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Molecular mechanism of transcription inhibition by phage T7 gp2 protein

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Abstract

E. coli T7 bacteriophage gp2 protein is a potent inhibitor of host RNA polymerase (RNAP). Gp2 inhibits formation of open promoter complex by binding to the β' jaw, an RNAP domain that interacts with downstream promoter DNA. Here, we used an engineered promoter with an optimized sequence to obtain and characterize a specific promoter complex containing RNAP and gp2. In this complex, localized melting of promoter DNA is initiated but does not propagate to include the point of the transcription start. As a result, the complex is transcriptionally inactive. Using a highly sensitive RNAP beacon assay we performed quantitative real-time measurements of specific binding of the RNAP-gp2 complex to promoter DNA and various promoter fragments. In this way, the effect of gp2 on RNAP interaction with promoters was dissected. As expected, gp2 greatly decreased RNAP affinity to downstream promoter duplex. However, gp2 also inhibited RNAP binding to promoter fragments that lacked downstream promoter DNA that interacts with the β' jaw. The inhibition was caused by gp2-mediated decrease of the RNAP binding affinity to template and non-template strand segments of the transcription bubble downstream of the -10 promoter element. The inhibition of RNAP interactions with single-stranded segments of the transcription bubble by gp2 is a novel effect, which may occur via allosteric mechanism that is set in motion by the gp2 binding to the β' jaw.

Keywords

RNA polymerase; transcription inhibition; T7 bacteriophage; open promoter complex; protein beacon assay

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Introduction

RNA polymerase (RNAP) is amongst the most highly regulated enzymes in the bacterial cell. Bacteriophages have developed ingenious mechanisms to modify host RNAP to make it serve viral needs. Interactions between phage-encoded factors and host RNAPs have long served as a rich source of paradigms for transcriptional regulation (reviewed in^{1,2}). One such example is provided by *E. coli* bacteriophage T7. The transcription of T7 phage genome proceeds in three stages. Early T7 genes are transcribed from several very strong promoters by *E. coli* RNAP containing primary σ^{70} subunit ($E\sigma^{70}$), whereas middle and late T7 genes are transcribed by phage-encoded RNAP³. The product of the middle T7 gene 2, a 7 kDa protein called gp2, binds to the β' jaw domain of the host RNAP and inhibits it^{4,5}. Only one gp2 molecule participates in tight interaction with an RNAP molecule⁶ and point mutations in as well as deletions of the β' jaw abolish this binding⁵. Gp2 was originally thought to simply switch phage gene transcription from host to viral RNAP. However, its essential biological role is to prevent transcription by host RNAP into those parts of T7 genome that are normally transcribed by viral RNAP. The slow-moving *E. coli* RNAP slows down the fast-moving T7 RNAP, causing the latter enzyme to pause, which initiates aberrant processing of T7 replicative intermediates, leading to accumulation of improperly packaged, defective phage particles^{7,8}.

The RNAP site of gp2 binding, the β' jaw, is part of a trough that accepts double-stranded DNA downstream of the transcription start point⁹. An RNAP mutant lacking the β' jaw (RNAP- $\beta'\Delta$ jaw) is active but forms unstable transcription initiation complexes⁹. Binding of gp2 to the β' jaw prevents formation of detectable promoter complexes by $E\sigma^{70}$ (5,10). Gp2 has no effect on (and does not bind to) preformed open promoter complexes (RP_o) or transcription elongation complexes⁵. Thus, the β' jaw interactions with downstream DNA and gp2 appear to be mutually exclusive, suggesting that the mechanism of inhibition is based on steric blockade of the β' jaw interactions with DNA by bound gp2.

It is commonly accepted that RP_o formation is initiated when $E\sigma^{70}$ contacts with DNA upstream of the transcription start point are established. Since gp2 binds the β' jaw, it is expected to inhibit a late stage of RP_o formation. Thus, gp2 is predicted to trap an intermediate complex *en route* to RP_o¹⁰. However, promoter complexes containing gp2, if they exist, are unstable and standard methods such as footprinting failed to reveal the interaction of the preformed complex of $E\sigma^{70}$ and gp2 ($E\sigma^{70}$ -gp2) with promoter¹⁰. Here, we used an engineered promoter with optimized sequence to obtain and partially characterize a specific transcriptionally inactive promoter complex containing $E\sigma^{70}$ and gp2. DNase I footprinting and KMnO₄ probing of this complex reveal that gp2 prevents establishment of downstream DNA contacts and interferes with DNA melting near the transcription start site but has no effect on RNAP interactions with DNA at and upstream the -10 promoter element. Using a recently developed fluorescence RNAP beacon assay¹¹ we performed quantitative real-time measurements of specific binding of the $E\sigma^{70}$ -gp2 complex to promoter DNA and various promoter fragments. In this way, the effect of gp2 on RNAP interaction with promoters was dissected. The data show that gp2 not only greatly decreases RNAP affinity to downstream promoter duplex but also reduces RNAP affinity to non-template strand segment of the transcription bubble located between the -10 element and the transcription start site (called “discriminator” on some promoters). Further, gp2 appears to impede the entry of the template strand into the active center cavity. The inhibition of RNAP-discriminator interactions by gp2 is a novel effect, which may be either direct or indirect, i.e., due to allosteric changes caused by gp2 binding to the β' jaw. We propose that promoter complexes trapped by gp2 correspond to an intermediate of promoter complex formation. Availability of such complexes, which are very transient in the absence of gp2, opens way for their functional and structural characterization.

Results

Experimental set-up: use of the RNAP beacon assay to monitor the inhibition of *E. coli* $E\sigma^{70}$ interactions with promoter DNA by T7 gp2

T7 gp2 binding to the jaw domain of the *E. coli* RNAP β' subunit decreases $E\sigma^{70}$ affinity to promoter DNA and prevents formation of catalytically competent $RP_o^{5,10}$. $E\sigma^{70}$ binding to promoter DNA and various promoter fragments can be measured using fluorescence protein beacon assay¹¹. The beacon assay relies on the detection of fluorescence signal from RNAP holoenzyme containing the σ^{70} subunit with fluorescent label site-specifically incorporated in region 2.3, close to the part of σ that recognizes the -10 promoter element. The ground level fluorescence of such RNAP beacon is low because aromatic σ amino acids quench the fluorescence of the label. When RNAP beacon binds promoter DNA or a promoter fragment specific interactions between σ region 2.3 and DNA are established. Since aromatic amino acids that quench the label participate in interactions with promoter DNA, the fluorescent probe becomes “unquenched” leading to increased fluorescence.

As can be seen from Fig. 2a, the fluorescence signal generated upon the addition of T5 N25 promoter-containing DNA fragment to the RNAP beacon preincubated with excess gp2 is considerably lower than that observed in the absence of gp2. The gp2 effect was insignificant when gp2 was added to preformed complex of RNAP beacon and T5 N25 promoter DNA (Fig. 2a). The results are consistent with standard abortive transcript synthesis assay (Fig. 2b): transcription from T5 N25 was strongly inhibited when gp2 was added to RNAP before the addition of promoter DNA (lane 2), but no effect was observed when gp2 was added to preformed open complexes (lane 3). Thus, the beacon assay allows one to follow the inhibitory action of T7 gp2 on RNAP interactions with promoter.

A stable ternary complex containing $E\sigma^{70}$, gp2, and promoter DNA can form when RNAP interactions with promoter are optimized

Gp2 also decreased to the binding of RNAP beacon to control non-promoter DNA fragment (Fig. 1) that contained multiple substitutions in the -35 and -10 regions and lacked transcription activity *in vitro*. Curiously, the signal observed upon the addition of T5 N25 DNA to $E\sigma^{70}$ -gp2 was higher than that observed upon the addition of non-promoter DNA (Fig. 2a). We interpreted this result by hypothesizing that some specific interactions between $E\sigma^{70}$ -gp2 complex and promoter are established. However, we could not detect specific binding of T5 N25 DNA to RNAP in the presence of gp2 using $KMnO_4$ probing or DNase I footprinting techniques (data not shown). We hypothesized that this negative result may reflect insufficient stability of ternary (RNAP-gp2-T5 N25) complexes. To improve the putative ternary complex stability we prepared a derivative of -65 to $+35$ T5 N25 DNA fragment containing consensus -35 element, an optimized UP element¹², and the extended -10 element (Fig. 1). We expected that RNAP binding to this promoter, which we call N25cons, would be strengthened due to improved interactions upstream of the -10 promoter element. As expected, the DNA fragment containing N25cons generated significant fluorescence signal when combined with RNAP beacon in the presence of gp2 (Fig. S1a), indicating that specific complex between $E\sigma^{70}$ -gp2 and N25cons forms on this promoter. In the absence of gp2, $E\sigma^{70}$ was highly active on N25cons in the abortive transcription initiation assay (Fig. 1b, lane 1), yet, despite the considerable fluorescence signal observed in the beacon assay, transcription from N25cons was highly sensitive to the addition of gp2 prior to promoter complex formation (Fig. S1b, lane 2). Gp2 had no effect on preformed open complexes on N25cons (Fig. S1b, lane 3).

$E\sigma^{70}$ -gp2 formed complexes with the N25cons promoter that were readily detectable and clearly distinct from complexes formed by $E\sigma^{70}$ alone or when gp2 was added after

promoter complex formation (Fig. 3). First, DNase I footprinting demonstrated that while $E\sigma^{70}$ strongly protected DNA up to +18 (with weaker protection extending approximately up to position +25), no protection downstream of position +8 was observed in complexes formed by $E\sigma^{70}$ -gp2 on either DNA strand (Fig. 3, compare lanes 2 and 3, and 11 and 12). In contrast, the upstream portion of the footprint was identical in all complexes. Second, Exo III treatment of promoter complexes revealed that the downstream boundary of $E\sigma^{70}$ promoter complex, located at position +18 in the absence of gp2, was mostly shifted to around position +3 in the case of $E\sigma^{70}$ -gp2 complex (Fig. S2). The upstream boundary, located approximately at position -42, was slightly shifted downstream in the presence of gp2, whether added before or after DNA (Fig. S2, compare lanes 6, 7, and 8). While this observation suggests that gp2 may modify RNAP interactions upstream of the -35 promoter element, this effect is weak, as experiment shown in Fig. 7 (see below) demonstrates. $KMnO_4$ probing revealed that in the absence of gp2, thymine bases at positions from -11 to +3 on the template strand are highly accessible to permanganate, with thymine at position +4 and cytidine at position -14 exhibiting weak sensitivity to the modifying reagent (Fig. 3). In complexes formed by $E\sigma^{70}$ -gp2, the reactivity of bases located from the upstream edge of the transcription bubble up to -6 was altered insignificantly, while intensity of $KMnO_4$ -sensitive bands further downstream was decreased (Fig. 3, compare lanes 15 and 16). On the non-template strand, strong reactivity at and around the transcription start point was observed in the absence of gp2 (Fig. 3, lane 6). This reactivity was strongly decreased in $E\sigma^{70}$ -gp2 complexes (Fig. 3, lane 7). We explain the residual $KMnO_4$ reactivity at and around the transcription start site seen in reactions containing $E\sigma^{70}$ -gp2 complexes by the presence of a small fraction of $E\sigma^{70}$ -promoter complexes in the reactions. The presence of such complexes is also evident from Exo III footprinting (a minor fraction of exonuclease stops at position of +18, Fig. S2, lane 3) and residual abortive initiation activity (Fig. S1b, lane 5). We therefore suspect that in $E\sigma^{70}$ -gp2 complexes *per se*, the transcription start site is closed explaining why these complexes are transcriptionally inactive. The $KMnO_4$ reactivity decreased insignificantly upon a 2-min challenge of the $E\sigma^{70}$ -gp2-N25cons complex with 50 μ g/ml of DNA competitor heparin (data not shown).

Overall, the results indicate that when interactions with the upstream promoter part are strong, RNAP forms a specific, stable, yet transcriptionally inactive promoter complex in the presence of gp2. The complex is only partially open and formation of contacts in the downstream portion of the transcription bubble, as well as contacts with double-stranded DNA downstream of the transcription initiation start point appears to be compromised. In the ternary complex, the DNA trajectory downstream from the -10 element must differ considerably from that in RP_o . A similar complex may form on the wild-type T5N25 promoter, however it is too transient to be detected by footprinting.

Quantitative dissection of the gp2 inhibitory effect using model promoter fragments

$E\sigma^{70}$ specifically binds upstream and downstream fork junctions--partially double stranded DNA containing short non-template strand overhangs with the -10 promoter element sequence--and this interaction can be monitored by the beacon method^{11,13,14}. By "upstream" and "downstream" we here mean the location of double-stranded segments relative to the non-template strand overhangs. Gp2 inhibits $E\sigma^{70}$ binding to downstream fork junction probes, whereas RNAP binding to upstream fork junction probe [-38/-11] [-38/-12] (shown in Fig. 1) containing a short single-stranded extension corresponding to the -11A base is not affected¹⁴. These results indicate that downstream fork junctions contain promoter segments whose recognition by RNAP is targeted by gp2 and, therefore, these probes can be used to study the gp2 inhibition mechanism in more detail. We decided to quantitatively measure $E\sigma^{70}$ binding to various downstream fork junctions and some other probes (shown in Fig. 1) in the presence of gp2. To reach binding

saturation, much higher concentrations of downstream fork junction probes were required in the presence of gp2 than in its absence (Fig. 4a, see also¹⁴). However, the binding observed in the presence of gp2 was specific, since control probes bearing a C to T substitution at the highly conserved -7 position did not generate any noticeable signal (see, for example, Fig. 4b). The equilibrium dissociation constant (K_d) for $E\sigma^{70}$ -gp2 binding to [-12/+20][+4/+20] downstream fork junction probe (with junction point positioned at +4) was calculated by fitting the experimental dependence of the fluorescence signal amplitude on probe concentration to a chemical equilibrium equation (e.g. by titration assay¹¹) (Fig. 4a). The resulting value was 120 nM. Free $E\sigma^{70}$ bound the same probe with a K_d of less than 0.1 nM (Fig. 4a). Thus, gp2 decreases RNAP affinity to the downstream fork junction promoter fragments by more than 1000-fold.

As shown in Fig. 4b, probes with junction points at +2 and +3 bound the $E\sigma^{70}$ -gp2 less efficiently than the [-12/+20][+4/+20] probe. The apparent dependence of downstream fork junctions affinity to $E\sigma^{70}$ -gp2 on the junction position is somewhat surprising, since negligible difference in affinity of these probes to $E\sigma^{70}$ was detected¹⁴.

The effect of gp2 on RNAP interaction with a segment of template strand adjacent to the transcription start point was estimated by comparing the binding of [-12/+20][+4/+20] or [-12/+9][+4/+9]SP, a short hairpin downstream fork junction, and their derivatives bearing 2–7 nt-long non-complementary extensions of template strand. Fluorescence signal changes observed upon $E\sigma^{70}$ -gp2 interaction with these probes are shown in Fig. 5a and Fig. S4. The kinetic curves in Fig. 5a indicate that much higher fluorescence levels are observed when $E\sigma^{70}$ -gp2 is mixed with parent [-12/+20][+4/+20] probe than with derivative probes containing template strand overhangs. We determined the K_d value for RNAP-gp2 binding to one such probe, NC(-3/+4). Low signal level made it impossible to determine the K_d value for this probe by titration assay. Therefore, an estimate of RNAP-gp2 binding to NC(-3/+4) ($K_d > 0.6 \mu\text{M}$) was obtained from a competition binding assay¹¹ by measuring RNAP-gp2 binding to -12/+3 oligo with and without 0.2 μM NC(-3/+4) (Fig. S3). In contrast, the mutant $\beta'\Delta$ jaw RNAP has higher affinity to downstream fork junction probes bearing template strand extensions than to parent probes (Fig. 5b, Table 1). Thus, the inhibitory effect of single-stranded template strand extension on binding of the [-12/+20][+4/+20] derivatives is specifically caused by the gp2 binding to RNAP.

Upon incubation with $E\sigma^{70}$ -gp2, [-12/+9][+4/+9]SP-based downstream fork junction with template strand extension generated saturated signal similar to that observed with parental [-12/+9][+4/+9]SP; however, the kinetics of signal increase was significantly slower with the probe bearing the extension (Fig. S4). These data are in marked contrast with the situation observed in the absence of gp2, when an overhang from +3 to -4 actually improves the affinity of short fork junctions to $E\sigma^{70}$ by ~40-fold (Fig. S5). These results indicate that the interactions of template strand overhangs with RNAP are affected by gp2 in the $E\sigma^{70}$ -gp2 complexes with downstream fork junctions.

If gp2 inhibited transcription solely by targeting interactions with the double-stranded DNA downstream of the transcription start point, then no effects on the interaction with single-stranded DNA in the transcription bubble should be expected. To determine if gp2 effect of transcription bubble formation is direct or indirect, we studied the effect of gp2 on the interaction of RNAP with oligonucleotides containing sequences matching the -10 promoter element consensus sequence and corresponding to single-stranded non-template extensions of downstream fork junction probes. $E\sigma^{70}$ specifically binds such oligonucleotide probes¹⁵ and the interaction can be measured by the RNAP beacon assay¹¹. We found that gp2 inhibits binding of -12/+2 oligo 27-fold (Fig. 6A, Table 1). The effect was specific since *i*) only very small decrease in RNAP binding to -12/+2 was observed in the presence of R58E

gp2 mutant having impaired affinity to RNAP¹⁰, and *ii*) no inhibition of $-12/+2$ oligo binding was observed upon gp2 addition to RNAP beacon containing RNAP- $\beta'\Delta$ jaw¹⁴ (data not shown).

$E\sigma^{70}$ binding to $-12/-3$ oligo was inhibited by gp2 ~9-fold; the binding of $-12/-6$ oligo was not affected (Figs. 6b, 6c). The results suggest that gp2 hinders $E\sigma^{70}$ interactions with the $-5/+2$ segment of the non-template strand. Consistent with this conclusion, gp2 also decreased binding of upstream fork junction probes bearing longer ($-11/+2$ and $-11/-3$) non-template strand overhangs, whereas the binding of an upstream fork bearing a short $-11/-7$ overhang was not affected (Fig. S6).

Comparison of K_d values of $E\sigma^{70}$ -gp2 binding to $-12/+2$ oligo and $[-12/+20][+4/+20]$ fork junction shows that the $+3/+20$ segment increases affinity 37-fold. In contrast, the $+3/+20$ segment increased $E\sigma^{70}$ affinity 9000-fold¹⁴. Thus, gp2 greatly decreased RNAP affinity to downstream promoter duplex. We also determined K_d values for binding of RNAP beacon containing RNAP- $\beta'\Delta$ jaw to $-12/+2$ oligo and $[-12/+20][+4/+20]$ probe (Table 1). In agreement with previous results, RNAP- $\beta'\Delta$ jaw binds the downstream fork junction probe much weaker than wild-type RNAP¹⁴; however, the mutant RNAP affinity is ~30-fold higher than that of $E\sigma^{70}$ -gp2. The data also show that unlike the addition of gp2, deletion of the β' jaw has only a slight effect on the $-12/+2$ oligo binding (Table 1).

As noted above, the Exo III footprinting data suggested that gp2 might affect RNAP-promoter interactions at around position -42 . Moreover, this effect must be distinct from the “normal” function of gp2 as it is observed whether gp2 is added before or after promoter complex formation (Fig. S2). To determine the significance of this new effect, we measured the consequences of gp2 addition on RNAP binding to $[-59/-12]$ probe. A control, T4 AsiA protein, which is known to inhibit RNAP interaction with the -35 element¹⁶, strongly inhibited RNAP binding to this probe (Fig. 7). In contrast, gp2 had very little or no effect on the binding of this probe to $E\sigma^{70}$ (Fig. 7). We conclude that gp2 does not noticeably affect RNAP-promoter interactions upstream of the -10 promoter element.

Effect of gp2 on RNAP interaction with double-stranded promoter fragments truncated downstream of the transcription start point

The DNase I footprinting of the ternary $E\sigma^{70}$ -gp2-N25cons complex suggests that gp2 blocks RNAP-promoter contacts downstream from approximately $+8$ position. A set of double-stranded probes was used to assess the influence of promoter segments downstream of the $+6$ position on promoter interactions with the RNAP-gp2 complex. The parent probe was a derivative of the $[-50/+14]$ fragment of the T5 N25 promoter containing consensus -35 element and the extended -10 element (Fig. 1). The binding of $[-50/+14]$ and three other derivative probes truncated at $+6$, $+8$, and $+12$ was assessed by measuring RNAP beacon signals upon the addition of *i*) gp2 to preformed $E\sigma^{70}$ complexes with each probe or *ii*) DNA probes to the $E\sigma^{70}$ -gp2 complex. Upon reaching equilibrium binding, signal amplitudes should be the same independent of the order of DNA and gp2 addition. As can be seen in Fig. 6a, the binding curves were strikingly different when gp2 was added to preformed $E\sigma^{70}$ -DNA complexes. The fluorescence intensity decrease observed with the $[-50/+14]$ and $[-50/+12]$ probes was very slow. In contrast, signal amplitudes rapidly reached equilibrium values upon the addition of gp2 to $E\sigma^{70}$ complexed with $[-50/+6]$ or $[-50/+8]$ probes. Moreover, abortive transcript synthesis from $[-50/+6]$ was inhibited when gp2 was added either prior or after this probe (Fig. 6b, compare lanes 2 and 3). Combined with DNase I footprinting and structural modeling¹⁰ data, these results suggest that $E\sigma^{70}$ interactions with duplex DNA downstream of ca. position $+9$ and with gp2 are mutually exclusive. On the other hand, $E\sigma^{70}$ -DNA contacts upstream of ca. position $+9$ are

compatible with gp2 binding, however, the interaction with gp2 leads to formation of an inactive $E\sigma^{70}$ -gp2-promoter complex.

Discussion

The T7 gp2 is a powerful inhibitor of promoter complex formation on most promoters. RNAP interactions with gp2 and promoter were thought to be mutually exclusive and promoters that are resistant to gp2, such as the *galP1* promoter⁵, escape inhibition by displacing gp2 from RNAP via optimized interactions of downstream DNA with the enzyme⁹. The principal result of this work is the demonstration that at conditions when RNAP contacts with promoter elements are strong, a complex between RNAP σ^{70} holoenzyme, T7 gp2, and promoter can form. This complex is transcriptionally inactive, however, based on DNase I and ExoIII footprints and $KMnO_4$ probing, it resembles “normal” RP_o at and upstream of the -10 promoter element. Downstream of the -10 element, the complex containing gp2 differs from RP_o : the transcription start point is not melted and contacts with DNA downstream of position $+8$ are lacking (in RP_o , strong downstream protection extends to position $+18$). Thus, it appears that in the presence of gp2, melting of promoter is initiated normally but does not proceed to the transcription initiation start point. The inhibition of promoter melting near the transcription start site is consistent with a proposal based on a structural model of the gp2-RNAP complex¹⁰. While promoter complex we describe here was obtained on a specially designed promoter, we surmise that similar complexes may be formed on other promoters. However, these complexes are too transient to be detected by standard biochemical assays.

It could have been expected that gp2 prevents RP_o formation simply by sterically blocking loading of downstream DNA into the enzyme trough formed by the β' jaw and several other RNAP domains^{9,10}. However, the fact that gp2 inhibits transcription from open complexes formed on a model template, which lacks DNA downstream from the $+6$ position (Fig. 6b), suggests that the mechanism of gp2 inhibition is more complex. In fact, the absence of promoter opening at and immediately upstream of transcription start point may be caused by gp2 binding *per se*, rather than by the absence of downstream DNA loading. Indeed, gp2 inhibits RNAP binding to various promoter fragments lacking distal part of the downstream promoter duplex segment, including oligonucleotides and upstream fork junctions probes which bear no DNA bases downstream from the transcription start site. Since these effects occur in the absence of β' jaw-DNA contacts, they, therefore, indicate that gp2 inhibits RNAP interaction with downstream part of the transcription bubble between positions $-6/+2$. Since the site of the binding of gp2, a small protein, is located away from RNAP sites interacting with single-stranded promoter DNA, we believe that this inhibition may be of allosteric nature. For example, the binding of gp2 to the β' jaw may interfere with movements of the RNAP β' clamp domain that may be required for establishment of interactions with single-stranded DNA segments^{17,18}. In the RNAP holoenzyme, σ^{70} subunit region 1.1 is positioned inside the RNAP active-site channel from where it is displaced by the downstream promoter segment upon RP_o formation^{19,20}. Recent results suggest that efficient inhibition of $E\sigma^{70}$ by gp2 requires the σ^{70} region 1.1⁶. Thus, the inhibition of RNAP interactions with single-stranded DNA by gp2 may also involve the σ^{70} region 1.1. Additional biophysical experiments will be required to test these ideas.

The high affinity for RNAP and the two-pronged mechanism of inhibition of open promoter complex formation pathway, first, by affecting promoter opening, and second, by interfering with the β' jaw contacts with downstream DNA, make gp2 a very efficient inhibitor. This is a biologically relevant property, since any leaky transcription from early phage promoters, particularly the A3 promoter⁸, lead to transcriptional interference between the slow-moving

host RNAP and fast-moving T7 RNAP, causing abnormal progeny phage genome processing and packaging.

Melting of the transcription start site is an obviously critical step during the formation of the catalytically-competent open promoter complex. Apparently, different factors and/or changes in the RNAP structure can affect promoter melting near the start site, without having an effect on the nucleation of promoter melting further upstream. The complex formed in the presence of gp2 appears to be similar to previously described complex formed by RNAP lacking the β lobe²¹. However, the mutant RNAP complexes establish proper downstream contacts in the presence of transcription substrates and are transcriptionally active. An antibiotic myxopyronin binds to a pocket inside the RNAP β clamp domain and blocks promoter melting propagation towards the active site downstream from the -3 position^{18,22}, again resulting in a complex that may be similar to the gp2-containing complex. Some promoters having GC-rich discriminator sequences are known to form unstable open complexes, a property that is relevant for their specific inhibition during aminoacid starvation^{2,23,24}. It is possible that the stringent control dependent destabilization of complexes formed on these promoters is due to the same mechanism that is used by gp2. Future work will have to establish how such partially opened promoter complexes are related to intermediate complexes on the pathway to RP_o formation^{25–27}.

Our work demonstrates that protein beacon assay is a powerful approach to uncover mechanistic details of transcription factor action. The high sensitivity and the ability to probe RNAP interactions with upstream, downstream, and central (corresponding to the transcription bubble) regions of a promoter allows the use of the beacon assay to study even such potent transcription initiation inhibitors as T7 gp2. In addition, because the beacon assay signal relies on unquenching of σ^{70} region 2.3 aromatic residues with promoter DNA, it allows one to study the local environment of the sigma 2 aromatic cluster in response to different inputs. Other transcription regulators and inhibitors affecting RNAP-promoter interactions, including numerous factors encoded by bacteriophages, can be informatively analyzed by this technique.

Materials and Methods

Proteins

RNA polymerase holoenzyme containing σ^{70} derivative labeled at position 211 with fluorescent label 5-TMR ([211Cys-TMR] σ^{70}), was prepared as in¹¹. T7 phage gp2 wild type and R58E mutant, *E. coli* core RNAP containing a deletion (1149–1190) in the β' subunit, and T4 phage AsiA protein were prepared as described previously^{5,10,16}.

DNA probes

DNA oligonucleotides and modified oligonucleotides containing the SP18 spacer were synthesized by Integrated DNA Technologies. Fork junction and double stranded DNA probes were prepared as in^{11,14}. A control non-promoter DNA fragment consisted of mutated $-65+35$ T5 N25 strands in which -10 and -35 promoter element sequences were changed to CGCGGC and CCACTT, respectively.

Fluorometric assays

Fluorescence measurements were performed using a Quanta-Master QM4 spectrofluorometer (PTI) in transcription buffer (40 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol, 1 mM DTT and 10 mM MgCl₂) containing 0.02% Tween 20 at 25°C. Final assay mixtures (800 μ l) contained 1 nM labeled RNAP holoenzyme and DNA probes at various concentrations. The TMR fluorescence intensities were recorded with an excitation

wavelength of 550 nm and an emission wavelength of 578 nm. Time-dependent fluorescence changes were monitored after manual-mixing of RNAP beacon (800 μ l) and a DNA probe (<20 μ l) in a cuvette; the mixing dead-time was 15 s.

To obtain equilibrium dissociation constants (K_d) of the RNAP beacon with oligonucleotides and fork junctions, the experimental dependence of the fluorescent signal amplitude on DNA probe concentration was fitted to a chemical equilibrium equation (i.e. titration assay) as described in¹¹, unless otherwise noted.

The complex of $E\sigma^{70}$ with gp2 ($E\sigma^{70}$ -gp2) was prepared by mixing 1 nM $E\sigma^{70}$ beacon with 200 nM gp2 followed by 10 min incubation at room temperature. Increasing gp2 concentration up to 600 nM did not influence binding of DNA probes to $E\sigma^{70}$ -gp2 under conditions of the measurements.

***In vitro* abortive initiation assay**

Abortive transcription reactions were performed in a final volume of 10 μ l and contained 200 nM of *E. coli* σ^{70} -holoenzyme (Epicentre), 500 nM of gp2 (where indicated), and 50 nM of various DNA templates prepared as described above in transcription buffer. Reactions were mixed as indicated, incubated for 10 minutes at 37 °C, followed by the addition of CpA RNA dinucleotide primer (200 μ M), cold UTP (20 μ M) and [α -³²P] UTP (3000 Ci/mmol). The reactions were incubated for a further 10 minutes at 37 °C and then terminated by the addition of an equal volume of urea-formamide loading buffer. The reaction products were resolved on a 20% (w/v) polyacrylamide denaturing gel and visualized using a PhosphorImager.

Footprints

For *in vitro* footprinting experiments, the double-stranded DNA promoter fragments were prepared as in¹¹ using radioactively 5' end-labeled with [³²P]- γ ATP oligonucleotides corresponding to either non-template or template strand. Next, the labeled promoter fragments were purified on Micro Bio-spin 6 columns (BioRad) and used for the assays.

DNase I footprinting—Promoter complexes were formed in a final volume of 10 μ l and contained 200 nM of *E. coli* σ^{70} -holoenzyme (Epicentre), 2 μ M of gp2 (where indicated), and 50 nM of a DNA template in transcription buffer. Reactions were mixed with the order of addition of particular components as indicated, incubated for 10 minutes at 37 °C, followed by the addition of 0.2 units of DNase I (New England Biolabs). The reactions were proceeded for 30 s at 37 °C and were terminated by the addition of 20 μ l of stop buffer (10 μ g of calf thymus DNA and water) followed by ethanol precipitation. Samples were dissolved in 8 μ l of urea-formamide loading buffer and resolved in 7% polyacrylamide denaturing gel.

KMnO₄ probing—Reactions were set up as described for DNase I footprinting. Promoter complexes were treated with 1 mM KMnO₄ at room temperature for 30 s. Reactions were terminated by the addition of 300 mM β -mercaptoethanol followed by ethanol precipitation and 20-min treatment with 10% piperidine at 95 °C. Reaction products were next treated with chloroform to remove piperidine, ethanol precipitated, dissolved in 8 μ l of urea-formamide loading buffer and resolved in 7% polyacrylamide denaturing gel.

Exo III footprinting—Reactions were set up as described above. Promoter complexes were treated with 0.5 units of Exo III (New England Biolabs) for 2 minutes at 37 °C. Reactions were terminated by the addition of 20 μ l of stop buffer (10 μ g of calf thymus

DNA and water), and precipitated with ethanol. Samples were dissolved in 8 μ l of urea-formamide loading buffer and resolved in 7% polyacrylamide denaturing gel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

RNAP	RNA polymerase
Eσ^{70}	RNAP holoenzyme containing primary σ^{70} subunit
RP_o	open promoter complex
K_d	equilibrium dissociation constant

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Figure 2. T7 gp2 inhibits RNAP interaction with the T5 N25 promoter

(a) Time dependence of the change in fluorescence upon mixing 1 nM [211Cys-TMR] σ^{70} RNAP holoenzyme with 2 nM -65/+35 T5N25 (black) or a non-promoter DNA fragment (red) in the presence or in the absence of gp2. The upper curve (labeled as “(RNAP +T5N25)+gp2”) was obtained in experiment where gp2 was added to the RNAP beacon preincubated with 2 nM T5N25 promoter fragment for 30 min. In other experiments, gp2 was added to preformed RNAP-T5N25 or RNAP-non-promoter DNA complexes.

(b) Inhibition of abortive transcript synthesis from T5N25 promoter fragment by gp2. Reactions contained $E\sigma^{70}$ alone (lane 1) or $E\sigma^{70}$ with gp2 added before (lane 2) or after (lane 3) open complex formation. An autoradiograph of denaturing polyacrylamide gel is presented.

Figure 3. $E\sigma^{70}$ forms complexes on the N25cons promoter in the presence of gp2

Reactions containing indicated proteins and N25cons promoter DNA fragment terminally labeled at the template ("t") or non-template ("nt") strands were footprinted with DNase I or probed with KMnO₄ as described in Materials and Methods. Gp2 was added prior to (lanes 3, 7, 12, 16) or after (lanes 4, 8, 13, 17) open complex formation. Reaction products were resolved by denaturing PAGE and revealed using Phosphorimager.

Figure 4. Binding of downstream fork junction probes to the $E\sigma^{70}$ -gp2 complex

(a) Titration of $E\sigma^{70}$ and $E\sigma^{70}\text{-gp2}$ with $[-12/+20][+4/+20]$ downstream fork junction. The K_d values were determined by fitting the dependence of relative fluorescence signal amplitude (F/F_0) on probe concentration to a chemical equilibrium equation¹¹. (b) The effect of fork junction point position on $E\sigma^{70}\text{-gp2}$ interaction with downstream fork junction probes. Time dependence of the increase in fluorescence upon mixing the (RNAP beacon-gp2) complex with 50 nM of $[-12/+20][+2/+20]$, $[-12/+20][+3/+20]$, $[-12/+20][+4/+20]$, $[-12/+20][+5/+20]$, and $[-12/+20; -7C][+4/+20]$ probes abbreviated as +2, +3, +4, +5, and +4;-7C, correspondingly. The upper curve corresponds to RNAP beacon binding to 50 nM $[-12/+20][+4/+20]$ probe in the absence of gp2.

Figure 5. Binding of downstream fork junction probes with template strand overhangs to the $E\sigma^{70}$ -gp2 complex and $\Delta\beta(1149-1190)E\sigma^{70}$

(a) Time dependence of the fluorescence intensity in samples containing 1 nM [211Cys-TMR] σ^{70} holoenzyme, 200 nM gp2 and 50 nM of indicated downstream fork junction probes.

(b) Time dependence of the fluorescence intensity upon the addition of 3 nM of indicated downstream fork junction probes to 1 nM [211Cys-TMR] σ^{70} holoenzyme bearing the $\Delta\beta$ (1149–1190) deletion.

(a)

(b)

(c)

Figure 6. Binding of non-template oligos containing the -10 promoter element consensus sequence to free $E\sigma^{70}$ and the $E\sigma^{70}$ -gp2 complex

Titration of [211Cys-TMR] σ^{70} holoenzyme and the gp2-[211Cys-TMR] σ^{70} holoenzyme complex with oligonucleotide probes. Solid lines correspond to nonlinear regression fit of the data.

Figure 7. The effect of T7 gp2 and T4 AsiA on $E\sigma^{70}$ binding to [-59/-12] probe
 2 nM of [-59/-12] probe was combined with RNAP beacon (1 nM) preincubated for 15 min with or without 0.2 μM T7 gp2 or T4 AsiA and increase in fluorescence was monitored.

Figure 8. The effect of gp2 on Eσ⁷⁰ interaction with double-stranded promoter fragments truncated at various positions downstream of the transcription start point

(a) Time dependence of fluorescence intensity in samples containing 1 nM [211Cys-TMR] σ⁷⁰ holoenzyme, 200 nM gp2 and 2 nM of either of [-50/+14], [-50/+12], [-50/+8], or [-50/+6] fragments. Upper curves correspond to experiments in which gp2 was added to the RNAP beacon preincubated with a DNA probe for 30 min; bottom curves show fluorescence intensity changes upon addition of the DNA probes to the RNAP beacon preincubated with gp2. The addition of gp2 or DNA probe is indicated by vertical arrows. (b) Inhibition of abortive transcript synthesis from the [-50/+6] probe by gp2. Reactions contained Eσ⁷⁰ alone (lane 1) or RNAP with gp2 added before (lane 2) or after (lane 3) open complex formation.

Table 1

Dissociation constants for the binding of promoter fragments to RNAP beacon, RNAP beacon complex with gp2 and $\Delta\beta'(1149-1190)$ RNAP beacon.

DNA probe	K_d , RNAP, μM	K_d , (RNAP- gp2), μM	K_d , (RNAP- $\beta'\Delta\text{jaw}$), μM
[−12/+20][+4/+20]	$<10^{-4}$	0.12	$3.4\text{--}10^{-3}$
NC(−4/+3)	$<10^{-4}$	>0.6	$1.0\text{--}10^{-3}$
−12/+2	0.16	4.4	0.46
−12/−3	1.8	17	
−12/−6	80	87	

The structures of DNA probes are shown in Figs. 3A, 4A and 5. The K_d values presented are averages from 2–3 individual experiments; the error is $\pm 15\%$.