Photochemical crosslinking of transcription complexes with psoralen. I. Covalent attachment of in vitro SV40 nascent RNA to its double-stranded DNA template

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ABSTRACT

14C-labeled SV40 DNA has been transcribed with E. coli RNA polymerase using 3H-labeled ribonucleotide triphosphates as precursors. The resulting transcription complexes were then photochemically crosslinked with the psoralen derivative, 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), at 37°C and analyzed in SDS-sucrose gradients. It was found that the photochemical crosslinking procedure caused the nascent RNA chains to co-sediment with their double-stranded (helical) SV40 templates in the denaturing sucrose gradient. This result and several control experiments suggest that covalent linkages have formed between nascent RNA and helical DNA after the photochemical reaction. The crosslinking phenomenon was observed to be independent of the superhelical state of the DNA used as the template. Prior addition of EDTA to stop the transcription is not required for successful crosslinkage.

INTRODUCTION

Psoralens are a class of planar furcocumarins that photoreact with biological systems. Several of them are being used widely to treat skin diseases (1). Their interaction with cellular components, mostly with nucleic acids, have been studied on the molecular level for the past decade. Similarly to other drugs such as ethidium bromide psoralens react with DNA by first intercalating between two adjacent base pairs of the DNA double helix (2,3). After irradiation with long wavelength UV light (320-380 nm), the psoralens react covalently with the pyrimidine bases (4,5). If the intercalation site of psoralen in the DNA helix contains pyrimidines on opposite strands, a pyrimidine-psoralen-pyrimidine covalent linkage can be generated photochemically. The two DNA strands are no longer separable by hydrogen bond-disrupting agents such as heat or alkaline pH (6,7,8,9).

In fact, psoralens are able to covalently crosslink double helices of RNA and DNA-RNA hybrid as well. By denaturation electron microscopy and other biochemical studies, we have shown recently that interstrand cross-links form in double-stranded reovirus RNA as well as in DNA-RNA hybrid.
prepared in vitro on bacteriophage fd DNA template (10,11). The latter observation led us to attempt the crosslinking of transcription complexes using the psoralen-nucleic acid photoreaction.

The sequence of double-stranded DNA template is transformed into RNA through the Watson Crick base pairing mechanism during transcription. This suggests that inside the active transcription machinery there must exist a short DNA-RNA hybrid region where the nascent RNA is base paired transiently to its parental DNA strand before it is displaced from the DNA template. It is interesting to determine if psoralens can intercalate into this hybrid region and crosslink the nascent RNA to the helical DNA template. If successful the technique would provide a unique method of fixing the structure of transcription complexes in vitro and in vivo.

MATERIALS AND METHODS

Materials. $^{14}$C-labeled SV40 I (supercoiled form, 4000 cmp/μg) was kindly supplied by Hisao Yokota and Dr. James Bartholomew. E. coli RNA polymerase containing the σ subunit (the holoenzyme) is a gift from Drs. Tim Kovacic and James C. Wang. Unlabeled ribonucleotide triphosphates and $^3$H-labeled cytosine triphosphates were purchased from ICN Inc. AMT has been synthesized by Isaacs et al., as described before (10).

Nicking of SV40 I. $^{14}$C-SV40 II DNA (nicked form) was generated by incubating 6.6-10.0 μg$^{14}$C-SV40 I in 1 ml of 0.4 μg/ml DNAase I (Worthington), 3 mM MgCl₂, 10 mM Tris•HCl (pH 8.0), and 1 mM EDTA at 0°C for 70 minutes. The reaction mixture was made 4 mM in EDTA, extracted stepwise with phenol and ether, and then dialyzed against 0.01 M Tris•HCl (pH 8.0), 0.001 M EDTA.

In Vitro Transcription of SV40 DNA. A typical reaction mixture contains 9 μg/ml (SV40 I) or 4.5 μg/ml (SV40 II) of $^{14}$C-labeled DNA (diluted with cold SV40 DNA to appropriate specific activity), 37 μg/ml RNA polymerase, 200 μM of each of the four unlabeled ribonucleotide triphosphates, 25 μM of $^3$H-CTP (100 Ci/mole), and 200 μg/ml of bovine serum albumin in 10 mM Tris•HCl (pH 7.5), 50 mM KCl, and 10 mM MgCl₂. After incubation of the reaction mixture at 37°C for an appropriate amount of time, the transcription was terminated by adding EDTA (0.5 M, pH 8.0) to a final concentration of 10 mM. The extent of transcription was assayed by using the DEAE cellulose paper—0.5 M sodium phosphate washing procedure (11).

Photochemical Reaction of Transcription Complexes with 4'-Aminomethyl-4,5',8-trimethylpsoralen Hydrochloride (AMT). In most cases, a transcription complex of volume 150 μl was mixed with 150 μl of 450 μg/ml AMT in 10
mM Tris·HCl (pH 7.5), 50 mM KCl. This solution was then put into a quartz cell and irradiated for 20 minutes with long wavelength UV light (100 mW/cm², 340-380 nm) as described before (10,11). The transcription mixture had been maintained at 37°C throughout the mixing and irradiation procedures.

Analysis of Transcription Complexes in SDS-Sucrose Gradients. The (AMT + UV)-treated complexes as well as the controls were dialyzed at 4°C against 1 M NaCl, 10 mM Tris·HCl (pH 8.0), 1 mM EDTA for 24 hours, two changes, and then against 10 mM Tris·HCl (pH 8.0), 1 mM EDTA. After dialysis, 180-220 μl of each sample was made 1% in sodium dodecyl sulfate (SDS), heated at 37°C for 1 minute, and quenched to room temperature. They were layered onto 5.2 ml 5%-23% sucrose gradients in 100 mM LiCl, 10 mM EDTA, 0.1% SDS (pH 7.6) (12) and sedimented at 4°C, 45,000 rpm, for 4 hours in Beckman SW 50.1 or SW 50L rotors. Fractions of volume 230-300 μl were collected from the bottom of the tubes and burned to completion in a Packard Sample Oxidizer. The resulting H₂O and CO₂ were separated, dissolved in 15 ml monophase-40 and (9 ml carbo-sorb + 12 ml permafluor-V), respectively, and counted in a Beckman LS-230 liquid scintillation counter.

RESULTS

Co-sedimentation of the DNA and RNA of SV40 Transcription Complexes in SDS-Sucrose Gradients After Photochemical Crosslinking. SV40 I or SV40 II DNA has been transcribed by E. coli RNA polymerase in vitro for 1 minute and 5 minutes, respectively. Approximately the same amount of RNA was produced in each case: RNA (nucleotides)/DNA (base pairs) = 3.8.

One half of each transcription mixture had AMT added to it and was UV irradiated. The crosslinked and untreated samples were then dialyzed and sedimented in 0.1% SDS-5%-23% sucrose gradients. The sedimentation profiles of untreated transcription complexes of SV40 I and SV40 II are shown in Figures 1(a) and (c), respectively. The ¹⁴C-DNA separated well from the ³H-RNA in both cases, with the ³H-RNA sedimenting near the top of the gradients. From the positions of D. melanogaster 18S and 26S ribosomal RNAs in these gradients (P. Wollenzien, D. Youvan, unpublished results), we have estimated the sedimentation coefficients of SV40 I and SV40 II to be 21-23S and 16-17S, depending on the details of the procedures. These S values agree with those reported in the literature (13).

After crosslinking, however, both SV40 I and SV40 II sediment much faster (Figs. 1(b) and 1(d)). For SV40 I, the DNA seems to have fallen into two populations of molecules—one sediments at 26S and the other has an
Fig. 1. 0.1% SDS—5-25% sucrose gradients of (a) SV40 I transcription complexes; (b) (AMT + UV)-treated SV40 I transcription complexes; (c) SV40 II transcription complexes; (d) (AMT + UV)-treated SV40 II transcription complexes. DNA and RNA have been labeled with \(^{14}C\) (o-o-o) and \(^{3}H\) (e-e-e), respectively. For convenience, only fractions number 1 to 20 are shown in this figure as well as in Figures 2, 3, 4 and 5.
average $S$ value greater than 33S. All of the SV40 II has sedimented to the bottom of the tube after the photochemical crosslinking. Accompanied with these observations was the increase of $S$ values of a significant fraction of the tritium counts that originally sedimented near the top of the gradient. Furthermore, except in the 16S-23S region of the SV40 I gradient, the radioactivity of these fast-sedimenting RNA species is associated intimately with the DNA counts in the gradient. From the specific activities of DNA and RNA, it can be calculated that for the bottom fractions (fraction 1), approximately 1.1 pg and 1.6 pg of RNA are associated with 1 pg of SV40 I (Fig. 1(b)) and SV40 II (Fig. 1(d)), respectively.

The above results suggest strongly that after treatment of transcription complexes with AMT and UV, covalent linkages are generated between the nascent RNA chains and the double-stranded SV40 DNA template (Form I or II). However, they do not show whether AMT or long wavelength UV light alone will create this effect.

A Combination of AMT and Long Wavelength UV Light Is Necessary for Covalent Crosslinking of Nascent RNA and DNA Template. Psoralens are known to react covalently with DNA only when long wavelength UV light is present. To test whether the crosslinking of RNA and DNA in the SV40 transcription complexes was caused by the combined effect of UV light and AMT, transcription complexes have been treated at 37°C with either AMT or UV and analyzed in the SDS-sucrose gradient. It can be seen from Figure 2 that, in neither reaction was any nascent RNA crosslinked to the DNA template. This means a photoreaction involving the double-stranded DNA, the nascent RNA, the AMT molecules, and possibly RNA polymerase is required to generate the covalent linkage between DNA and RNA of transcription complexes. We will discuss the possible involvement of RNA polymerase in the crosslinking reaction in the DISCUSSION section.

Intact Structure of the Ternary Complexes is Required for the Crosslinking Reaction to Occur. The crosslinking phenomenon appears to be specific for native transcription complexes. A transcription complex of SV40 I was prepared as described above. It was made 0.1% in SDS, the protein-denaturing agent, and incubated at 37°C for 1 minute before the addition of AMT and irradiation with UV light. In this case, there was essentially no $^3$H-radioactivity associated with the $^{14}$C-counts (Fig. 3(a)).

Another control experiment in which a transcription mixture containing no RNA polymerase was photoreacted with AMT. The $^{14}$C-radioactivity separated well with the $^3$H-radioactivity in SDS-sucrose gradient (Fig. 3(b)).
Fig. 2. 0.1% SDS—5–25% sucrose gradients of SV40 I transcription complexes that have been treated with either UV light or AMT. (a) Transcription complexes irradiated with long wavelength UV light (100 mW/cm²) at 37°C for 20 minutes; (b) Transcription complexes incubated with 225 µg/ml of AMT at 37°C for 20 minutes.

Nascent SV40 RNA Is Crosslinked to DNA Template in the Presence of Low Concentrations of AMT. The photoreaction of SV40 transcription complexes described above utilized high concentrations of AMT (Figs. 1,2, and 3). During the irradiation, the molar ratios of AMT to DNA base pairs were 114:1 for SV40 I and 223:1 for SV40 II (225 µg/ml of AMT; 4.5 µg/ml of SV40 I and 2.3 µg/ml of SV40 II). Because such high concentration of AMT may disturb the structure of the transcription complex as well as other cellular components, for in vivo crosslinking experiments in the future, it is important to see if low concentrations of AMT are able to induce covalent linkages between nascent RNA and the helical DNA templates.

Transcription complexes of ¹⁴C-SV40 II have been prepared and treated with (AMT + UV) as before except that the AMT concentration was reduced to 3.3 µg/ml. This corresponds to a AMT to DNA (in base pairs) ratio of 3.3:1.
Fig. 3. (a) SV40 I transcription complexes were denatured by SDS, photochemically crosslinked with AMT, and analyzed in 0.1% SDS—5—25% sucrose gradient. (b) A transcription mixture of SV40 I containing no RNA polymerase was incubated at 37°C for 1 minute, treated with (AMT + UV) at 37°C for 20 minutes, and analyzed in 0.1% SDS—5—25% sucrose gradient.

As can be seen from Figure 4, the photoreaction again brought all the DNA and a fraction of the RNA toward the bottom of the tube as in Fig. 1(d). The quantity of RNA covalently associated with the DNA in the gradient is 1.51 µg and 1.49 µg per µg of DNA for fractions numbers 1 and 2, respectively.

A Dynamic Feature of the AMT—Transcription Complex Interaction. All the above experiments have been done by first stopping the transcription with EDTA before the photochemical reaction. It turns out that the termination step is not required for efficient crosslinking between DNA template and nascent RNA to occur.

A transcription mixture of SV40 I has been prepared and incubated at 37°C. After one minute, instead of adding EDTA, 225 µg/ml of AMT was added and the incubation at 37°C was continued for another minute before the UV irradiation. Several things have been observed: 1. The amount of RNA synthesized after the first minute was similar to the other transcription
Fig. 4. 0.1% SDS—5–25% sucrose gradient of SV40 II transcription complexes that have been irradiated with long wavelength UV light in the presence of 3.3 μg/ml AMT.

experiments under the same conditions; 2. The transcription continued after the addition of AMT, the rate of RNA synthesis being not significantly reduced; 3. Most of the transcription activity was shut off as soon as the system was irradiated with the intense UV light; 4. After photochemical crosslinking, over 90% of the DNA sedimented faster than the uncrosslinked \(^{14}\text{C}-\text{SV40 I}\) (Fig. 5). Similar to the experiment in Fig. 1(b), two populations of molecules were again observed, one had a broad distribution around 26–27S and the other sedimented down to the bottom; 5. For fraction number 1, approximately 1.75 μg of RNA were co-sedimenting with 1 μg of DNA.

DISCUSSION

The psoralen-DNA photoreaction is becoming an important structural probe for many biological systems both because of its covalent nature and because of the high permeability of cells, nuclei, and even virus particles to psoralens. It has been utilized to study the chromatin structure of *Drosophila melanogaster* (14,15,16), and mouse (17), to stabilize imperfect inverted repeats of viral DNA (18,19), and to study the sequence organization of *Drosophila* satellite DNAs *in vitro* as well as *in situ* (9,16). We have proposed recently (11) that psoralens could be useful agents for fixing transient DNA-RNA hybrids in chromosomes or in other biological structures. This report has demonstrated clearly that one of the psoralen derivatives,
Fig. 5. 0.1% SDS—5-25% sucrose gradient of SV40 I transcription complexes that have been treated with (AMT + UV) without the addition of EDTA to stop transcription before the photochemical reaction. The details are described in the text.

AMT, photoreacts with in vitro prepared SV40 transcription complexes and crosslinks the nascent RNA chains to the helical DNA template efficiently. This crosslinking phenomenon of transcription complexes by AMT could be a general one for all transcription systems since a similar observation has been made with T7 transcription complexes in vitro (Hsieh, T.-S., Shen, C.-K. J., Wang, J. C. and Hearst, J. E., unpublished results).

Several control experiments have indicated that genuine covalent linkages are generated between nascent RNA and their DNA templates when intact transcription mixtures are photochemically reacted with AMT. First, no crosslinking was seen if the transcription complexes had not been treated with both AMT and long wavelength UV light (Figs. 1(a), 1(c), 2(a), 2(b)). These crosslinkages were not caused by aggregation of the DNA, precursors, nascent RNA, RNA polymerase, and the positively charged AMT molecules, as shown by the control experiments described in Figs. 3(a) and 3(b). Furthermore, the crosslinking occurs irrespective of the physical state of the DNA templates. As can be seen in Figs. 1(b) and (d), the photoreaction has caused the nascent RNA to co-sediment with either the supercoiled DNA (SV40 I) or the nicked DNA template (SV40 II).

Reproducibly, two populations of molecules were seen in the SDS-sucrose gradients when SV40 I transcription complexes were crosslinked with AMT and UV
We interpret the faster one as the supercoiled SV40 DNA and the slower one as nicked SV40 molecules, both of which have nascent RNA chains covalently attached. The slower population was likely generated by the introduction of single-stranded breaks into a fraction of the originally supercoiled SV40 DNA molecules during the irradiation and dialysis procedures. This nicking phenomenon is a minor side reaction of the photoreaction of psoralens with DNA (Wiesehahn, G. and Hearst, J. E., unpublished results). It is not clear why a portion of the RNA radioactivity becomes faster-sedimenting compared to the controls during the photoreaction of transcription complexes (Figs. 1(b), 5). Renaturation of some complementary RNA into double-stranded RNA is not possible because the transcription of SV40 I by *E. coli* RNA polymerase is asymmetric (20). Further experiments are needed to clarify this point.

In the gradients of crosslinked SV40 II transcription complexes, the DNA sedimented to the bottom of the tubes (Figs. 1(d), 4) similar to the fast-sedimenting fraction of crosslinked SV40 I transcription complexes (Figs. 1(b), 5). This is probably caused by the covalent attachment of more RNA molecules to each SV40 II molecule than SV40 I since the initial mole ratio of RNA polymerase to DNA is twice as large in the SV40 II transcription mixture. Indeed, the bottom fractions of the gradient of crosslinked SV40 II transcription complexes (Figs. 1(d), 4) have 1.4 fold more RNA per DNA molecule than that of crosslinked SV40 I transcription complexes (Figs. 1(b), 5).

The detailed structural features of the ternary transcription complexes are still not resolved (for a review, see Ref. 21). Under optimal conditions, the RNA polymerases bind strongly to certain regions of DNA (promoters) forming so called "open binary complexes." The helices of DNA are unwound by approximately 4 base pairs upon binding of each holoenzyme at the promoter site (22). This "open binary complex" then reacts with the four riboprecursors to initiate RNA synthesis and the unwinding of 4 base pairs per RNA polymerase persists during the elongation step. The transcription probably proceeds with the continuous opening of base pairs in the region being transcribed and the displacement of the newly synthesized portion of the RNA (23, 24, 25). The transcription can be terminated by a variety of factors including the addition of EDTA to chelate the magnesium ions. There is only indirect evidence showing that a portion of the nascent RNA chain is hydrogen-bonded to one strand of the DNA template. Circular dichroism studies have suggested the existence of some A-form DNA or DNA-
RNA hybrid regions in the ternary complex (26). Approximately 20 nucleotides or less of each nascent RNA chain are protected from nuclease digestion of the complex (25,27).

The transient DNA-RNA hybrid regions mentioned above are the most likely places for psoralen-crosslinking reaction to occur. We have shown that AMT and two other psoralen derivatives crosslink DNA-RNA helices very efficiently (11). One possible model of crosslinking of transcription complexes is, therefore, that AMT molecules diffuse into the RNA polymerase bound regions of the ternary complex and intercalate between base pairs of the transient DNA-RNA helices. Upon irradiation with long wavelength UV light, covalent interstrand crosslinks form between the DNA and RNA (Fig. 6(a)). Another model is that the nascent RNA is crosslinked by AMT and UV

![Diagram](image)

Fig. 6. Two possible models for the covalent attachment of nascent RNA to the helical DNA template. (a) Nascent RNA is directly crosslinked to one strand of the helical DNA template by the AMT molecules. (b) Both the nascent RNA chain and one strand of the DNA template are crosslinked by AMT to the RNA polymerase (RNAP). For simplicity, the fact that RNA polymerase contains subunits (21) has been ignored. In either model, the two strands of the helical DNA template are crosslinked by AMT.
to an RNA polymerase subunit which has also been photochemically crosslinked to the DNA template (Fig. 6(b)). We are currently characterizing the nature of the covalent linkages formed between the nascent RNA and its helical DNA template.

Glutaraldehyde fixation has been developed by Delius et al. (28) as a successful method of stabilizing in vitro transcription complexes of SV40 DNA. However, the psoralen crosslinking technique described here has the potential advantage of being an in vivo probe as we have mentioned earlier. Nascent RNA chains inside cells or nuclei could be covalently attached to their DNA templates. The portion of DNA with covalently bound nascent RNA chains could be isolated from the rest of the genome on the basis of its higher buoyant density or molecular weight. This may provide a unique tool for probing the structure of in vivo transcription complexes as well as for studying the genetic regulation of cells at the transcriptional level.

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