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A specific and efficient photoreaction between *E.coli* RNA polymerase and T_{+1} in the *lac*UV5 or *deo*P1 promoter

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Abstract

Upon irradiation of the RNA polymerase-lacUV5 or deoP1 promoter complex with short wavelength ultraviolet light ($\lambda \leq 300$ nm) the polymerase is covalently crosslinked at an efficiency of > 10% to the first transcribed base of the template DNA strand when this is a thymine. The temperature dependence of this RNA polymerase-T_{+1} photoreaction strongly indicates a relation to the formation of the open complex. It is suggested that open complex formation is preceeded or accompanied by a specific contact between the RNA polymerase and the first transcribed base of the DNA template.

Introduction

The E. coli RNA polymerase is an oligomeric enzyme with the subunit composition $\alpha_2\beta\beta'\sigma$ (1). Following sequence specific binding of E.coli RNA polymerase to promoter DNA a conformational change takes place, resulting in an "open complex" in which the two DNA strands become unwound over a ~ 12-17 bp region near the transcription initiation site (2-3). Chemical probing has revealed important contact points between the RNA polymerase and bases in the -10 and -35 regions of the promoter DNA (3). Ultraviolet irradiation of RNA polymerase and 5-bromouracil substituted DNA has resulted in crosslinking of the β-subunit with position +3 in the non-template strand, and crosslinking of σ-factor to the -3 position of the same strand (4). Chemical crosslinking using partly depurinated DNA has yielded a general picture of the DNA contacts of the various RNA polymerase subunits and has allowed the formulation of a model of the RNA polymerase promoter complex (5). We now report that ultraviolet irradiation ($\lambda < 300$nm) of E.coli RNA polymerase bound in an open complex, results in a specific crosslink at T_{+1} in the template

strand of the deoP1 or the lacUV5 promoter. This result indicates that open complex formation is preceeded or accompanied by a specific contact between RNA polymerase (presumably a lysine residue, tentatively assigned to the β-subunit) and the first transcribed base of the DNA template.

Materials & Methods

Either a 231 bp BamHI-BglII fragment (from the plasmid pGD11 (6) containing the region from -101 to +104 around the transcription initiation site of the deoP1 promoter) or a 320 bp BamHI-PvuII fragment from pCJ202 was used. pCJ202 was constructed by ligating a BamHI-EcoRI fragment from pGD11 (containing the deoP1 sequence from -101 to -10) with a synthetic EcoRI-SalI fragment (containing the deoP1 promoter sequence from -10 to +8 except for a T to C change at +1) into the BamHI-SalI sites of pUC19. The fragments were 3'-end-labeled with α^{32}P-dGTP at the BamHI site and purified using standard techniques. For the experiments with the lacUV5 promoter, the 190 bp EcoRI-DdeI fragment (3'-^{32}P-labeled at the EcoRI site) of the plasmid pIVS11 (7) was used. The E.coli RNA polymerase (RNP) was purified according to Burgess & Jendrisak (8).

Photoreaction. ~0.1-0.2 pmol ^{32}P-fragment and 5 pmol of RNP were mixed in 100 µl binding 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°C for 10 min, the samples were placed at the desired temperature (30°C unless otherwise indicated) for 5 min before irradiation. (The binding of RNP to the deoP1 promoter under these conditions has been verified by DNaseI footprinting (9)). The samples were irradiated from above in Eppendorf tubes for 20 min using a Philips TL20W/12 fluorescent light tube (λ~300 nm, 30nm band width, 24 J s^{-1} m^{-2}). The DNA was recovered by EtOH precipitation, treated with 1M piperidine at 90°C for 20 min, precipitated with n-butanol and analyzed on 6% acrylamide, 50% urea gels in TBE-buffer followed by autoradiography.

Nitrocellulose binding. After irradiation, 200 µl denaturation buffer (8 M urea, 2.5 mM EDTA, 50 mM Tris-HCl, pH 7.0) was added. The sample was cooled on ice for 5 min and then filtered through nitrocellulose. The filter was washed with 3 x 5 ml 2 M NaCl in TE and 2 x 5 ml TE. The DNA was then liberated from filterbound

protein by extraction of the filter with 1 ml TE for 1 h at 50°C. The DNA was precipitated with EtOH (+ 10 μg tRNA) and treated as described above.

Identification of photocrosslinked RNP subunit. Two μg purified BamHI-BglII fragments were self-ligated using T_4 DNA ligase. (The self-ligation of the promoter fragment was carried out in order to minimize artifacts originating from RNP binding to the ends of the DNA, to increase the size of the DNA making the RNP-DNA crosslinked product more "DNA-like", and to prevent the DNA from migrating into the gel thereby interfering with the protein staining.) This deoP1 DNA was mixed with 150 μg RNP in 1 ml binding buffer. After incubation at 37°C for 10 min, the sample was divided into two. One half was irradiated at 30°C while the other was kept in the dark during this time. Sarcosyl and EDTA were then added to 1% and 10 mM final concentrations, respectively, and the samples were cooled on ice. After four chloroform/isoamylalcohol (24:1) extractions (4°C), 0.4 vol of 5 M NH_4Ac was added followed by a chloroform extraction and precipitation with 2 vol of isopropanol. The lyophilized pellet was taken up in gel-loading buffer , heated to 80°C for 10 min and finally analyzed on 10% polyacrylamide/SDS gels according to Thomas & Kornberg (10)

Results and Discussion

Photochemical reactions have successfully been used in studies of protein/DNA interactions, both for crosslinking of protein to DNA (4, 11-12) and for footprinting of protein/DNA-contacts (13, 14). In the course of our investigations of probes for photo-footprinting of protein binding sites on DNA (9, 15-16), we observed that irradiation with 300 nm light of a ^{32}P-endlabeled DNA fragment - containing the deoP1 promoter (17) - in the presence of E.coli RNA polymerase results in the formation of a base labile site at position T_{+1} of the template DNA strand (Fig.1, lane 6, Fig.4, lane 5). No other polymerase dependent photoreaction of any base of either DNA strand was observed. Since the photoreaction occurs at a T-residue the well described photoreaction of thymines with primary amines including lysine residues in proteins (18, 19) comes to mind. This reaction involves a nucleophilic attack of the amine on the excited thymine

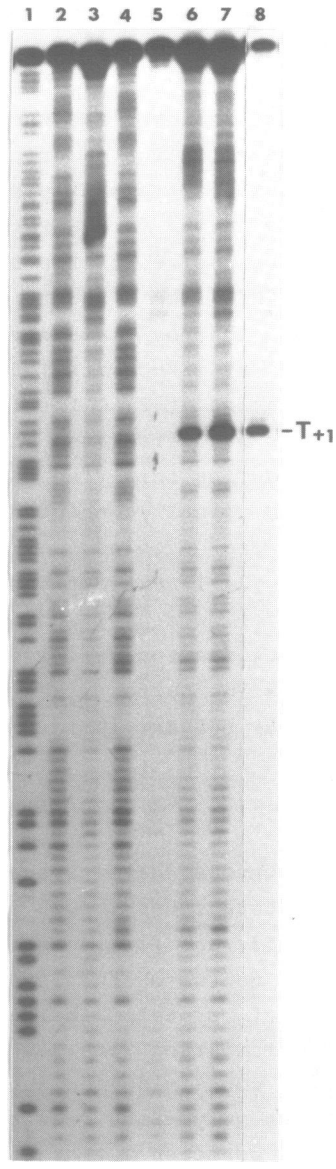

Fig.1 Cleavage at T_{+1} in the coding strand of a deoP1 promoter fragment. Lane 1: A+G sequence reaction. Lane 2: no irradiation (hv), no RNA polymerase (RNP). Lane 3: +RNP, no hv. Lane 4: +hv, no RNP. Lane 5: +RNP, hv, no piperidine treatment. Lane 6: +RNP, +hv (16°C). Lane 7: +RNP, +hv (30°C). Lane 8: DNA covalently linked to protein after irradiation in the presence of RNP as determined by nitrocellulose binding under denaturing conditions.

Table I

Cut-off wavelenght (10% transmission)	relative T_{+1} photocleavage#
none	1.0
250 nm	0.8
285 nm	0.6
305 nm	0.03

Effect of irradiation wavelength on the T_{+1} photoreaction. The experiment was performed in quartz tubes using an SP 200 super pressure mercury lamp equipped with cut-off transmission filters but otherwise as described in Fig.1a, lane 6. Irradiation was for 2 min (~800 J · m^{-2} · 5^{-1} without filter and ~ 600 J · m^{-2} · S^{-1} with 305 nm cut off filter). Quantification was done by densitometric scanning of the autoradiogram.

We estimate an overall photocrosslinking efficiency of > 10%.

thereby opening the thymine ring and in case of a protein-DNA interaction creating a covalently crosslinked protein-DNA intermediate. This intermediate easily undergoes rearrangement resulting in thymine transfer to the protein leaving the glycosylic thymine nitrogen as a free amino group on the deoxyribose (19).

A covalent protein-DNA intermediate is indeed formed in the case of the photoreaction between RNA polymerase and T_{+1} in the deoP1 promoter as seen from the retention of the T_{+1}-labile DNA on nitrocellulose under denaturing conditions (Fig.1, lane 8). (A similar enrichment of the T_{+1} band was achieved by ethanol precipitation of the interphase of a phenol/SDS extraction of the deoP1 DNA-RNA polymerase photoproduct (data not shown)). The specific retention of the T_{+1}-fragment on the nitrocellulose as compared to other DNA fragments (Fig.1: lane 8 versus lane 7) strongly indicates that the protein-dependent photoreaction is restricted to T_{+1}. The mild treatment (50°C,1h) needed to release the base-labile DNA from the nitrocellulose bound protein, and the wavelength dependence of the T_{+1} photoreaction (Table I) showing the highest efficiency at wavelengths overlapping the thymine absorption also strongly argue in favour of the proposed mechanism.

The specificity of the polymerase-T_{+1} interaction is stressed by the fact that the deoP1 promoter sequence contains

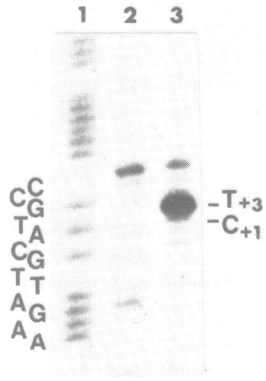

Fig.2 Effect of substituting T_{+1} of the deoP1 promoter with a C. Lane 1: A+G. Lanes 2 & 3: irradiation in the absence or presence of RNP, respectively.

several T-residues in close proximity to T_{+1}, namely T_{-2} and T_{+3} of the template strand and T_{+2} of the non-template strand. Of these potentially photoreactive thymines only T_{+3} reacts to a small degree (~5 % of the T_{+1} reaction) with the RNA polymerase. The amino acid reacting weakly with T_{+3} is probably the same as that reacting with T_{+1}. If T_{+1} is substituted with a cytosine using recombinant DNA technique no cleavage at this position takes place, but instead an enhanced cleavage at T_{+3} is observed (Fig.2, lane 3).

Analogous results were obtained using a lacUV5 promoter that has thymines at positions +1 and +2. An efficient photoreaction between RNA polymerase and T_{+1} is observed whereas the reaction with T_{+2} is more than 5 fold less efficient (Fig. 3, lane 3).

Two experiments were performed to correlate the T_{+1} cleavage with a functional state of the polymerase-promoter complex. When the four nucleoside triphosphates were included in the reaction mixture to initiate transcription, virtually no T_{+1} cleavage was detected (Fig.4, lane 6). These results show that the T_{+1} crosslink is dependent on polymerase bound at the promoter. The formation of the T_{+1} product was, furthermore, temperature dependent (Fig.4) in a manner (20) that strongly suggests a relation to the open complex. As can be seen in Fig.4,

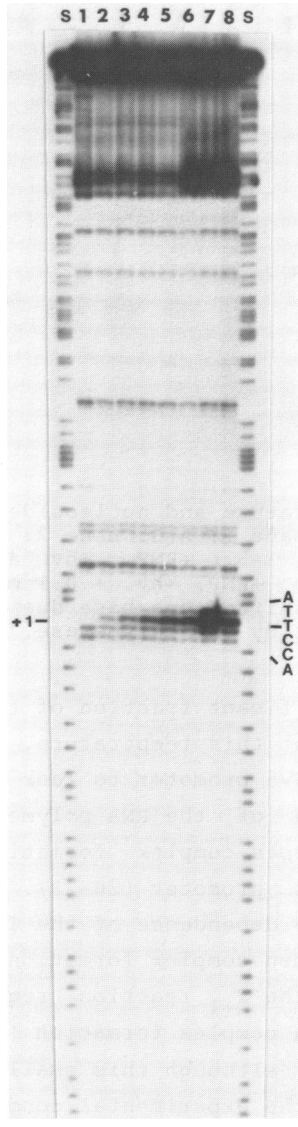

Fig.3 Effect of temperature on T_{+1} reaction of lacUV5. Lanes S: A+G formic acid sequence reaction. Lane 1: no RNP (25°C). Lanes 2-8: Irradiation of RNP-lacUV5 complex at 10, 12.5, 15, 17.5, 20, 22.5 and 25°C, respectively.

lane 3 no T_{+1} reaction is detected in the deoP1 promoter at low temperature (4°C) where an open complex is not formed. An intermediate reaction level is observed at 16°C (compared to 30°C)

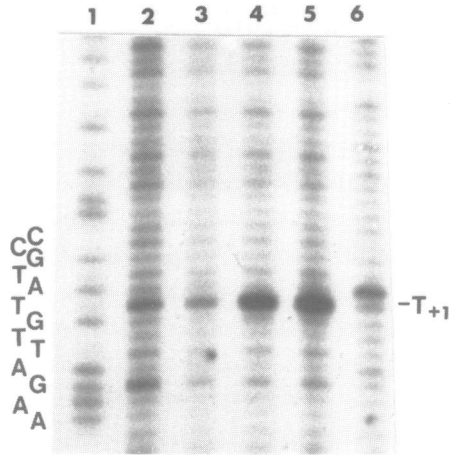

Fig.4 Effect of temperature and nucleoside triphosphate on T_{+1} cleavage of deoP1. Lane 1: A+G. Lane 2: irradiation in the absence of RNP. Lanes 3-5: +RNP, +hv at 4°, 16° & 30°C, respectively. Lane 6: +RNP, +hv, + 0.1 mM rNTP's. The bands in lane 6 of the gel are shifted one base due to filling out of the end of the DNA fragment by RNA polymerase.

suggesting that a significant fraction of the complexes exist in a closed conformation at this temperature.

We used the lacUV5 promoter to look more closely at the temperature dependence of the RNA polymerase-T_{+1} photoreaction since the kinetics of open complex formation has been studied extensively with this promoter (20-22). We find correspondence between the temperature dependence of the T_{+1} photoreaction (Fig.3) and that of open complex formation (20). Our results indicate, however, that the T_{+1} reaction requires slightly lower temperature than open complex formation ($T_{1/2}$~ 18°C versus 22°C for open complex (20)); although this small difference could be due to differences in the experimental conditions.

Although the crosslinked polymerase-DNA complex is quite labile, an attempt was made to identify the protein component of the complex taking advantage of the expected tendency of a crosslinked subunit to follow the DNA in repeated chloroform/isoamylalcohol extractions and subsequent isopropanol precipitation. Using this approach we tentatively assign the crosslink to the β-subunit (Fig.5). The faint β' band in the gel is ascribed to incomplete dissociation of the RNA polymerase

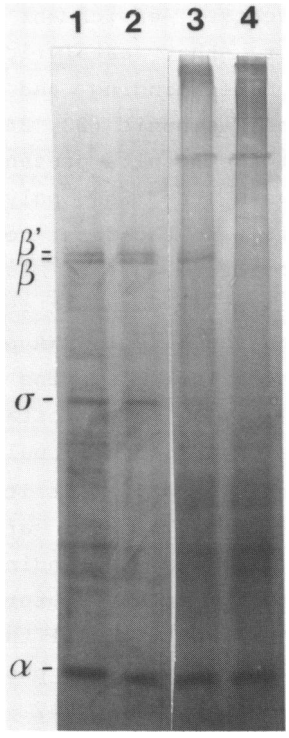

Fig.5 Silver staining of gel showing protein copurifying with DNA. Lane 1-2: marker lanes containing 230 & 70 ng purified RNA polymerase. Lane 3: extracted protein after irradiation of RNP in the presence of deoP1 promoter DNA. Lane 4: control (as 3) without irradiation.

during the extraction and the significant amount of the α-subunit to incomplete extraction of this polypeptide under the applied condition. Although we cannot exclude that the α-subunit is also crosslinked to the DNA, this is highly unlikely since previous experiments using both chemical (5) and photochemical crosslinking (4, 23) have shown no evidence of contacts between the α-subunit and the DNA. It should be mentioned that less than 1% of the β-subunit is recovered in crosslinked form although the yield of the photoreaction is estimated as > 10% (judged from densitometric scanning of the autoradiograms of Figs. 1 & 3). We ascribe the low recovery yield to the relative instability of the covalent protein-DNA complex. We can not at this point rule out that crosslinks to parts of the DNA molecule other than T_{+1} may

contribute to the selective enrichment of the β-subunit. It should be noted, however, that catalytic activities such as ribonucleoside triphosphate binding and rifampicin resistance have been assigned to the β-subunit (22, 24, 25).

We conclude from the results presented in this paper that a specific contact between the RNA polymerase (presumably a lysine of the β-subunit) and the first coding base (N_{+1}) of the template DNA is formed prior to, or at least concomitant with open complex formation. At this point it is not known whether the proposed contact between the E. coli RNA polymerase and T_{+1} has any functional importance. It is tempting to speculate that this contact, which is presumably an electrostatic interaction with phosphate at N_{+1} of the DNA backbone, could actually participate in open complex formation. The fact that it is seen for two different promoters (deoP1 and lacUV5) argues in favour of a biological significance, as does the finding that RNA polymerase from Salmonella typhimurium also photoreacts with T_{+1} of the deoP1 promoter (data not shown). It will therefore be of great interest to determine how general the photoreaction is in terms of other promoters and RNA polymerases, e.g. from enkaryotic sources.

If the proposed mechanism for the reaction between E.coli RNA polymerase and T_{+1} in the deoP1 promoter is correct, a thymine transfer (19) to a lysine residue in the RNA polymerase should take place. Assuming that this lysine is at or near the active site, this highly efficient photoreaction can be used to photoaffinity label and ultimately identify the active site of the RNA polymerase (employing e.g. DNA containing radiolabeled thymine at position +1).

Finally, we believe that T_{+1}-RNA polymerase photoreaction is ideally suited for time resolution studies of promoter open complex formation by rapid mixing flash irradiation techniques (12). These aspects are now being pursued.

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