termination sites were precisely mapped. Monoadducts on the coding strand and cross-links induced termination two bases away from the covalent adduct, but monoadducts on the noncoding strand did not half RNA polymerase.

DNA molecules can be targeted by photoreactions mediated by several sensitizers such as psoralens, acridines and promazines [1, 2]. Psoralens constitute a class of compounds widely used in the phototreatment of vitiligo and psoriasis. They photobind to DNA forming monoadducts and interstrand cross-links [3, 4]. Promazine derivatives (PZD) are photosensitizers which initiate diversified damage to biomolecules. Several types of lesions result from the photosensitization reactions mediated by PZD; including alkali-labile bonds [5], true single-strand breaks [6] and covalent photoaddition products at guanine residues [7]. Both psoralen and promazine derivatives are lethal to cells, bacteria and viruses upon near-u.v. irradiation [1, 2]. One of the causes of the lethal effect of PZD or psoralen photoadducts is the inhibition of DNA replication [7–9]. However, nothing is known about the interference of these photoadducts with transcription.

The aim of this work is to investigate the photoeffects on DNA of five promazine derivatives (promazine, chlorpromazine, triflupromazine, methoxypromazine and acepromazine) and of a water soluble derivative of psoralen (4′-hydroxymethyl-4,5′,8-trimethylpsoralen, HMT) on *in vitro* transcription of SV40 DNA by *E.coli* RNA polymerase. *In vitro* transcription of SV40 FI DNA by *E. coli* RNA polymerase has been extensively studied by a variety of different methods to gain insight on the molecular mechanisms which drive the initiation of transcription, the elongation of the RNA molecule, the polymerase pausing and the termination, of transcription [10–12]. Employing a defined low molar ratio between SV40 DNA and RNA polymerase and at 18°, it is possible to initiate transcription at a preferred site on the SV40 genome [10].

In this work, we show that the DNA photosensitization mediated by promazine derivatives inhibits RNA transcription. Promazine adducts on guanine moieties seem to be bypassed by RNA polymerase. HMT cycloadducts, on the other hand, promote termination of transcription. These results allow some precision on the SV40 DNA-*E. coli* RNA polymerase transcriptional complex which is the distance between the leading unwindase activity and the catalytic site of *E. coli* RNA polymerase as defined in the model proposed by Gamper and Hearst [11].

EXPERIMENTAL PROCEDURES

Chemicals. PZD (promazine, PZ; chlorpromazine, CPZ; methoxypromazine, MTPZ and acepromazine, ACPZ) were from Specia (Paris, France) except triflupromazine (TFPZ) which was from M.S. Chemicals (Milano, Italy). They were used without further purification. HMT was from HRI (Emeryville, CA) and was used as received. ATP, CTP, GTP and UTP were from Boehringer. Adenyl-(3′,5′)adenosine (ApA) and the 3′-O-methyl analogues of NTP were from P-L Biochemicals. [^{3}H]-CPZ (22 Ci/mmole) was from New England Nuclear. [^{3}H]-HMT (2.0 Ci/mmole) was from HRI (Emeryville, CA, USA). α-[^{32}P]-ATP (400 Ci/

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mmole) and γ-[^{32}P]ATP (3000 Ci/mole) were from Amersham.

Enzymes and DNA. *E. coli* RNA polymerase (EC 2.7.7.6) and horseradish peroxidase (EC 1.11.1.7, HRP) were from Boehringer. Superhelical SV40 DNA (form I) was isolated from African green monkey kidney cells following a modification of the Hirt extraction, essentially as described by Hallick *et al.* [13].

Photosensitization of SV40 DNA. When PZD were used as sensitizers, mixtures containing SV40 DNA (40 μg/ml) and 0.5 mM PZD dissolved in TE buffer (20 mM Tris–HCl, 5 mM EDTA, pH 7.5) were irradiated using a Xenon lamp (Osram XBO150) equipped with a WG305 filter ($\lambda > 290$ nm transmitted, Schott, Germany). When HMT was used as a sensitizer, two different irradiation conditions were used: (i) Mixtures containing 40 μg/ml SV40 DNA and 0.2 mM HMT dissolved in the TE buffer were irradiated using a Xenon lamp (Varian 300 watt) equipped with a liquid filter (0.21 M $CuCl_2$, 1.06 M $CaCl_2$, pH 1.0, 3 cm, $\lambda > 395$ nm transmitted). (ii) Mixtures containing 40 μg/ml SV40 DNA and 54 μM HMT dissoved in the TE buffer were irradiated with the XBO150 lamp equipped with the WG305 filter coupled with a KG1 filter (λ between 290 and 1000 nm transmitted, Schott, Germany). The photosensitization of SV40 DNA by HMT was carried out under such conditions that the number of HMT molecules bound per SV40 genome (determined as described below) was limited to no more than 12. At various irradiation times, aliquots (0.4 μg SV40 DNA) were removed from the irradiated mixtures. The free sensitizer molecules were eliminated by two chloroform extractions and three ether extractions and the DNA was ethanol precipitated. When the modified DNA was reirradiated, DNA pellets were resuspended into 10 μl of the TE buffer and again irradiated using the XBO150 lamp equipped with a WG360 filter ($\lambda > 310$ nm transmitted, Schott, Germany) and then ethanol precipitated.

Reaction of SV40 DNA with PZD cation radicals. The five PZD cation radicals (PZD^+) were enzymatically generated in the dark, in the presence of SV40 DNA (0.4 μg) using the method of Duran *et al.* [14] as described by Merville *et al.* [7]. After reaction, the mixtures were phenol extracted prior to being treated as described for the irradiated samples.

RNA synthesis on reacted SV40 DNA. Transcription assays were carried out as described by Reisbig and Hearst [10], except that the unwinding buffer described by Gamper and Hearst [11] was used (40 mM HEPES, 10 mM $MgCl_2$, 20 mM KCl, 0.1 mM EDTA, 0.1 mM Dithiotreitol, pH 8.0; UW buffer). All the solutions were preincubated at 18°, and transcription experiments were performed at the same temperature.

The DNA pellets (0.4 μg) were resuspended into 4 μl of the initiation mixture (165 μM ApA, 10 μM GTP, 20 μM UTP and 0.8 μCi α-[^{32}P]-ATP dissolved in the UW buffer). The initiation step was begun by adding 1 μl RNA polymerase (70 ng). The elongation step was started by adding 5 μl of the elongation mixture (320 μM ATP, 310 μM UTP, 320 μM GTP, 330 μM CTP and 200 μg/ml heparin dissolved in the UW buffer). After 25 min, elongation was stopped by adding 50 μl of stop solution (20 mM Tris–HCl, 200 mM NaCl, 10 mM EDTA and 0.5% SDS; pH 8.0). The RNA transcripts were ethanol precipitated, the pellets were washed with 70% ethanol and resuspended into 7 M urea, 0.05% bromophenol blue, 0.05% xylene-cyanol dissolved in the TBE buffer (0.1 M Tris, 0.1 M boric acid and 2 mM EDTA). [^{32}P]-ATP incorporation in the RNA has also been measured by trichloroacetic acid (5% w/v) precipitation on filters during 2 × 20 min. The filters were washed with ethanol and liquid scintillation counting.

The measurement of the rate of initiation was carried out without ApA but using γ-[^{32}P] ATP as radiolabel. The [^{32}P] incorporation into the transcript has been measured 5 min after the addition of the enzyme and nucleotides by trichloroacetic acid precipitation on filters.

Sequencing of the RNA transcripts. In order to determine the nucleotide sequence of RNA transcripts, we have used the inhibitor method [15] as described by Reisbig and Hearst [10]. The intiation step was made by mixing 2 μg SV40 DNA in the initiation solution (given above) in a total volume of 25 μl and then adding 1.8 μg RNA polymerase (0.23 μg/μl). After 5 min, the mixture was divided in five aliquots of 5 μl each: one for the control and four for analyzing the position of each of the four NTPs. Elongations were started by adding 5 μl of the elongation solution (given above) to the control reaction and 5 μl of 160 μM ATP, 200 μg/ml heparin to each of the other reactions plus (i) when ATP was analyzed, 160 μM of each of the three other NTPs and 95 μM 3′-O-methyl ATP; (ii) when GTP was analyzed, 160 μM CTP, 160 μM UTP and 50 μM 3′-O-methyl GTP; (iii) when CTP was analyzed, 10 μM CTP, 160 μM GTP, 160 μM UTP and 25 μM 3′-O-methyl CTP; (iv) when UTP was analyzed 160 μM CTP, 160 μM GTP and 100 μM 3′-O-methyl UTP. Elongations were stopped after 25 min and samples were treated as described above.

Polyacrylamide gel electrophoresis of the RNA transcripts. Before loading on the gels, the RNA transcripts were denaturated by heating for 1 min at 90° and then chilling in an ice bath. RNA transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (40 cm long, 0.4 mm thick, 7 M urea, 8% acrylamide). The gels were run at constant power (60 Watts) until the bromophenol blue ran off the end of the gels. Autoradiography of the gels was carried out using Fuji-X-ray films at −80° with a Kodak intensifying screen.

^{3}H-CPZ and ^{3}H-HMT covalent binding to the SV40 DNA. All the reactions (CPZ photosensitized, CPZ cation radical mediated or HMT photosensitized) were carried out as described above except that, (i) 25 μCi [^{3}H]-CPZ, supplemented with 0.46 mM unlabeled CPZ were used in the photosensitized reaction; (ii) 5 μCi [^{3}H]-CPZ, supplemented with 0.49 mM unlabeled CPZ were used in the CPZ cation radical mediated reaction; (iii) the concentrations of [^{3}H]-HMT were the same as those used for the two irradiation conditions described above. After the reactions, samples were worked up as described above except that after the last ethanol precipitation,

the pellets were resuspended in the TE buffer and [^{3}H] was determined by scintillation counting.

Relaxation of SV40 RFI DNA by digestion with Eco RI. 4.8 μg SV40 DNA has been digested by 6 U of Eco RI in the presence of ethidium bromide (140 μg/ml) during various times (0, 1, 5, 15 and 60 min) at 37°. Aliquots were withdrawn at these times mixed with EDTA (100 mM final concentration) and split in two. One is used to measure the percentage of relaxation by agarose gel electrophoresis and scanning photodensitometry; the other half is used as substrate of transcription after cleaning up the DNA by phenol extraction and ethanol precipitation.

RESULTS

Transcription of SV40 DNA photosensitized by PZD

In order to initiate transcription of SV40 DNA at a single position (at nucleotide 2556, numbered according to Reddy *et al.* [16]), all the transcription experiments are carried out at 18° and with a constant molar ratio of 1.5 between RNA polymerase and SV40 DNA [10]. The effects of the various PZD photoreactions on the template activity of SV40 DNA for transcription are determined by measuring the amount of α-[^{32}P]-ATP incorporated into the RNA transcripts. PZD photoreactions clearly lead to an inhibition of the amount of [^{32}P] incorporated in the RNA molecules, but with efficiencies varying with the derivative. CPZ appears to be the most active when ranked by kinetic rate constants (k = 0.16 min^{-1}). MTPZ (k = 0.06 min^{-1}), PZ (k = 0.03 min^{-1}) and TFPZ (k = 0.02 min^{-1}) are less active, whereas ACPZ is without measurable activity. Photoexcited PZD are known to form covalent adducts with DNA [7] and these adducts are demonstrated to block *E. coli* DNA polymerase when copying single-stranded DNA [7]. Polyacrylamide gel electrophoresis of the RNA transcripts are then carried out to investigate whether a similar phenomenon is responsible for the inhibition of transcription. The analysis of the products of transcription performed on SV40 DNA photosensitized with MTPZ, PZ, CPZ and TFPZ lead to similar results, ACPZ is without effect (data not shown). On the autoradiography it can be observed in the channels corresponding to unirradiated samples that the transcripts have a high molecular weight and remain at the top of the gel. In addition, as described by Reisbig and Hearst [10], the natural pausing sites of the RNA polymerase are observable. In the channels corresponding to irradiated samples, the amounts of RNA transcripts of high molecular weight decrease in proportion to the α-[^{32}P]-ATP incorporation measurements. The bands corresponding to the natural pausing sites of the RNA polymerase are also observable, but the intensity of these bands decrease with the irradiation time. However, the PZD photoreactions do not lead to the appearance of any new bands on the gel corresponding to adduct induced termination of RNA synthesis (data not shown). These results seem somewhat surprising because under the conditions of phototreatment used covalent photobinding of PZD on SV40 DNA occurs. Figure 1 shows a time course of CPZ photoaddition

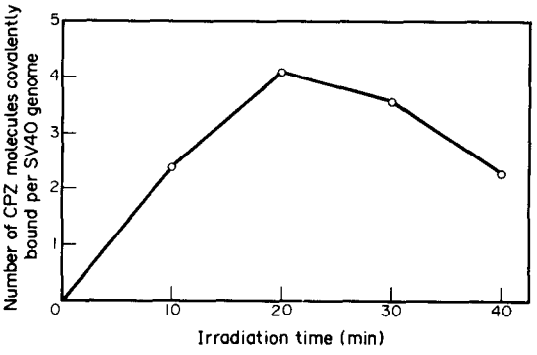

Fig. 1. Kinetics of photoaddition of [^{3}H]-chlorpromazine (CPZ) to SV40 DNA. The amount of covalently bound drug per SV40 genome is plotted versus the irradiation time (min). Aliquots are withdrawn during irradiation and phenol-, chloroform- and ether extracted, then ethanol precipitated before counting.

to SV40 DNA. During the reaction the amount of covalent adduct increases linearly up to 20 min leading to the addition of 4 CPZ covalently bound per SV40 genome. We conclude that the covalent adducts formed during the photoreaction are probably not involved in the inhibition of transcription observed on photoreacted DNA.

A possible explanation of this loss of template activity could be that the photoreactions lead to a relaxation of the superhelical SV40 DNA. Superhelical DNA was shown to be a better template for transcription than its corresponding allomorphic forms [17]. Agarose gel analysis of the SV40 DNA during the course of the photosensitization reaction reveals that the amount of SV40 FI DNA decreases gradually with a parallel increase of relaxed SV40 FII DNA. Among the various derivatives used, CPZ is the most active DNA breaker and ACPZ is without measurable effect.

In order to ask whether the conversion of the supercoiled template into a relaxed form could be the event reponsible for the inhibition of transcription, the SV40 DNA is nicked using another technique. A gradual conversion into SV40 FII DNA is obtained by digesting the SV40 FI DNA using Eco RI in the presence of ethidium bromide [18]. SV40 DNA with various FI concentrations is then used as substrate for transcription using *E. coli* RNA polymerase. The determination of α-[^{32}P]-ATP incorporated in the transcripts is presented in Fig. 2. These results clearly show that the progressive loss of supercoiled template does not lead to an inhibition of transcription. Moreover these transcripts are analyzed by denaturing polyacrylamide gel electrophoresis and do not reveal any differences in the length of the synthesized RNA molecules (data not shown). These data demonstrate that the inhibition of transcription recorded during the PZD photoreactions cannot be due to the observed SV40 DNA relaxation.

To find out whether or not the observed inhibition induced by the photosensitized reaction could be attributed to a defect in the initiation of the transcription, experiments are performed using γ-[^{32}P]-ATP as initiating nucleotide. In these conditions, the

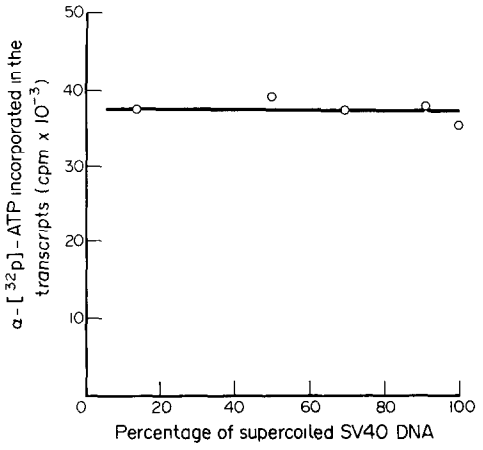

Fig. 2. Effect of the SV40 relaxation on the level of transcription. SV40 FI DNA is partially relaxed using Eco RI in the presence of ethidium bromide. The percentage in the contain of supercoiled template is measured by agarose gel electrophoresis and scanning photodensitometry. The amount of α-[^{32}P]-ATP incorporated in RNA is measured by TCA precipitation and scintillation counting.

amount of radioactivity incorporated in the transcripts is a measure of the rate of initiation [19]. The use of SV40 FI DNA photosensitized with PZD as substrate for transcription shows that the percentage of γ-ATP incorporated in RNA gradually decreases with the irradiation time (Fig. 3). The inhibition of γ-ATP incorporation in the transcripts follow single hit kinetics. CPZ is the most efficient derivative whereas ACPZ is without effect. The photosensitization reactions mediated by PZD inhibit the incorporation of the first nucleotide into the RNA thus lead to a defect in the initiation.

To demonstrate definitively that promazine covalent adducts do not lead to site-specific termination of transcription, it is necessary to perform transcription on a superhelical template containing covalent PZD adducts.

Transcription on SV40 DNA reacted in the presence of PZD cation radicals

PZD cation radicals were demonstrated to be the photochemical intermediate of PZD photoaddition to DNA [7]. Moreover, these cation radicals can be generated *in situ* in the dark by the enzymatic action of HRP in conjunction with isobutyl alcohol and oxygen. These radical species are shown to be rather poor DNA breakers (except for the MTPZ cation radical [6]). PZD cation radicals are produced in the dark and in the presence of SV40 DNA. Under these conditions it is possible to obtain a covalent addition up to 22 CPZ molecules per SV40 genome. Transcription experiments are carried out on SV40 DNA reacted with the various PZD cation radicals. The analysis of the transcription products by denaturing polyacrylamide gel electrophoresis does not reveal the presence of termination bands. Thus, even in the absence of single-strand breaks and DNA unwinding, PZD adducts do not block RNA polymerase during transcription (data not shown).

Two hypotheses could account for why PZD adducts do not stop RNA polymerase: (1) PZD adducts, like other kinds of base adducts, do not interfere with the forward progress of RNA polymerase ternary complexes; (2) PZD adducts are in a conformation such that they were unable to stop the RNA polymerase.

In vitro transcription on HMT photoreacted SV40 DNA

In order to ascertain whether DNA base adducts could block RNA polymerase transcription, we use a psoralen derivative (HMT) because this molecule is known to form, upon near-u.v. irradiation, monoadduct on thymine residues which are almost perpendicular to the DNA helical axis [20]. After absorption of a second photon, psoralen monoadducts can undergo a reaction with a pyrimidine residue to the opposite strand in an adjacent base pair leading to the formation of a cross-link [3, 21].

By using long near uv wavelengths ($\lambda > 395$ nm) to irradiated DNA–psoralen complexes, it is possible to favour monoadducts over crosslinks [22]. In the first set of experiments, SV40 DNA is irradiated in the presence of HMT using a $\lambda > 395$ nm irradiation source. Under these conditions, HMT monoaddition occurs mainly at the 3′ side of the thymine [23]. No single-strand breaks are detectable by agarose gel electrophoresis of SV40 DNA photoreacted under such conditions (data not shown). Figure 4 shows a polyacrylamide gel electrophoretic analysis of the products obtained when transcription experiments are carried out on SV40 DNA containing predominantly HMT monoadducts. In the channel corresponding to unirradiated sample, the radioactivity remains at the top of the gel and corresponds to full length transcript. With increasing irradiation times, new bands can be detected on the gel and are attributed to termination of RNA synthesis opposite HMT adducts. Moreover, the intensity of these

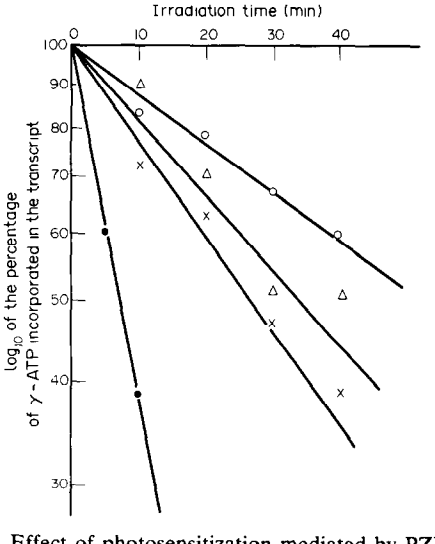

Fig. 3. Effect of photosensitization mediated by PZD on the initiation of transcription. Incorporation of γ-[^{32}P]-ATP in the transcript is determined after 5 min. SV40 DNA is photosensitized during various times in the presence of (●) CPZ, (×) MTPZ, (△) PZ and (○) TFPZ. ACPZ is without effect on initiation.

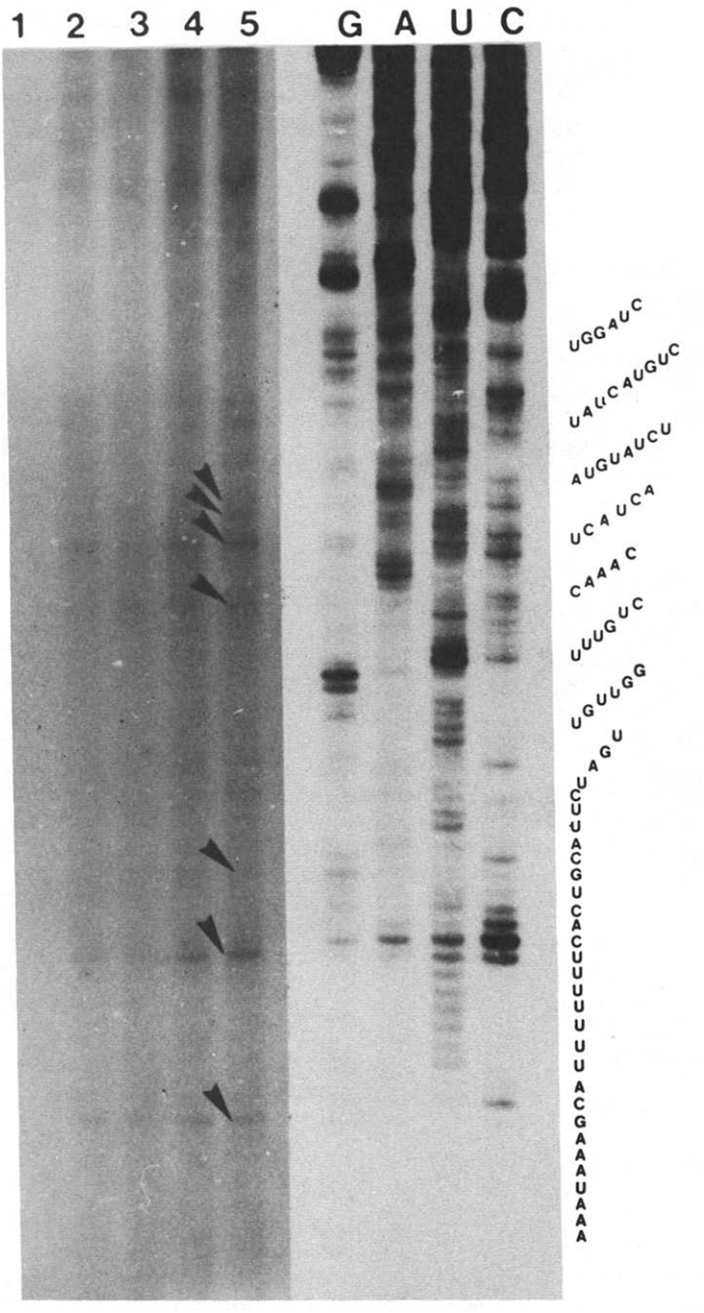

Fig. 4. Denaturating polyacrylamide (8%) gel analysis of the products synthesized by RNA polymerase when transcribing SV40 DNA irradiated in the presence of 0.2 mM HMT (selecting $\lambda > 395$ nm). The SV40 DNA FI is irradiated for 0 min (1) 60 min (2), 120 min (3), 180 min (4) and 240 min (5). The arrows show the position of the bands with intensities which increase with irradiation times. The *O*-methyl sequencing performed on unmodified template is running alongside as reference with the usual G, A, U and C channels.

bands increases with the irradiation time. The last nucleotide incorporated in the RNA transcripts can be determined by running alongside an *O*-methyl nucleotide sequencing procedure on unreacted SV40 DNA following the method of Axelrod *et al.* [15]. Figure 5 reports the position of the termination bands in the RNA nucleotide sequence. All the termination sites occur two bases away from thymine residues situated on the SV40 DNA coding strand. From these results, it is concluded that a HMT adduct oriented inside the DNA helix could promote termination of transcription.

Moreover, a second set of transcription experiments are performed on SV40 DNA molecules photoreacted under conditions where HMT can cross-link the two DNA chains. In the presence of a psoralen diadduct, the RNA polymerase should be unable to propagate the "bubble" as described by

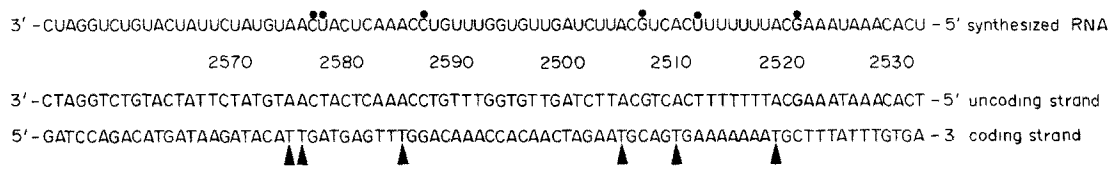

Fig. 5. Position of the RNA synthesis termination sites with reference to the sequence of the two SV40 DNA strands. The bases indicated by (●) are the last RNA bases incorporated in the RNA transcripts when RNA polymerase transcribed the SV40 DNA photoreacted with HMT + light >395 nm. The arrows indicate the position of the most probable HMT adduct which leads to the termination of transcription.

Gamper and Hearst [11] and the progress of the enzyme along the DNA should be stopped. SV40 DNA is photoreacted in the presence of HMT using 360 nm light. Photoaddition to HMT to superhelical SV40 DNA causes a decrease in its electrophoretic mobility on agarose gels. This indicates an unwinding of the supercoiled SV40 DNA similar to that reported by Wiesehahn and Hearst [24]. Figure 6 shows the relationship between mobility and the number of HMT molecules covalently bound per SV40 molecule. These results point out the limit between which the transcription experiments have to be performed to avoid a decrease in template activity due to unwinding. This is the reason why SV40 DNA photoreaction with HMT is limited to irradiation conditions leading to a minimal level of HMT covalently bound per SV40 DNA. Denaturing polyacrylamide gel electrophoresis of the transcription products carried out on SV40 DNA modified under these conditions, reveal the presence of several bands having intensities which increase with the irradiation time. These bands correspond to terminations of transcription. By using the *O*-methyl sequencing procedure, the positions of the termination sites induced by HMT photoreaction are precisely mapped on the SV40 genome. Figure 7 gives the positions of these termination sites. Three classes of stops can be differentiated: (1) one which occurs, as described above, two bases before a monoadduct on the 3' side of a thymine (noted (▲) in Fig. 7); (2) four stops where RNA polymerase is halted one base away from monoadducts probably situated on the 5' side of a thymine residue (noted (■) in Fig. 7); and (3) six stops where the terminations are likely due to cross-links situated on the 5' side of both thymines, one on the coding strand and one on the noncoding strand (noted (★) in Fig. 7).

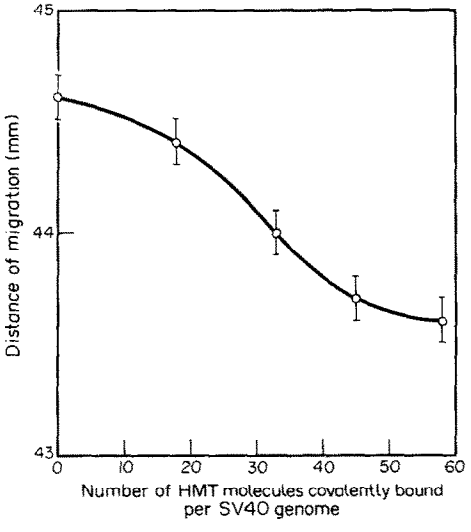

Fig. 6. Variation of the electrophoretic mobility of SV40 FI DNA on a neutral agarose gel (1%) as a function of the amount of HMT covalently bound per SV40 genome. SV40 DNA FI is irradiated in the presence of [^{3}H]-HMT for various periods of time with $\lambda = 360$ nm. The number of [^{3}H]-HMT covalently bound is then determined by scintillation counting after phenol-, chloroform- and ether-extraction and ethanol precipitation. The various aliquots of SV40 DNA were then analyzed by agarose gel electrophoresis and photographed after ethidium bromide fluorescence detection. The distance of migration is then determined.

From these data, it appears that all the observed termination sites are (i) due to cycloaddition of HMT to thymine residues situated on the coding strand, and (ii) situated two bases before the HMT adduct. Thus 3' side adducts cause a stop two bases away from the modified thymine while 5' side adducts cause a stop one base away from the modified thymine.

DISCUSSION

Superhelical SV40 DNA is an especially good template to study RNA transcription [10, 12] and to investigate the effects of chemical modifications on the rate of transcription [25]. In this work, we have demonstrated that various promazine derivatives induce upon irradiation SV40 DNA modifications causing an inhibition of *in vitro* transcription by *E.coli* RNA polymerase. The inhibition of transcription does not seem to be correlated with the induction of single-stranded breaks recorded during the PZD photosensitization reactions [6]. A progressive relaxation of the SV40 supercoiled template into a relaxed form cannot induce a decrease in the level of transcription. This result contrasts with the conclusions presented in the literature [17, 26–28] where RNA initiation is shown to increase as template acquired more negative superhelical turns. Under our experimental conditions, the SV 40 promoter turns out to be still utilized efficiently even on the relaxed SV40 DNA. However, the incorporation of the first nucleotide in the RNA molecule during

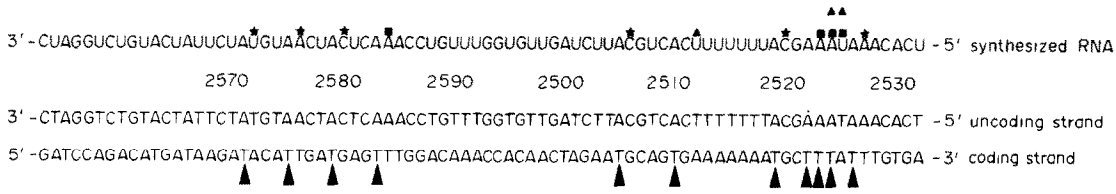

Fig. 7. Position of the RNA synthesis termination sites with reference to the sequence of the two SV40 DNA strands. The bases indicated by the symbols are the last RNA bases incorporated into the RNA transcripts. The terminations are interpreted as to be due to HMT cross-links (★), to HMT monoadducts on the 5′ side of thymine residues (■) and HMT monoadducts on the 3′ side of thymine residues (▲). The arrows indicate the position of the adducted bases responsible for the termination of RNA synthesis.

the initiation appears to be strongly affected by PZD photoreaction. Thus, the photosensitized reaction causes a defect in the initiation of the RNA synthesis. Similar results have been obtained when a PM2 DNA is reacted with a single-stranded specific reagent such as *N*-cyclohexyl-*N'*-β-(methylmorpholinium) ethyl carbodiimide [29].

Covalent addition on guanine residues has been demonstrated to be an important DNA lesion induced during the PZD photoreaction [7, 30]. These adducts can be introduced into the DNA molecule through the photosensitizing reaction or in the dark by an enzymatic reaction which can produce a high steady state concentration of PZD cation radical. The generation of the covalent adduct on the guanine residue of SV40 DNA by these two procedures does not seem to inhibit RNA transcription. To explain this lack of inhibition, it could be postulated that the promazine adduct on guanine residues do not destabilize the DNA helix. Since no DNA unwinding can be detected after both the photoreaction or the enzymatic reaction, a possible conformation of the adduct could be that the guanine base is stacked inside the helix with the promazine ring unstacked outside the helix and located in the DNA groove. Experiments are now in progress to elucidate the stereochemistry of the promazine–guanine adduct in DNA. Regardless of the conformational details of the PZD adduct, it still remains to find out whether or not the bypass of the PZD adduct by RNA polymerase could be relevant. The hypothesis that the PZD adduct is in a non-intercalative position is reinforced by the results obtained with HMT photoreacted SV40 DNA. Indeed, these HMT adducts, which are situated between base pairs and due to this kind of stereochemistry are able to promote termination of transcription.

HMT monoadducts are able to terminate RNA synthesis when they are situated on the coding strand. All the terminations attributable to HMT monoadducts occurred before adenine residues in the RNA molecules, thus induced by HMT adducts to thymine on the coding strand. As HMT reacted equivalently with the two DNA strands, it can be concluded that the adducts on the noncoding strand do not terminate synthesis or even cause RNA polymerase pausing (data not shown). From the data presented above, it turns out that RNA polymerase stops two bases away from the adducts. HMT can form two possible diastereoisomeric forms of the furan side monoadduct, one on the 5′ side and the other on the 3′ side of the pyrimidine residues. The distribution of the RNA termination sites due to HMT monoadducts could be explained by postulating that RNA polymerase stops (i) two bases away from thymine residues with the HMT cycloadduct at the 3′ side of the base, and (ii) one base before the thymine residues with the HMT adduct on the 5′ side. By analyzing carefully the SV40 DNA sequences upstream to the RNA synthesis stops we can conclude that RNA polymerase also stops two bases before HMT diadducts.

Independent crystallographic and solution studies [31] indicate that single intercalating agents, such as psoralens, shows a marked preference for CpG and TpG sequences in short self complementary oligonucleotides. This binding selectivity is probably one of the events which is responsible for the preferential HMT adduction to the 3′ side of the thymine [23]. However, in the data presented above, the transcription termination sites seem to be promoted by putative psoralen adducts not only on the 3′ side of thymine but also by HMT adducts on the 5′ side of thymine.

Psoralen photoaddition have been shown to introduce appreciable modification in the structure of DNA, such as lengthening the helix by the equivalent of about a base pair per photobound adduct [2, 32]. It has also been shown that the thymine and psoralen moieties remain planar even after the reaction and do not introduce appreciable bends in DNA [33]. Thus from the results presented above, it can be concluded that it is mainly the addition of the psoralen adduct in between the two base pairs which caused a halt in the progression of the ternary complex. The RNA polymerase molecule could not bypass the adduct, then stops its progression on the DNA chain and leaves the DNA molecule with a collapsing of the bubble.

When encountering a psoralen cross-link, RNA polymerase cannot unwind and separate the two DNA strands to propagate the local opening along the substrate. However, as shown above, the enzyme also terminates synthesis two bases before the HMT linked to the two DNA chains. From the measurements of the unwinding angle during the progression of the RNA polymerase, it has been argued [11] that the size of the unwound region is rigidly controlled by the enzyme. The most straightforward interpretation of our observations is that under our conditions the most probable distance between the leading unwindase and the catalytic site of the RNA

polymerase could be equivalent to the distance between the adduct and the last nucleotide read by the enzyme, thus around 6.8 Å.

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