Exposure of T7 RNA Polymerase to the Isolated Binding Region of the Promoter Allows Transcription from a Single-stranded Template*

Alexander Kukarin‡§, Minqing Rong‡§, and William T. McAllister‡¶

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While the binding region of the T7 promoter must be double-stranded (ds) to function, the non-template strand in the initiation region is dispensable, and a promoter that lacks this element allows efficient initiation. To determine whether the binding region serves merely to recruit the RNA polymerase (RNAP) to the vicinity of a melted initiation region or provides other functions, we utilized a GAL4-T7 RNAP fusion protein to provide an independent binding capacity to the RNAP. When the GAL4-T7 RNAP was recruited to a single-stranded (ss) promoter via a nearby Gal4 recognition sequence, no transcription was observed. However, transcription from the ss promoter could be activated by the addition, in trans, of a ds hairpin loop that contains only the binding region of the promoter. The same results were obtained in the absence of the GAL4 recognition sequence in the template and were also observed with wild type enzyme. Gel-shift experiments indicate that exposure of the RNAP to the isolated binding region facilitates recruitment of the ss template, but that the binding region is displaced from the complex prior to initiation. We conclude that exposure of the RNAP to the isolated binding region reorganizes the enzyme, allowing it to bind to the ss template. These findings have potential implications with regard to mechanisms of promoter binding and melting.

To initiate transcription, RNA polymerases (RNAP) must locate and bind to a promoter region, separate the two DNA strands near the start site, and position the template strand in the active center. Understanding how this process occurs is important to our knowledge of the transcription process. As is the situation with other RNAPs, promoters for T7 RNA polymerase have a bipartite structure that consists of an upstream binding region that is recognized in a sequence-specific manner and an initiation region that must be melted open prior to initiation (1). The binding region (~17 to ~5) appears to function independently of the initiation region, and a double-stranded (ds) DNA fragment that contains only this region of the promoter is tightly bound by the RNAP (2). The binding region is recognized only in the form of duplex DNA, and removal of either the template (T) or non-template (NT) strands in this region prevents polymerase binding (3). In contrast, the NT strand in the initiation region is not required for initiation, and a partially single-stranded (pss) promoter in which this strand has been removed downstream of ~4 (essentially a "premelted promoter") allows efficient and accurate initiation (3).

A variety of lines of evidence suggests that the binding and initiation regions function independently and that it may be possible to physically separate the two while retaining promoter function. In the crystal structure of a T7 RNAP-promoter complex, as well as in an initiation complex in which the first 3 bases in the template strand have been transcribed, the two promoter domains are observed to interact with separate regions of the RNAP (4, 5). Furthermore, a number of structural changes that disrupt the linkage between the binding and initiation regions do not prevent initiation. These include the introduction of non-nucleoside linkers into the T strand between these two regions, as well as interruption or removal of a portion of the T strand that lies between the binding region and the start site of transcription (nicked or gapped promoters) (6, 7).

The question arises, then, as to whether the binding region serves merely to recruit the RNAP to a (melted) region of the template that is suitable for initiation or whether its interaction with the RNAP is required for other functions during initiation and isomerization to a stable elongation complex. To answer this question, we took advantage of a Gal4:T7 RNAP fusion protein in which the yeast Gal4 binding domain is fused to the amino terminus of T7 RNAP, thereby conferring an independent binding capacity upon the RNAP (8). We found that recruitment of the Gal4:T7 RNAP protein to a ss promoter via a Gal4 binding site is not sufficient to activate transcription, but that transcription could be activated by the addition, in trans, of a ds hairpin loop that contained only the binding region. Strikingly, the same results were obtained with wild type (WT) enzyme, and in the absence of the Gal4 DNA binding site. Gel-shift experiments indicate that exposure of the RNAP to the isolated promoter binding region facilitates recruitment of the ss template and that the binding region is displaced prior to initiation.

EXPERIMENTAL PROCEDURES

RNA Polymerase and Templates—His-tagged wild type T7 RNAP was purified as described previously (9, 10). The Gal4:T7 RNAP fusion protein (8) was purified as described for unmodified WT RNAP (9) except that the chromatography step on DEAE-cellulose was omitted. Oligonucleotides were purchased from Macromolecular Resources and
purified by reverse phase chromatography. The sequences of all oligonucleotides are presented in Table I. Where indicated, synthetic oligomers were labeled with "32P" utilizing T4 polynucleotide kinase (11) and purified from free label by chromatography on QuickSpin columns (Qiagen). To assemble synthetic templates, DNA oligomers were taken up in transcription buffer (see below) to a final concentration of 0.5 μM each, heated to 70 °C for 10 min, and allowed to cool slowly to room temperature (2–3 h).

RESULTS

Recruitment of T7 RNAP to a Single-stranded Template via an Auxiliary DNA Binding Motif Is Not Sufficient to Activate Transcription—The Gal4:T7 RNAP fusion protein binds to the Gal4 site with an affinity that is greater than that of T7 RNAP for its promoter, yet it retains catalytic activity and is able to initiate transcription specifically at a T7 promoter (2, 8, 12). To recruit the fusion protein to a ss promoter template, we tethered a ds Gal4 recognition site to the template via a 5′ “tail” that is complementary to a region located 43 nt upstream from the promoter (Fig. 1, A and C, template b). Gel-shift experiments demonstrate assembly of the composite template and binding by Gal4:T7 RNAP (Fig. 1B). At the same time, we examined a similar construct in which the upstream binding region of the T7 promoter was tethered to the template in the same manner (Fig. 1, A and C, template c).

As shown in Fig. 1, C and D, recruitment of the RNAP to the ss template via the Gal4 binding motif (template b) was not sufficient to activate transcription. In contrast, the presence of a ds promoter binding region tethered upstream (template c) resulted in efficient transcription, giving rise to a spectrum of products that was nearly identical to that observed from a control template in which the target promoter was rendered double-stranded by annealing of the complementary NT strand (template d).

A potential explanation for our failure to observe efficient transcription from the ss template using the Gal4 binding site is that the geometry of the Gal4 site relative to the initiation region of the promoter is not appropriate and prevents correct placement of the initiation region into the active site. This explanation is diminished, however, by the finding that the Gal4 binding site did not interfere with initiation by the fusion protein when the promoter was rendered double-stranded by annealing of a complementary NT strand (template e). The presence of an additional promoter binding region upstream of the ds promoter also had little effect on initiation at the ds promoter (template f).

Recruitment of T7 RNAP to the Isolated Binding Region of the Promoter Activates the Enzyme to Transcribe a Single-stranded Template—The observation that the tethered promoter binding region activated transcription from the ss promoter, whereas the tethered Gal4 binding site did not, suggests that the promoter binding region provides other functions in addition to its ability to recruit the RNAP to a nearby initiation site. To examine this, we asked whether the addition of the isolated promoter binding region in trans (as an untethered ds hairpin loop) could activate transcription by an RNAP that was bound to the ss template via the Gal4 binding site (Fig. 2). Two stem-loop structures were employed in these studies. RC12 contains the upstream binding region of the T7 promoter from −5 to −23; RC13 has a similar stability and structure but contains the upstream binding region of the T7 promoter.

As shown in Fig. 2, lane 2 versus lane 4. Even more striking, however, were the observations that stimulation also occurred with the WT enzyme (lane 1 versus lane 3) and in the absence of the Gal4 binding site (template b, lanes 7 and 8). The addition of RC13 (the control stem loop structure) did not lead to activation (lanes 9 and 10) nor did the addition of another stem-loop structure that deviates from the consensus binding site only at position −8 (which prevents promoter function (13, 14)) (data not shown).
The Entire Promoter Sequence Must Be Present in the Single-stranded Template for Activation to Occur

In the templates described above, the target promoter in the ss template contained the entire consensus sequence from −17 to +6. To explore what features of the target promoter are required to allow activation (e.g. is the presence of the binding region required, or is the presence of a ss initiation region sufficient?), we constructed templates in which various portions of the binding region in the target promoter were deleted or replaced (Fig. 3). Truncation of the original template (MJ1) to 24 nt upstream from the start site (AK18) did not prevent activation by RC12 nor did substitution with a different sequence upstream of −17 (AK24). However, commencing at −15, substitution of the binding region with an unrelated sequence prevented activation. While necessary, the binding region is not sufficient, as additional flanking sequences upstream are also required (compare templates c and f).

A Model for Activation of the RNAP by Exposure to the Isolated Binding Region of the Promoter—The affinity of T7 RNAP for a promoter that lacks a ds binding region is very low (Refs. 2, 3, and 5 and see Fig. 5). Activation of the RNAP to transcribe such a template must therefore result (at least in part) from an increased ability to bind to and/or retain the template. A possible explanation for the observations reported here is that exposure of the RNAP to the isolated binding region reorganizes the enzyme, allowing binding of the ss template and its entry into the active site. While in principle this could involve only transient exposure of the RNAP to the binding region, the affinity of the polymerase for the isolated ds binding region is even greater than that of the enzyme for a
Fig. 3. The entire promoter sequence must be present in the ss template for activation to occur. Left panel, the ds control template (template a) was formed by annealing together oligomers AK22 and AK18, as in Fig. 1. Single-stranded templates (b–i) contained the same sequence in the T strand (template b) or had substitutions (templates c–e) or deletions (templates f–i) in the region upstream of the promoter, as indicated. Right panel, the templates indicated were transcribed by WT T7 RNAP in the presence or absence of RC12, as noted, and the products were resolved by electrophoresis as in Fig. 1D. The position of the 10 nt run-off product is indicated.

complete promoter (2, 16, 17), suggesting that once formed, the binary complex may be quite stable.

For these reasons, and for reasons that will be explained below, we suggest that binding of RC12 results in the formation of a binary complex that subsequently binds the single-stranded template. Three alternative pathways that could account for continued retention of the ss template and activation of transcription are presented in Fig. 4. First, formation of a stable ternary complex (consisting of RNAP, RC12, and the ss template) might be sufficient to allow initiation through cooperative interactions between the binding and initiation regions. However, this does not appear to be consistent with the observation that the ss template must also contain the sequence of the upstream binding region for activation to occur (see Fig. 3). Alternatively, the binding region present in RC12 may be denatured within the complex. Annealing of the T strand portion of the binding region to its complement in the ss template would then result in the formation of a ds binding region in the target promoter (i.e., a ss promoter; annealing model). In a third alternative, the ss template might displace the ds binding region, allowing it to interact with both the promoter binding region of the RNAP and with the active site (displacement model). The latter complex might mimic some of the features of an elongation complex in which promoter clearance has occurred.

The 23-bp ds stem portion of RC12 is expected to be quite stable (predicted \( \Delta G = -34.6 \text{kcal/mol} \)) and to rapidly self-annex even if transiently denatured. Heating and annealing of RC12 to the ss target sequence in solution did not result in significant hybridization of the binding region to the ss template, even when the template was present at a 10-fold molar excess (data not shown). We therefore conclude that spontaneous annealing of RC12 to MJ1 cannot account for the observed activation. (However, on the basis of this experiment alone we cannot rule out the possibility that the polymerase might facilitate separation and annealing of the strands of RC12 within the ternary complex.)

To investigate the alternative pathways shown in Fig. 4 more directly, we used a gel-shift assay to examine binding of RC12 and the ss template (Fig. 5). All three of the models predict that RC12 will enhance binding and retention of the ss template. As expected, adding increasing amounts of RC12 to reactions that contained a fixed concentration of RNAP and labeled ss template resulted in enhanced binding of the ss template (Fig. 5, A and B).

While the annealing model and the cooperative interaction model predict that adding increasing amounts of the ss template to complexes that have bound RC12 should not result in displacement of the ds binding region (and might even enhance its retention in the case of the annealing model), the displacement model predicts that addition of increasing amounts of the ss template should result in loss of the ds binding region from the complexes. As shown in Fig. 5, C and D, the latter result is observed. Here, binding of RC12 was observed in the absence of the ss template, but as the concentration of the ss template was increased, RC12 was displaced. Moreover, the transcription activity of the complexes increased with MJ1 concentration and remained at a high level even when nearly complete displacement of RC12 was observed. These observations are in direct contradiction of the annealing and cooperative interaction models, but are in agreement with the displacement model.

DISCUSSION

In this work, we have shown that recruitment of T7 RNAP to a ss template that contains the consensus promoter sequence is not sufficient to permit initiation of transcription. Strikingly, the presence of the ds binding region, either tethered to the ss template some distance away from the promoter or added in trans as a stem loop structure, activates the RNAP to initiate transcription on such a template. Gel-shift assays demonstrate that exposure of the RNAP to the isolated binding region enables the RNAP to bind to the ss template, but that the binding region is displaced prior to initiation.

From these experiments, we conclude that while T7 RNAP is ordinarily unable to bind a ss promoter template, exposure of the RNAP to the ds binding region of the promoter alters the enzyme in such a way as to enable it to bind and initiate transcription on the ss template. The observation that the ss template must contain the upstream binding sequence to be activated (Fig. 3) indicates that the ss template must replace some of the contacts made by the ds binding region, not just displace them. Most of the base-specific contacts made between the RNAP and the binding region of the promoter involve the template strand (4, 18, 19). Perhaps these interactions are important in the displacement process or in activation.

Why is the RNAP unable to bind to the ss template unless it is first exposed to the binding region? Some insight into this question may come from studies with bacterial RNAPs, which also have promoters with a bipartite structure comprising an upstream binding region and a downstream initiation region. The transition to an open complex involves multiple stages, in which promoter bound as duplex DNA (a closed complex) is locally melted, and the T strand is led down into the active site (to form an open complex). Recent structural analyses indicate
that in the open complex the T strand of the initiation region is enclosed in a channel that is surrounded on all sides by protein and that movement of region 1.1 of the transcription initiation factor /H9268 is required to permit entry of the T strand and of downstream DNA into their respective binding sites (20–22).

In view of the observations reported here, it would be of interest to determine whether exposure of the multisubunit RNAPs to the isolated binding regions of their promoter might induce conformational changes that are associated with open promoter formation.

The formation of an open complex by T7 RNAP also involves multiple steps. The initial closed complex (ED C) isomerizes to an intermediate form (ED O1) in which the promoter is melted from /H11002 to /H11002 and subsequently to a fully open complex (EDO2) that is melted to /H11001 (23). Kinetic studies indicate that the enzyme may exist in two conformations, which differ in their rates of promoter binding and open promoter formation, with the fast form predominating in most RNAP preparations (24).

It has not yet been determined whether exposure of the enzyme preparation to the isolated binding region alters the distribution of these two forms.

Based upon these observations, we suggest that unliganded T7 RNAP is unable to bind to and/or insert the T strand in the initiation region into the active site and that coordinated changes in the structure and organization of the transcription complex in response to base-specific contacts in the binding region are required to capture the T strand or otherwise stabilize the open conformation. A number of structural elements in T7 RNAP are known to be involved in promoter binding and melting. The specificity loop (amino acid residues 740–769) interacts in a sequence-specific manner with bps in the binding region from /H11002 to /H11002 (4, 13, 14, 25). In addition, an AT-rich recognition loop (residues 93–101) interacts with the promoter 13–17 nt upstream from the start, and a /H9252-hairpin intercalating loop (residues 245–250) interacts with the promoter between /H11002 and /H11002 and is thought to be important for promoter melting and directing the T strand into the active site) (4, 5). Whereas the positions of the AT-rich recognition loop and the specificity loop remain largely unchanged before and after promoter binding and during the early stages of initiation, the /H9252-hairpin intercalating loop is disordered and becomes visible in the crystal structure only after promoter binding (4, 26, 27).

Speculating that the unformed /H9252-hairpin loop might occlude the binding site or otherwise inhibit insertion of the T strand,

**Fig. 4. Potential models for activation of T7 RNAP by exposure to the isolated binding region of the promoter.** Exposure of T7 RNAP (ellipse) to the isolated binding region of the promoter (RC12) allows binding of the ss template (T). Once formed the ternary complex may be able to support transcription through cooperative interactions. Alternatively, denaturing of the ds binding region in RC12 might allow reannealing with the upstream binding region in the ss template, leading to the formation of a pss template (Annealing model). Last, binding of the ss template may result in displacement of the ds binding region (Displacement model).

**Fig. 5. Binding of the ss template results in displacement of the ds binding region.** A and B, 32P-labeled MJ1 (50 nM) and T7 RNAP (100 nM) were incubated with increasing concentrations of RC12, as indicated, and the complexes were analyzed by electrophoresis in 5% polyacrylamide gels under non-denaturing conditions. The fraction of MJ1 bound (open squares) as a function of increasing RC12 concentration is presented in the bottom panel (B). C and D, as above, except that the concentration of RC12 was 50 nM in all samples, and the concentration of MJ1 increased from 0 to 100 nM (as indicated). The fraction of RC12 bound (open squares) as a function of increasing MJ1 concentration is presented in the bottom panel (D). Transcription activity in the samples (filled circles) was determined by measuring the production of the 10-nt run-off product as in Fig. 3 and is plotted as a function of MJ1 concentration.
we examined whether mutant RNAPs in which this element had been disrupted might be able to initiate transcription from a ss template. Neither of the mutant RNAPs that we tested (DelbH, in which residues 236–240 are deleted (28), or Δ231–241, in which residues 231–241 are deleted (29)) provided this effect (data not shown); perhaps more substantial changes in this region are required. Other regions of the RNAP whose organization changes after promoter binding include the tip of the thumb domain, and a portion of the fingers domain, which forms part of the downstream DNA binding site in the elongation complex (4, 26, 27, 30). We have not tested the effects of mutations in the latter regions.

The templates used in the experiments described here extend only 10 nt downstream from the start site for transcription. While useful for studies of transcript initiation, the lengths of these templates are not sufficient to allow isomerization and that transcription on promoters that lack the NT strand downstream of ~4 (pss promoters) results in enhanced release of products in this size range (7, 31). To examine transcription from ss templates in more detail, we utilized templates that extend 20 nt downstream and found that the pattern of transcription was nearly identical to that of the pss promoters (data not shown).

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