The pathway of *E. coli* RNA polymerase-promoter complex formation as visualized by footprinting

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ABSTRACT

The pathway of *E. coli* RNA polymerase-promoter complex formation was probed by characterization of low temperature intermediates at the major coat protein promoter of phage fd DNA. Three different complexes could be distinguished. One of them represents the active 'open' complex, the other two have to be regarded as 'closed'. The promoter contacts of the lower temperature complexes were totally comprised within the contacts of the higher temperature complexes. Increase in temperature led to extension of contacts into the downstream direction, while the upstream border of the complexes remained virtually unchanged. Only in the 'open' complex contacts were extended beyond the start site of transcription.

INTRODUCTION

The DNA sequences which signal RNA polymerases to bind to them and initiate transcription are termed promoters (1). The mechanism by which bacterial RNA polymerase locates a promoter site and initiates transcription is still only fragmentarily understood. A two-step model for the interaction between *E. coli* RNA polymerase and a promoter region was originally proposed by Zillig and co-workers (2). It postulated initial binding of the enzyme to the intact duplex followed by subsequent partial unwinding of the DNA strands to act as a template in the transcription process via Watson-Crick base pairing with the ribonucleoside triphosphates. In fact, unwinding of DNA upon binding of RNA polymerase could be verified (3-5). The unwound or 'open' region was demonstrated to be located approximately between positions -10 and +3 (6-8). The comparison of promoter sequences (for a recent compilation see ref. 9) yield two moderately to highly conserved regions, each about 6 bp (base pairs) in length, around positions

-35 and -10 (the so-called -35 and -10 regions). Their functional importance is confirmed by many promoter mutations (9). In a stable complex capable of initiation of transcription RNA polymerase was found to protect the segment between approximately positions -20 and +20 against extensive digestion by DNAase I. However, the enzyme was unable to re-bind to this fragment (10-12). These observations suggested that, while the -10 region is essential for formation of the stable 'open' complex, the -35 region might serve as a recognition signal for RNA polymerase to form an initial complex in which the duplex possibly has not yet been unwound, the so-called closed complex (13). Direct experimental evidence for the existence of closed promoter complexes was first reported by Williams and Chamberlin (14) who observed by electron microscopy that RNA polymerase bound to the same sites of a promoter-containing DNA fragment at 37°C and 0°C. However, the regions of the promoter and RNA polymerase involved in the low temperature interaction remained unknown. Therefore, we attempted to characterize the promoter sites involved in closed complex formation.

MATERIALS AND METHODS

Enzymes

Calf intestine alkaline phosphatase (grade I), Klenow enzyme, and Hpa II were from Boehringer Mannheim; Hae III, Hinf I, Taq I, and T4 polynucleotide kinase were from Bethesda Research Laboratories; E. coli RNA polymerase holoenzyme was from New England Biolabs; bovine pancreas DNAase I was from Worthington.

DNA, nucleotides, and other substrates

Synthesis of the double-stranded fd DNA was described previously (15). After deproteinization and ethanol precipitation ccc DNA was isolated by acid phenol extraction (16). NTPs and dNTPs were from Boehringer Mannheim, γ -³²P-ATP (3000 Ci/mMol) and α -³²P-dCTP (3000 Ci/mMol) were from New England Nuclear, α -³²P-UTP (400 Ci/mMol) was from Amersham. BSA was from BRL. Heparin (grade II) was from Sigma.

Preparation of labeled DNA fragments

In general, conditions for restriction digests were as recommended by the enzyme purchasers. After the first of two subsequent di-

gests the fragments were dephosphorylated and deproteinized as described (17). Individual fragments were isolated via PAGE (polyacrylamide gel electrophoresis). The elution procedure was a modification of the Maxam and Gilbert protocol (18,17). P VIII was isolated on a 302 bp Hpa II/Hae III fragment, P X on a 157 bp Taq I/Hinf I fragment. The template strand of the 302 bp fragment was labeled by 'filling in' of the Hpa II end with Klenow enzyme (19) in the presence of α - 32 P-dCTP and 3 cold dNTPs. The non-template strands of the fragments, which had been selectively 5'-dephosphorylated (see above), were labeled with kinase and γ - 32 P-ATP essentially as described by Maxam and Gilbert (18). Subsequently, their sticky ends were blunted by incubation with Klenow enzyme and cold dNTPs (see above).

Footprinting

In 40 μ l of 20 mM Tris·HCl, pH 8.0 / 10 mM MgCl₂ / 120 mM KCl / 0.1 mM DTE / 0.1 mM EDTA / 5% glycerol (binding buffer) and 0.1 mg/ml BSA approximately 10 fmol of DNA fragment and 5 pmol of RNA polymerase holoenzyme were incubated at 7°C, 17°C, or 37°C. In Hha I protection experiments this enzyme concentration had been found to be necessary to approximately saturate the promoter at 37°C under similar conditions. At times 10 μ l aliquots were withdrawn, mixed with 0.5 μ l of DNAase I, and incubated for 0.5 min at the same temperature. Final concentrations of DNAase I were 2.5 μ g/ml at 7°C, 1.0 μ g/ml at 17°C, 0.25 μ g/ml at 37°C. Reactions were stopped by addition of 9 μ l of 30 mM EDTA / 70% formamide / 0.05% xylene cyanol / 0.05% bromphenol blue / 1 x TBE (18). Samples were immediately incubated at 90°C for 1 min, kept on ice for 1 min, concentrated in vacuo to 12 μ l and layered directly on a 12% polyacrylamide / 8.3 M urea / 2 x TBE slab gel (40 cm x 0.4 mm). After electrophoresis gels were exposed at -70°C using Kodak XAR-5 films and Cronex Hi-Plus intensifying screens. Restriction fragments in combination with the (dTMP)_n ladder (20) were used as chain length markers. Lengths are estimated to be correct within $\pm$ 1 nucleotide. In some experiments denatured DNA (to 13 μ g/ml) was added after 11 min to visualize complex decay.

Assay for initiation complexes

Approx. 1 fmol of DNA fragments and 0.5 pmol of RNA polymerase

were incubated in 30 μ l of binding buffer (see "Footprinting") containing 0.1 mg/ml BSA and 0.5 mM GTP for 10 min at 7°C, 17°C, or 37°C. Then 500 μ l of ice-cold binding buffer containing 1 M KCl were added. After vortexing weakly the mixture was left on ice for about 0.5 min and then filtered with gentle suction through cellulose-nitrate (Sartorius SM 11378; pore size: 0.15 μ m, diameter: 25 mm), which had been pre-soaked in ice-cold binding buffer containing 1 M KCl. The filter was washed with 250 μ l of binding buffer without KCl, dried, and counted in a toluene-based scintillation cocktail. Blank values (about 7% of input cpm) were determined accordingly in the absence of GTP.

Assay for rapid starter complexes

DNA and RNA polymerase were incubated for 10 min at 7°C, 17°C, or 37°C as described under "Footprinting". The complexes were then challenged with a mixture of heparin (final concentration: 5 μ g/ml) and the NTPs (final concentration: 100 μ M each for G, A, C; 1 μ M for α -³²P-UTP), left for 1 min at the indicated temperatures, and incubated for 1 h at 37°C. Reactions were stopped, and samples (10 μ l) were denatured, electrophoresed, and visualized as described (see "Footprinting").

RESULTS

The major coat protein promoter (P VIII) of *E. coli* phage fd was isolated on a 302 bp Hpa II/Hae III fragment. Its interaction with *E. coli* RNA polymerase holoenzyme at 7°C, 17°C, and 37°C was investigated by 'footprinting' with DNAase I (21). At all temperatures a specific interaction could be detected with both strands of the DNA, but the extensions of the footprints varied significantly (figures 1,2).

At 7°C perturbations of the DNAase I cleavage pattern were observed between positions -57 and -33 of the non-template strand and between -59 and -48 or (rather weakly) -31 of the template strand. Promoter binding appeared to be completed at 0.5 min.

At 17°C the footprint extended from position -57 to approximately -4 (non-template strand) and from -61 to approximately -12 or -1 (template strand). The downstream borders were not sharp, so the positions given are somewhat arbitrary. There was no change in

Figure 1: Footprints of RNA polymerase on the template strand of P VIII at different temperatures. Temperatures, incubation times with RNA polymerase, and some positions of the sequence are indicated. -46, e.g., defines a fragment cleaved 5' of position -46 in the template or 3' of position -46 in the non-template strand (figure 2). The extensions of the footprints are marked by brackets.

the footprint between 0.5 min and 10 min.

At 37°C a footprint was observed between positions -61 and +20 (non-template strand) and -61 and +15 (template strand). The time required for footprint formation was between 0.5 and 3 min. The footprints are diagrammed schematically in figure 3.

To correlate footprints and functional properties of the complexes, the stability of the complexes (in the presence of polyanions: denatured DNA), their ability to initiate transcription,

Figure 2: Footprints of RNA polymerase on the non-template strand of PVI at different temperatures. "T" indicates that this lane contains a (dTMP) ladder (20). Note that at 37°C denatured DNA was added after 1 min to visualize complex decay. For further details see legend to figure 1.

and their ability to rapidly initiate transcription were determined. Complex decay could be monitored by footprinting in the presence of denatured DNA, which traps the dissociated polymerases. (Denatured DNA did not interfere with cleavage of the promoter fragment by DNAase I.) At 7°C and 17°C the footprints had disappeared completely after 0.5 min, the earliest value determined (not shown). At 37°C after 0.5 min the footprint was only slightly reduced. It took about 10 min to vanish (figure 2).

Figure 3: Schematic representation of the footprints. The DNA double strand is symbolized by two straight lines side-by-side. Protrusions or indentations, respectively, in the double strand represent suppression or enhancement, respectively, of cleavage by DNAase. Broken lines indicate weak effects. Temperatures are indicated on the left side. The DNA sequence (22) is shown at the bottom of the figure. The upper is the non-template strand. +1 is position 103 from the left (*Hpa* II) end of the 302 bp fragment. The -35 and -10 regions are overlined.

Although these experiments allow no estimation of the relative stabilities of the 7°C and 17°C complexes, they demonstrate a drastic increase in complex stability when the temperature was raised from 17°C to 37°C.

The ability of the complexes to initiate transcription was verified by assaying for initiation complexes in the presence of GTP. These complexes contain a short G-chain. The major start site in vitro is at position -2 in figure 3 (23). The relative concentrations of these complexes were determined in a filter retention assay. RNA polymerase and DNA complexed by this enzyme are retained on cellulose nitrate filters (24). All non-initiation complexes were destroyed prior to filtration under high salt/low temperature conditions (25). After 10 min of incubation the ratio of concentrations was 10:8:1 (37°C : 17°C : 7°C). This indicates that only at 7°C initiation is virtually impossible. As open complexes are able to initiate, this result indicates that at least the 7°C complex has to be regarded as closed.

The ability to rapidly initiate transcription is a more stringent

Figure 4: Transcription of 'rapid starter' complexes from P VIII at different temperatures. Complexes were formed at 7°C (lane 1), 17°C (lane 2), or 37°C (lane 3). Lane 4 contains marker DNA fragments. Some of their lengths (in nucleotides) are indicated. Arrows on the left side mark P VIII-specific transcripts. The short products are due to a termination or pausing event observed at low UTP concentrations.

criterion for open complexes (26), because in the assay described above, closed complexes which are in equilibrium with the open form are measured as 'open'. The relative concentrations of 'rapid starter' complexes were determined by challenging the preformed complexes with a mixture of the NTPs and heparin. Closed complexes rapidly dissociate in the presence of polyanions which trap the free polymerases (26). Therefore, only complexes initiating rapidly upon addition of NTPs (i.e. open complexes) are measured in this assay. Transcription was allowed to proceed to the fragment terminus, the reaction mixtures were subjected to PAGE, and the amounts of ^{32}P -labeled P VIII-specific RNAs were quantitated densitometrically from the autoradiograms (figure 4) and taken as a measure of open complex concentrations. The ratio of the concentrations was 33:7:1 (37°C : 17°C : 7°C). This illustrates that both, the 7°C and 17°C footprints, predominantly characterize closed complexes.

These results were corroborated by footprints of initiation complexes. At 37°C the presence of GTP caused a slight overall increase in band suppressions (presumably reflecting increased

Figure 5: Footprints of RNA polymerase on the non-template strand of P VIII. Lanes 1-3: 17°C, in the presence of GTP (500 μ M). Lanes 4-6: 37°C, in the absence of GTP. For further details see legend to figure 1.

complex stability due to chain initiation), but no basic change of the footprint (not shown). As the vast majority - if not all - of the complexes had initiated transcription (see below) and an initiation complex necessarily has to be open, this result confirms that the footprints of the binary complex at 37°C in fact visualize an open complex. At 17°C the addition of GTP caused a significant change in the footprint (compare figure 2, centre, and figure 5). The region between -4 and +22, which was unaffected in the absence of GTP, now showed band suppressions throughout. Thus, the footprint became identical to the open complex footprint observed at 37°C (figure 5, lanes 4-6). At 7°C the presence of GTP did not lead to any change in the footprint (not shown). This is in keeping with the results of the filter retention and transcription assays.

Table 1: Characterization of RNA polymerase - P VIII complexes

Temp.	Footprint borders	Association	Dissociation ¹⁾	Initiation	Rapid Initiation
7°C	-60/-45	rapid	rapid	-	-
17°C	-60/- 5	rapid	rapid	+	-
37°C	-60/+20	medium	slow	+	+

¹⁾ In the presence of polyanions.

The characteristics of the polymerase-promoter complexes at the three temperatures are summarized in table 1.

The transition temperature for the conversion of the closed into the open complex depends on the promoter sequence (27,8). Consequently, the "transition temperature" between the respective footprints should also be promoter-specific. In an experiment with another strong fd promoter, P X, this could in fact be shown to be the case. Unlike P VIII, P X forms open complexes very efficiently even at 17°C (unpublished results). In agreement with this observation, in the absence of NTPs P X yields virtually identical footprints at 37°C and 17°C which show the location and extension of an open complex footprint (figure 6, lanes 1,3-5).

According to the DNA sequence and the initiation site of transcription (10,28) an RNA of chain length 7 should be synthesized at P X in the presence of G-, U-, ATP. Although the length of the transcript was not examined, we note parenthetically that the upstream and downstream borders of the 37°C footprints of P X in the presence or absence of these NTPs were identical (figure 6, lanes 2-3) suggesting that during this stage of RNA synthesis the enzyme maintains the original promoter contacts, as proposed by Carpousis and Gralla (29).

DISCUSSION

Our results demonstrate that RNA polymerase is able to form at least three different specific complexes at a promoter site, two of which are transcriptionally inactive.

For these latter complexes, trapped at reduced temperatures (7°C and 17°C), polymerase footprints are visible with the up-

Figure 6: Footprints of RNA polymerase on the non-template strand of P X. Lane 1: 37°C, without polymerase. Lane 2: 37°C, with polymerase and A-, U-, GTP. Lane 3: 37°C, with polymerase. Lane 4: 17°C, with polymerase. Lane 5: 17°C, without polymerase. For further details see legend to figure 1.

stream region of the promoter (beginning around position -60). Both complexes differ in the downstream extensions of their footprints which increase with temperature (from about -45 to the transcriptional start site). A further increase solely into the downstream direction (to about +20) is seen upon transition into the active (open) complex at 37°C. Thus the footprint regions of the lower temperature complexes are totally comprised within the footprint regions of the higher temperature complexes.

Taking together the stability of the three complexes, their initiation behaviour, and the locations of their footprints, we conclude that the 7°C and 17°C complexes are closed. The closed 17°C complex, however, is in equilibrium with the open form: initiation is possible at this temperature, and in the presence of GTP (which is sufficient for RNA chain initiation at this promoter) a conversion of the footprint into the pattern character-

Figure 7: Representation of the upstream region of the P VIII footprints on a cylindrical projection of B DNA with 10.5 bp/turn. T = template strand, NT = non-template strand. ● = suppression of cleavage, ○ = enhancement of cleavage.

istic of the open complex is observed.

For the 7°C complex the most prominent effects in footprinting are observed between -60 and -45. This rather unexpected result gives rise to a number of questions: Does the location of the footprint coincide with the region actually complexed by RNA polymerase? If so, what is the origin of the apparent specificity of the interaction? Are the footprints upstream and downstream from -45 caused by different polymerase molecules, as proposed for the *tyrI* promoter (35)?

Polymerase binding seems to not absolutely require DNA upstream from about -40 (e.g. 30), and with the *lac* UV5 and T7 A3 promoters an interference of phosphate and purine alkylation with complex formation has not been detected upstream from this position (7). Therefore, one has to consider that the DNAase pattern in this region might not result from physical proximity of the polymerase, but rather from conformational changes in the DNA (31) induced by a polymerase bound downstream from -45. We

regard this unlikely for two reasons. First, the presence of DNA upstream of -50 improves promoter function (32,33). Second, the footprint is asymmetric with respect to the two faces of the duplex (see below; figure 7). A conformational distortion would not be expected to be face-specific.

In the region comprised by the 7°C footprint neither promoter (point) mutations nor prominent sequence homologies have thus far been detected (9). However, a moderately conserved A is found at -45 (9). In a considerable number of promoters (especially of phage genomes) this is extended into an A-rich stretch (9). Its potential function is presently unknown. fd P VIII contains 5 As between -42 and -48 (figure 3). They might be involved in the specificity of the observed interaction. Additionally (or alternatively) the specificity might originate from contacts (e.g. with the -35 region) which are too labile to yield a distinct footprint. We note that very faint effects are visible down to about -32.

On the basis of their footprinting data Travers et al. (35) suggest that two RNA polymerase molecules bind to the tyrT promoter (P tyrT) at 37°C. They postulate a secondary binding site extending from -45 to -65 or -75. The location of the 7°C footprint on fd P VIII falls into this region. However, there are also remarkable differences between the interactions observed and the DNA sequences involved at the two promoters. The P tyrT secondary site is not complexed at 25°C, while at reduced temperature the respective P VIII region is preferentially bound. The 7°C footprint at P VIII covers not more than about 1.5 helical turns. Only P tyrT contains in its upstream region a pair of appropriately spaced putative -10 and -35 regions. Besides, association and dissociation kinetics with P VIII failed to reveal a different behaviour of the regions upstream and downstream from -45. Moreover, preliminary results indicate that at 37°C the entire footprint is stabilized against dissociation by RNA chain initiation in the presence of GTP. For these reasons we regard it more likely that at P VIII all footprints originate from the binding of a single polymerase molecule.

For closed complexes or the closed parts of the open complex the spatial arrangement of polymerase-promoter contacts may be des-

cribed on a B-form DNA duplex. At all temperatures the P VIII footprints show a fine structure of band suppressions and enhancements between positions -61 and -45. When drawn on a cylindrical projection of the duplex (figure 7) it becomes obvious that these two types of effects are located on different faces of the duplex. If one considers that, where polymerase tightly binds to the DNA, DNAase cannot cleave (suppressed bands) and that polymerase cannot directly occupy the sites where DNAase cleaves (unaffected and enhanced bands), the assumption suggests itself that the distribution of band suppressions and enhancements signals that polymerase is bound to the 'suppression face'. This face is identical for all three complexes. When polymerase extends its contacts around the duplex, this has to result in an increase in the number of suppressed bands as well as in a decrease in the sum of the numbers of unaffected and enhanced bands. This is in fact observed (figure 7). Therefore we suggest that the loss of band enhancements upon increase in temperature does not signal changes in, but rather additions of polymerase-promoter contacts.

Footprints of other polymerase-promoter complexes also show band enhancements at a few sites. We examined the data for which the exact positions either were given or could be identified (34-39, this paper). The sites of enhancements vary greatly.

Figure 8: RNA polymerase-promoter interaction at 37°C as visualized by different techniques. The data are depicted on cylindrical projections of B form DNA with 10.5 bp/turn. T = template strand, NT = non-template strand. a: Footprinting data, only enhancements of cleavage shown. Numbers aside the circles give the respective references. 1=(34); 2=(35); 3=(36); 4=(37); 5=(38); 6=(39); 7=(35); 8=this paper, fd P VIII; 9=this paper, fd P X. b: Chemical modification data (7), only phosphate contacts shown. c: Crosslinking data (40). All data were normalized as follows: Position 6 of the -10 region was placed at position -8. If necessary, the spacer between the -10 and -35 regions was adjusted to 17 bp. This was done either by adding a base pair between -19 and -20 or by deleting the base pair(s) at -20 or from -19 to -21. The face borders were defined by maximizing the number of cleavage enhancements (a) or contacts (b,c) upstream of the 'open region' (pos. -10) on one face. This leads to different face borders for a, b, c. Relative to a, the face borders in b and c are shifted by 60° or 30°, respectively, in the same direction. For c a second orientation is possible which almost coincides with b.

However, when depicted on a cylindrical projection of the duplex, virtually all positions appear on one face (figure 8a). Thus it seems that at least upstream from position -15 (where the downstream-most enhancement is observed) RNA polymerase predominantly contacts the 'lower' face (as defined by figure 8a) of all these promoters (comprising lac UV5). We compared this result to chemical modification (7) and crosslinking data (40) on the lac UV5 and T7 A3 promoters (figure 8a-c). Note that these data indicate the face which polymerase contacts. The 'binding faces' defined by these methods deviate from the 'binding face' inferred from the footprints by 30-60°C. There are indications that similar closed complexes as observed with fd P VIII are also formed at other promoters. Wang (41) mentioned that footprints of RNA polymerase on the T7 A3 promoter extended between -47 and +14 at 37°C and between -56 and +1 at 0°C. When lowering the temperature from 37°C to 25°C, Travers et al. (35) found that the tyrT promoter, not initiating efficiently any more, showed reduced protection against DNAase I downstream of +1. (They also observed a shift of the upstream border of the footprint from -75 or -65 to -45, but, as mentioned above, attributed this to the binding of a second enzyme molecule at 37°C.) These results qualitatively agree with ours in that upon increase in temperature the footprint is extended downstream. The differences possibly reflect variations in interaction due to the individual promoter sequences and reaction conditions, e.g. ionic strength. The reduced temperature intermediates must not necessarily be intermediates of the pathway of complex formation at 37°C. However, there are some indications that this might be the case. First, it was shown directly (by the addition of GTP) that the 17°C closed complex is a precursor of the 17°C open complex. The presence or absence of GTP does not seem to alter the pathway, since the footprint observed transiently (at 0.5 min) in the presence of GTP is very similar to the footprint obtained at equilibrium in the absence of GTP (reduced band suppressions downstream from position -4). Second, the footprint observed at 0.5 min and 37°C also shows reduced band suppressions in this region (visible in figures 1 and 5, less so in figure 2), thus

being similar to the pattern of the 17⁰C closed complex. Third, Siebenlist et al. (7) concluded from 'alkylation interference' experiments performed at 37⁰C (with the lac UV5 and T7 A3 promoters) that the contacts in the initial complex(es) are only a subset of those in the final complex. This is in keeping with the footprints obtained at the three temperatures.

Thus, if we assume that the reduced temperature intermediates are in fact precursors of the open complex at 37⁰C, the pathway of promoter binding could roughly be pictured as follows:

Step 1: Formation of specific contacts (H-bonds?) between polymerase and recognition site(s) within the promoter (around position -45 and/or at the -35 region?) accompanied by formation of probably less specific (ionic?) bonds to one face of the duplex between positions -60 and -50 or -45.

An interaction which involves different regions of the participating molecules and different types of bonds is favourable, because then a disturbance in one of these parameters will less frequently lead to complex decay.

Step 2: Formation of stronger contacts (intercalation?) with the same face of the duplex between positions -45 and +1.

This region potentially comprises promoter segments involved in step 1. Possibly transient breaking of bonds made in step 1 is required for formation of these stronger bonds. During such a process the contacts upstream from about -45 could serve to reduce complex decay.

Step 3: Partial unwinding of the promoter, which is accompanied or shortly preceded by the formation of additional bonds downstream to position +20.

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