Uranyl mediated photofootprinting reveals strong *E.coli* RNA polymerase–DNA backbone contacts in the +10 region of the *DeoP1* promoter open complex

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ABSTRACT

Employing a newly developed uranyl photofootprinting technique (Nielsen et al. (1988) FEBS Lett. **235**, 122), we have analyzed the structure of the *E. coli* RNA polymerase *deoP1* promoter open complex. The results show strong polymerase DNA backbone contacts in the –40, –10, and most notably in the +10 region. These results suggest that unwinding of the –12 to +3 region of the promoter in the open complex is mediated through polymerase DNA backbone contacts on both sides of this region. The pattern of bases that are hyperreactive towards KMnO_4 or uranyl within the –12 to +3 region furthermore argues against a model in which this region is simply unwound and/or single stranded. The results indicate specific protein contacts and/or a fixed DNA conformation within the –12 to +3 region.

INTRODUCTION

Transcription initiation by *E. coli* RNA polymerase involves two crucial steps. The RNA polymerase first binds to the promoter in a closed complex. Subsequently in a rate limiting step this RNA polymerase-promoter complex undergoes a conformational transition forming the open complex, in which the DNA helix is unwound ~ 1.7 helical turns and strand separation in the –12 to +3 region has taken place. At this stage the complex is ready to commence RNA synthesis (reviewed in e.g. (1) & (2)).

Genetic studies have identified bases in the promoter DNA which are essential for RNA polymerase promoter recognition (3). RNA polymerase contacts with promoter DNA have been identified by base methylation protection and interference as well as phosphate ethylation interference studies (4). These results have, however, given very few clues as to the mechanism by which the promoter DNA is unwound by the RNA polymerase, and as to how the strand separation leading to open complex formation is mediated.

Using a novel uranyl photofootprinting technique (5), we now report RNA polymerase promoter contacts in the +10 region. These results as well as the identification of uranyl and KMnO_4 hyperreactive sites in the –11 to +2 region give important information about protein-DNA interactions that are responsible for open complex formation.

MATERIALS & METHODS**Proteins**

E. coli RNA polymerase was purified according to Burgess and Jendrisak (6) and was a gift from Dr. Kaj Frank Jensen. λ -repressor was purified according to Johnson et al. (7) from an *E. coli* overproducer.

DNA

The 250 base pair *Bam*HI/*Hind*III fragment from plasmid pCJ200 containing the *deo*P1 promoter (8) was labeled with 32 P on either strand at the *Bam*HI site using standard techniques (9). A pUC19 derivative containing the O_R1 operator (10) was used for the λ -repressor experiments.

Uranyl photofootprinting

In these experiments 2.5 pmol RNA polymerase or 30 pmol λ -repressor were mixed in 100 μ l buffer (40 mM Tris-HCl pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 2 mg/ml Calf thymus DNA) containing 0.1–0.2 pmol 32 P-endlabelled DNA fragment. After incubation at 37°C for 10 min the samples were returned to room temperature.

Uranyl-EDTA footprinting was performed by addition of 5 μ l of a solution containing 20 mM $UO_2(NO_3)_2$ and 20mM EDTA (prepared immediately before use from 100mM stock solutions of EDTA and $UO_2(NO_3)_2$ in H_2O) and irradiation from above at RT for 20 min at a distance of ~ 2 cm from a fluorescent light tube (Philips TL 40 W/03) emitting around 420 nm. After irradiation 10 μ l of a 100 mM sodium citrate solution was added to complex the uranyl and the samples left at RT for 10 min before the DNA was precipitated with 2 vol. ethanol. The DNA pellet was washed with 70% ETOH and lyophilized. The samples were subsequently fractionated on polyacrylamide/50% urea sequencing gels and the cleavage products visualized by autoradiography.

The uranyl-citrate reactions were performed essentially as described for uranyl-EDTA substituting citrate for EDTA except that the irradiation time was 40 min and the light source was a Philips TL 20 W/12 emitting around 300 nm. (The choice of light source (300/420 nm) does, however, not influence the footprinting results).

KMnO₄ probing

The RNA polymerase promoter complex was formed under the condition described above. After incubation at 37°C for 10 min the samples were transferred to 30°C and treated with 5 μ l 20 mM $KMnO_4$ for 15 sec. The reactions were stopped by addition of 50 μ l ice-cold stop buffer (1.5 M NaAc pH 7.0, 1 M 2-mercaptoethanol) and immediately precipitated with 2 vol. ETOH. The pellets were resuspended in 250 μ l 0.3 M NaAc and reprecipitated. Following lyophilization the samples were treated with 0.5 M piperidine at 90°C for 20 min.

RESULTS

We have previously found that uranyl salts induce virtually random single strand nicks in DNA upon irradiation with long wavelength ultraviolet light (300 nm $< \lambda < 420$ nm) (5). In the presence of λ -repressor the operator O_R1 sequence is protected against uranyl attack at positions (5) (Fig. 1) closely corresponding to the protein-DNA backbone contacts previously identified by EDTA(FeII) footprinting (11) and predicted from the crystal structure (12,13).

In our original paper (5), we used UO_2Ac_2 or $UO_2(NO_3)_2$ directly. Subsequently, however, we found that alkaline conditions (pH > 7) in common organic buffers (e.g. Hepes, MOPS, Tris etc.; used for RNA polymerase binding to promoter DNA) cause rapid precipitation of yet unidentified uranyl complexes. It can therefore be difficult to reproducibly obtain the optimal concentration of uranyl for photofootprinting. In order to solve this problem we found it preferable to liberate the uranyl in situ from soluble complexes. We have used two such complexes in 1:1 molar ratio, namely uranyl-EDTA and uranyl-citrate. In buffers containing Mg^{2+} , the Mg^{2+} will displace uranyl from

Figure 1. a: Uranyl photofootprinting of λ -repressor bound to O_R1 operator DNA. Lane 1: A+G sequence reaction. Lane 2: control cleaved with uranyl in the absence of λ -repressor. Lane 3: in the presence of λ -repressor. The brackets denote the O_R1 operator sequence (I) and the DNaseI footprint (II).

EDTA. In contrast, uranyl complexed with citrate appears to be stable in the presence of Mg^{2+} . Since we changed our original procedure, it was important to verify that the photofootprint of λ -repressor bound to O_R1 operator DNA could be reproduced under these conditions. We therefore performed a footprinting experiment with λ -repressor under the exact same conditions as those used later for the RNA polymerase footprints. As can be seen from Fig. 1, uranyl-EDTA produces a footprint of λ -repressor similar to that obtained previously (5). Experiments with uranyl-citrate did not result in any footprint of λ -repressor and hardly any photocleavage of DNA (results not shown). Uranyl-citrate can, however, be used as a probe for structural changes in promoter DNA after binding of RNA polymerase (see below).

Uranyl-EDTA and uranyl-citrate photofootprinting analyses of *E. coli* RNA polymerase bound to *deoP1* promoter in an open complex produced the results shown in Fig. 2. Several regions of protection (-45 to $+11$ region) as well as sites of enhanced reactivity (-9 to $+2$ region) are observed on both strands using uranyl-EDTA. In contrast only hyperreactive sites on both strands are seen using the uranyl-citrate complex; most notably at position $+2$ of the template strand (Fig. 2).

Treatment of single stranded DNA with $KMnO_4$ leads to oxidation of thymines (14). Double stranded DNA, however, reacts very slowly with $KMnO_4$ unless it is somehow perturbed, e.g. by bending (15), or unwound by intercalators (16). In view of the observed uranyl hypersensitive sites, it was therefore of interest to probe the DNA in the polymerase open complex with $KMnO_4$. The results (Fig. 3) show that thymines in the -11 to -2

region of the template DNA strand and in the -6 to $+2$ region of the non-template DNA strand are hypersensitive to KMnO_4 oxidation (Fig.4).

To verify that the presence of uranyl does not interfere with open complex formation we also performed the KMnO_4 probing in the presence of uranyl (using the conditions for uranyl probing without irradiation). Results indistinguishable from those presented in Figure 3a were obtained indicating that the uranyl binding to DNA does not significantly perturb the open complex (data not shown).

DISCUSSION

Mechanism of uranyl photofootprinting

The mechanism of the uranyl photofootprinting reaction is not resolved at present. It is well known, however, that the excited uranyl(VI) ion is a strong oxidant (17) capable of oxidizing e.g. olefins (18). Due to the sequence neutrality of the uranyl photocleavage of DNA, it is therefore almost certain that photooxidation of the deoxyriboses of the DNA backbone is involved.

Uranyl(VI) furthermore forms complexes with inorganic phosphate (19) and we presume that analogous complexes form with DNA. Indeed, high concentrations of uranyl salts precipitate DNA (unpublished results). The uranyl(VI) ion usually complexes with two anions each occupying two coordination sites, as seen in e.g. uranyl nitrate ($\text{UO}_2(\text{NO}_3)_2$) and uranyl acetate ($\text{UO}_2(\text{CH}_3\text{COO})_2$), in a planar hexagon arrangement also involving two molecules of water (20, 21). Thus we expect an analogous uranyl complex formation with the two non-esterified oxygens of the phosphates of DNA. The geometry of the DNA helix does, however, not allow complex formation with two phosphates within the double helix across either the major or minor groove. Thus the other uranyl coordination site must be occupied by a ligand from the medium (acetate, nitrate, H_2O etc.).

It is also well known that uranyl oxidizes coordinated ligands (e.g. oxalic acid) (17). We therefore propose that photooxidation of the DNA backbone takes place at the deoxyribose proximal to the phosphate which is complexed to the uranyl ion. The 3' or 5' hydrogens are most accessible, and oxidation at either of these positions would result in spontaneous cleavage of the backbone.

Since the products of the uranyl photocleavage reaction have not been identified, the assignment of the cleavage sites to the DNA sequence is only accurate within one base. The assignment used here (Fig.4) is based on the observed comigration of the uranyl cleaved DNA fragments with those obtained by the chemical sequence reaction, which indicates that 3'- and 5'- phosphate termini are produced by uranyl photocleavage. Furthermore, a comparison of the uranyl photofootprinting results of λ -repressor bound to O_R1 (Fig.1 & ref.5) with previous phosphate ethylation interference studies (13) indicates that uranyl photocleavage of a base is caused predominantly by uranyl associated with the phosphate 3' of this base.

This model for uranyl photocleavage of DNA predicts that photofootprinting of ligand-DNA complexes by uranyl reports the accessibility of phosphates for complex formation,

Figure 2. Uranyl photofootprinting of RNA polymerase *deoP1* promoter open complex. a: non-template strand. b: template strand. Lane 1: A+G sequence reaction. Lanes 2 & 4: control without RNA polymerase. Lanes 3 & 5: in the presence of RNA polymerase. Lanes 2 & 3: Photofootprint using the uranyl/EDTA method. Lanes 4 & 5: Photofootprint using the uranyl/citrate method. +1 denotes start of transcription.

Figure 3. KMnO_4 probing of RNA polymerase-*deoP1* promoter open complex. a: KMnO_4 , template strand. b: KMnO_4 , non-template strand. Lane S: A+G sequence reaction. Lane +: in the presence of RNA polymerase. Lane -: control without RNA polymerase.

Figure 4. DNA sequence of the *deoP1* promoter (38) showing DNaseI footprint (brackets) (from ref.8), uranyl photofootprint (underlining), uranyl hypersensitive sites (from the uranyl/EDTA experiment) (bars) and KMnO₄ hypersensitive sites (T).

e.g. influenced by hydrogen- or electrostatic bonding to amino acid residues in proteins. This interpretation is consistent with the photofootprinting results of the λ -repressor/*O*_R1 complex, although uranyl photofootprinting, EDTA/FeII footprinting (11), phosphate ethylation interference (13) and X-ray crystallography (13) experiments do not yield identical results. This is not unexpected, however, due to the different principles of the techniques. X-ray crystallography reports proximity of atoms in a fixed complex (13) but not necessarily bonding, EDTA/FeII footprinting probes the accessibility of deoxyriboses, presumably from the minor groove (11), phosphate ethylation interference identifies phosphates that are indispensable for the formation of a stable protein-DNA complex, whereas uranyl photofootprinting presumably identifies phosphate that are accessible to complex formation with uranyl.

The inability of the uranyl citrate complex to photocleave DNA is presumably due to the stability of this complex compared to that of uranyl DNA. Thus in the presence of citrate, uranyl does not bind to DNA.

Backbone contacts of RNA polymerase

Ethylation interference studies using the *lacUV5* or the *T7A3* promoter (4) have identified polymerase DNA backbone contacts in the -35, -16 and -8 regions of the template strand and -32 of the non-template strand.

Our uranyl photofootprinting results are in accordance with these previous findings. In particular, the regions -10 to 12 and -31 to -32 of the template strand and regions -9 to -11 and -36 to -43 of the non-template strand are protected from photocleavage by uranyl (Fig.4). The observation that strong RNA polymerase-promoter DNA backbone contacts are present in the +2 to +11 region is, however, not detected in ethylation

Figure 5. DNA helix model showing the footprinting results described in Fig.4. T denotes KMnO₄ hypersensitive thymines. Arrow heads show uranyl hypersensitive sites, while the shaded areas of the DNA backbone indicate protection from uranyl photocleavage.

interference studies and may give a clue to the mechanism of DNA unwinding in connection with open complex formation (see below). It is obvious that any binding of the RNA polymerase downstream from the promoter should be to the DNA backbone, and most probably electrostatic without specific protein-base interactions since this DNA is part of the gene and contains no conserved sequence.

The reason why these downstream contacts were not identified in previous ethylation interference experiments could be that these studies depended on filter-binding to separate DNA bound to RNA polymerase from unbound DNA. Contacts, such as the ones in the +10 region which may be important for open complex formation but are not pertinently required for RNA polymerase binding will not be detected with a filter-binding assay.

As can be seen from Fig.5, the uranyl photofootprinting results also confirm previous conclusions (2,4,22,23) that RNA polymerase in the open complex is bound predominantly to one side of the promoter DNA helix.

Promoter unwinding and open complex formation

Open complex formation results in an unwinding of the promoter corresponding to ~ 1.7 helical turns (24,25). It is clear from various structure probing experiments that most of the unwinding is confined to the -12 to $+3$ region of the promoter. This region shows increased reactivity to phenanthroline/CuI (26), and it has been inferred from cytosine methylation studies that the ~ -9 to $+3$ region is truly single stranded (27,28). It is evident from the schematic model presented in Fig.5 how the RNA polymerase through strong backbone contacts on both sides of the unwound region could maintain the promoter DNA in such an otherwise unfavourable conformation.

We see two possibilities of how this complex can be formed. Either the RNA polymerase first establishes the backbone contacts, and subsequently via a conformational change of the polymerase produces the unwinding. Such a mechanism most probably involves one or more of the β, β' or σ subunits, these being in contact with the DNA in this region (22,29). Alternatively the RNA polymerase stays in a fixed conformation in which the protein-DNA contacts of the open complex constitutes an energy minimum. Thus, RNA polymerase can in this way 'catch' or 'freeze' the DNA in an unfavourable conformational state which under normal conditions does exist, but which is very scarcely populated. It has indeed been shown that the TATA box has lowered helix stability (30).

Structure of the unwound region

The highly selective reaction of the bases just around position +1 on the coding strand with the large uranyl-citrate complex suggests to us that these residues are engaged in a complex of unique structure. Since neither double nor single stranded DNA is photocleaved by uranyl citrate, we believe that a high affinity binding site (as compared to citrate) for uranyl is created around position +1 in the open complex. We speculate that the RNA polymerase could induce a 180° rotation of individual or several of these residues (using the DNA backbone as a hinge) thereby allowing RNA synthesis to takeplace on the outside of the DNA helix. The open complex that maintains the DNA in an unwound conformation could facilitate such a base rotation. Energy calculations have indicated that base pair opening by base rotation into the major groove requires ~ 28 kcal/mol. If, however, the DNA is bent, only ~ 7 kcal/mol is required to 'flip out' a base (31). RNA polymerase does indeed bend DNA (32), and we find it noteworthy that the center of bending is around position +3 in the open complex (33). The surprising finding that blockage of transcription occurs at the base right in front of a psoralen DNA interstrand crosslink (34), could also be taken as evidence of a transcription mechanism involving base rotation to the outside

of the DNA helix. The model is also compatible with the observation that RNA polymerase is blocked by a psoralen monoadduct on the template strand but is virtually unaffected by monoadducts on the non-template strand (35). We speculate that the number of 'flipped out' bases during RNA synthesis corresponds at least to the ones engaged in hydrogen bonding to the nascent RNA.

Since T_{+1} and T_{+3} of the template strand are protected from attack by KMnO_4 in the open complex, these bases must either be stacked or protected by direct association with the RNA polymerase. (The phosphates are accessible to uranyl, however). We have previously found that T_{+1} and to a lesser extent T_{+3} photoreact very efficiently with RNA polymerase indicating close protein-DNA interactions (36). These interactions could be involved in maintaining the region in the conformation that makes it hypersensitive to uranyl-citrate. More information than we are now able to extract from these results will emerge when the uranyl photocleavage mechanism is unraveled. It will be of great interest to compare different RNA polymerase-promoter complexes in order to see if the polymerase DNA contacts depend on the promoter sequence, maybe in connection to promoter strength, or if they constitute a general feature irrespective of the promoter sequence.

After completion of the present work, EDTA/FeII footprinting results of *E. coli* RNA polymerase bound to the *merR* promoters were reported (37). These results are qualitatively in accordance with the uranyl photofootprinting results presented here with respect to RNA polymerase DNA backbone contacts, but probing of the *merR* promoter with EDTA/FeII did not reveal any hyperreactive sites (37).

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