A Cryptic Promoter in the O_R Region of Bacteriophage λ

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A cryptic promoter, designated P_{α} , initiates transcription within the O_R region of bacteriophage λ . Transcription from P_{α} proceeds in the direction of the cI repressor gene from sites 46 and 48 bp preceding the P_{RM} transcription start site. P_{α} is likely to compete with both P_R and P_{RM} for formation of open complexes, since it is only active when P_R is mutated and can be suppressed by mutations that increase P_{RM} activity. In addition, transcription initiation at P_{α} is blocked by λ repressor. Kinetic analysis of relative abundance of the products of in vitro transcription indicated that P_{α} was approximately 1/3 as strong as P_{RM} . However, a P_{α}^- mutation had little effect on $K_{\alpha}k_f$ (the association rate constant) for P_{RM} . These observations can be explained by the finding that open complexes formed at P_{α} are relatively unstable (half-life = 20 to 25 min). Dissociation of RNA polymerase from P_{α} allows additional open complexes to form at P_R or P_{RM} , and thus the apparent strength of P_{α} decreases with increasing preincubation times.

In order to understand factors that affect open complex formation by RNA polymerase (RNAP) at closely spaced, divergent promoters, we have focused on the interaction between RNAP molecules during open complex formation at the P_R and P_{RM} promoters of bacteriophage λ . On templates in which the spacing between the transcription start sites is reduced from 82 to 81 bp, RNAP bound at P_R strongly interferes with (represses) open complex formation at P_{RM} . Surprisingly, this repression does not result from steric exclusion. Instead, RNAP bound at P_R facilitates formation of closed complexes at P_{RM} but inhibits their isomerization to open complexes (34). On templates with the normal spacing, the effects of mutations in P_R on P_{RM} activity are qualitatively similar, but are less dramatic quantitatively than the effects observed with 1 bp deleted (7, 14).

In the course of these experiments, we discovered a cryptic promoter, designated P_{α} , which is located between P_R and P_{RM} . Its presence was revealed by in vitro transcription experiments in which we examined open complex formation at P_{RM} on several different P_R^- templates. In each case, we observed a new transcript slightly longer than that initiating at P_{RM} . Primer extension analysis of RNA synthesized in vitro demonstrated that this transcript originates from sites 46 and 48 bp upstream of P_{RM} . Mutations in the putative -10 region of P_{α} inactivate the promoter, and consistent with the location of these sequences, we find that open complex formation at P_{α} is prevented by λ repressor.

The appearance of P_{α} activity on a variety of P_R^- templates demonstrates that P_{α} is not created by these mutations but is instead uncovered: its activity is dramatically increased by reduction or elimination of competition with P_R for RNAP binding. Similarly, competition between P_{RM} and P_{α} is suggested by the fact that up mutations in P_{RM} prevent open complex formation at P_{α} even in a P_R^- genetic background. The mutual interactions among P_{α} , P_R , and P_{RM} complicate mutational and kinetic analyses of transcription initiation in this region (7). Further complexity arises because open complexes formed at P_{α} are unstable, dissociating almost 2 orders of magnitude more rapidly than do open complexes at P_{RM} or P_R .

MATERIALS AND METHODS

Transcription in vitro. Mutant templates used for in vitro transcription assays were constructed as described previously (33) by oligonucleotide-directed mutagenesis (35). All DNA templates used were 1,180-bp *Bam*HI-*Pvu*II fragments containing 338 bp of λ DNA corresponding to sequences from 37,770 to 38,107 (6) inserted between efficient rho-independent transcription terminators. These fragments were excised from derivatives of pSW101N (33). Transcription from P_R terminates at the *ant* gene terminator of phage P22 (2), located 296 bp from the P_R transcription start site. The 460-nucleotide P_{RM} -specific transcript terminates at the phage T7 early terminator (15). For in vitro transcription experiments, RNAP and promoter-containing DNA (20 and 2 nM during preincubation, respectively) were incubated in standard transcription buffer (100 mM KCl, 40 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 10 mM dithiothreitol, 100 μ g of bovine serum albumin per ml) at 37°C to allow open complexes to form. Following incubation for the times indicated, substrates were added in 1/3 volume to yield final concentrations of 0.2 mM (each) ATP, CTP, and GTP, 0.04 mM [α -³²P]UTP (specific activity, 2.5 Ci/mmol), and 50 μ g of heparin per ml. Single-round transcription was allowed to proceed for 15 min and was stopped by addition of a 1/5 volume of termination mix (7 M urea, 0.1 M EDTA, 0.4% sodium dodecyl sulfate [SDS], 40 mM Tris-HCl [pH 8.0], 0.5% bromophenol blue, 0.5% xylene cyanol). Reaction products were analyzed by autoradiography following separation on 5% (wt/vol) polyacrylamide-7 M urea gels. Relative transcript abundance was estimated by using a Bio-Rad (Richmond, Calif.) video densitometer to scan autoradiographs produced on preflashed Kodak XAR-5 film.

Identification of transcription start sites by primer extension. RNAP (20 nM) and DNA (2 nM) were preincubated for 15 min in 150 μ l of transcription buffer at 37°C. To monitor specific transcription initiation, a 15- μ l aliquot was transferred to 5 μ l of a substrate-plus-heparin solution to yield the final concentrations outlined in the previous section. To assay for the presence of ATP as the initiating nucleotide, additional 15- μ l aliquots were transferred to 5 μ l of the same mixture modified so that the final mixture would contain [γ -³²P]ATP (0.04 mM; specific activity, 6.25 Ci/mmol) and

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0.2 mM UTP. To the remaining preincubation mixture, nonradioactive nucleoside triphosphates (NTPs) and heparin were added to final concentrations of 0.2 mM each NTP and 50 μ g of heparin per ml. After 15 min, DNase I (Sigma Chemical Co., St. Louis, Mo.) was added in transcription buffer to a final concentration of 20 μ g/ml and incubation was continued for 10 min at 37°C. Exposure to DNase I had no effect on the RNA produced in vitro (data not shown). Reaction mixtures were extracted twice with equal volumes of phenol-CHCl₃ (1:1) and once with CHCl₃. Next, 0.5 pmol of a 29-base ³²P-end-labeled oligonucleotide primer (specific activity, ~1.5 μ Ci/pmol) corresponding to λ cI *I*-strand sequences between nucleotides 37,819 and 37,847 (6) was added to the extracted RNA in the final aqueous phase and allowed to hybridize for 30 min at 37°C. (For unknown reasons, higher hybridization temperatures resulted in rapid degradation of the RNA template [data not shown].) RNA-primer complexes were precipitated by addition of a 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol in siliconized 1.5-ml Eppendorf tubes. After 30 min at -20°C, the precipitate was collected by centrifugation at 4°C, rinsed with cold 70% ethanol, and allowed to dry. The pellets were resuspended in 15 μ l of reverse transcriptase buffer (0.5 mM [each] deoxy NTP, 40 mM KCl, 50 mM Tris-HCl [pH 8.0], 6 mM MgCl₂). Primer extension was initiated by addition of 1 μ l (15 U) of avian myeloblastosis virus reverse transcriptase (U.S. Biochemical, Cleveland, Ohio). After 30 min at 37°C, reactions were terminated with 4 μ l of transcription stop solution (see above). This protocol was based on the suggestions of Boorstein and Craig (3).

Aliquots of the primer extension reaction mixtures were loaded onto 5% polyacrylamide-7 M urea gels prerun for 30 to 60 min in TBE buffer (18) alongside wells containing samples of dideoxy chain termination sequencing reactions (26) in which the same (nonphosphorylated) 29-base primer that was used in the reverse transcriptase assays was annealed to an appropriate derivative of M13mp18 (33) and extended by the Sequenase enzyme (U.S. Biochemical) in the presence of deoxyadenosine 5'-[α -³⁵S]triphosphate.

Assays of stability of open complexes. Heparin challenge experiments (5) were used to examine the relative stability of open complexes formed at P_{RM} , P_{α} , and P_R . RNAP (20 nM) and $P_{R\alpha 3}$ DNA fragments (2 nM) were combined in 105 μ l of transcription buffer. After 15 min at 37°C, 0.5 μ l of 10-mg/ml heparin was added to sequester RNAP not in open complexes. At various times thereafter, 15- μ l aliquots were removed and added to 5 μ l of NTP solution to yield the same concentrations as used in single-round transcription assays. Synthesis was allowed to proceed for 15 min and was then terminated with 4 μ l of stop solution.

Kinetic analysis. DNA templates (669-bp *HindIII-EcoRI* fragments from pSW101H derivatives) and reaction conditions used for abortive initiation assays were described by Woody et al. (33). Values of τ_{obs} , the average time required for formation of open complexes at P_{RM} , were determined by assaying abortive synthesis of UpApU in the presence of UpA and [α -³²P]UTP (11). Sigmaplot (Jandel Scientific, Corte Madera, Calif.) was used to perform a least-squares fit of the data to equation 1:

$$N = Vt - V\tau_{obs}[1 - \exp(-t/\tau_{obs})] \quad (1)$$

where N is the amount of product produced per mole of promoter, V is the final steady-state rate of abortive synthesis (mole product per mole promoter per minute), t is the reaction time, and τ_{obs} is the average time required for open

complex formation at a particular RNAP concentration (19). Values of τ_{obs} were plotted as a function of $1/[RNAP]$ to derive values of K_B (the equilibrium binding constant for closed complex formation) and k_f (the rate constant for the isomerization of closed to open complexes) on the basis of a least-squares fit to equation 2:

$$\tau_{obs} = 1/k_f + 1/k_f K_B [RNAP] \quad (2)$$

This analysis is based on the two-step model for open complex formation (19). In the case of competing promoters, the kinetic parameters determined in this way are only apparent (app) values. These are related to the corresponding intrinsic kinetic parameters for the individual promoters according to the following equation (17):

$$app K_{Bapp} k_f = K_{B1} k_{f1} + K_{B2} k_{f2} \quad (3)$$

(Numbers in the subscripts denote kinetic parameters specific for each promoter.)

Materials. RNAP holoenzyme was purified by the method of Burgess and Jendrisak (4) as modified by Lowe et al. (16). Enzyme preparations were 90 to 95% pure and 95% saturated with sigma subunit, as judged by SDS-polyacrylamide gel electrophoresis. The fraction of active enzyme, determined by titration of a DNA fragment containing only the λ late promoter P_R' (5), was 65 to 70%. Purified repressor was a gift from R. T. Sauer. Restriction enzymes (New England Biolabs, Beverly, Mass.), DNase I, and avian myeloblastosis virus reverse transcriptase were purchased from the indicated sources. UpA was purchased from Sigma, and radioactive substrates were obtained from Amersham/Searle (Chicago, Ill.).

RESULTS

Identification and localization of P_{α} . We have previously shown that on templates in which the distance between the P_{RM} and P_R transcription start sites is reduced from 82 to 81 bp by the mutation $P_{RM}\Delta 34$ (Fig. 1), RNAP in open complexes at P_R strongly inhibits open complex formation at P_{RM} (34). We were prompted by these results to examine the effects of mutations in P_R on the activity of P_{RM} on templates in which the normal spacing was maintained (7). Unexpectedly, each P_R template produced an additional transcript (designated P_{α} in Fig. 2) that is slightly longer than the 460-base P_{RM} transcript. Although (for reasons considered in the next section) the amount of P_{α} -specific transcript does not increase significantly as P_R promoter strength decreases (from left to right in Fig. 2), the appearance of the P_{α} band is clearly not a specific function of the nucleotide sequence of P_R . (The reduced activity of P_R caused by the $\alpha 3$ and 10G mutations is not apparent in this experiment because of the relatively long [15-min] preincubation period during which open complexes were allowed to form.)

These experiments also provide a fortuitous clue to the location of P_{α} . Because of a difference in the method of cloning, the t_{ant} -terminated transcripts from the P_{R10G} and P_{R7C} promoters are 13 bases longer than those initiating on the $P_{R\alpha 3}$, $P_{R\alpha 3}$, or $P_{R\alpha 3,7C}$ templates (34). Because the length of P_{α} transcript is the same on all templates, it must direct transcription in the same direction as P_{RM} (opposite to that of P_R). On the basis of the relation between known transcript length and migration distance, the initiation site for the P_{α} transcript was thus estimated to be 25 to 50 bp upstream from P_{RM} .

These conclusions were confirmed by mapping the tran-

FIG. 1. DNA sequence and genetic map of the P_{RM} - O_R - P_R region. The DNA sequence is numbered relative to the P_{RM} and P_R transcription start sites above the respective promoter sequences. Repressor binding sites within O_R are indicated by horizontal brackets above the sequence (see references 9 and 22). The consensus -10 and -35 sequences (5'-TATAAT-3' and 5'-TTGACA-3') are positioned between the strands, to indicate the corresponding P_{RM} and P_R sequences. The P_α transcription start site(s) is indicated by a bracket, and putative -10 and -35 sequences for P_α are underlined. Mutations used in this study are indicated below the sequence and are as follows: in P_{RM} , KM11 (8), $up-1$ (20), 116 (25), and X34 and $\Delta 34$ (33); in P_R , x3 (10), 10G, and 7C (7); in P_α , $\alpha 7C$, $\alpha 11T$, and $\alpha 35C$, which were constructed as described in Materials and Methods.

scription start sites of RNA synthesized in vitro. A ^{32}P -labeled oligonucleotide complementary to λ r -strand sequences in cI and oriented (5' to 3') toward P_{RM} was annealed to RNA produced in vitro and extended by avian myeloblastosis virus reverse transcriptase (see Materials and Methods). As expected, on a wild-type template (Fig. 3a and b, lane 1), only the P_{RM} -specific cI transcript is produced in vitro and only one major product of the reverse transcriptase reaction is detected. When the template is $P_R 7C/P_{RM}^+$ (Fig. 3, lanes 2), transcription of cI initiates from both P_{RM} and P_α . The reverse transcriptase-primer extension experiment reveals that the P_α transcript initiates at either of two A residues located 46 and 48 bp upstream of the P_{RM} start site. On a $P_R x3/P_{RM}$ template, for which the activities of both P_R and P_{RM} are reduced by mutation, cI transcription initiates only from P_α (Fig. 3, lanes 3). Assignment of the P_α transcription start site to either of the two A's at -46 and -48 of P_{RM} is consistent with the results of additional experiments in which [γ - ^{32}P]ATP was used to label transcripts synthesized in vitro. In addition to the P_{RM} and P_R transcripts, both of which initiate with ATP, the band

corresponding to the P_α transcript was also labeled in these experiments (data not shown).

Inspection of sequences upstream from the P_α start site(s) reveals two hexamers (underlined in Fig. 1) with reasonable similarity to the *Escherichia coli* consensus promoter sequences (12): the sequence 5'-TAAAT-3' 6 (or 8) bp upstream from the two P_α transcription start sites matches the -10 consensus sequence (5'-TATAAT-3') at five of six positions. This is separated by 19 bp from the sequence

FIG. 2. P_R^- mutations increase P_α activity in vitro. Transcription reactions were carried out as described in Materials and Methods, with the following P_{RM}^+ templates in lanes 1 to 5, respectively: P_R wild type, $P_R 10G$, $P_R x3$, $P_R 7C$, and $P_R x3, 7C$. DNA (2 nM) and RNAP (20 nM) were preincubated for 15 min prior to addition of heparin and ^{32}P -labeled NTPs. Transcripts initiating at P_R , P_{RM} , or P_α are indicated by arrows. The P_R transcripts on the 10G and 7C templates are 13 nucleotides longer than those initiated at the P_R^+ , $P_R x3$, or $P_R x3, 7C$ promoters because of differences in the method of cloning (33).

FIG. 3. Determination of the P_α transcription start site by reverse transcriptase-primer extension reactions. DNA (2 nM) and RNAP (20 nM) were combined in transcription buffer and preincubated for 30 min at 37°C to allow open complexes to form. (a) Aliquots removed from the preincubation reaction mixtures were added to substrates (including [α - ^{32}P]UTP) plus heparin to monitor specific transcription. (b) A ^{32}P -end-labeled oligonucleotide was annealed to the (unlabeled) RNA in the remaining reaction mixture and extended by avian myeloblastosis virus reverse transcriptase as described in Materials and Methods. The products were separated on a denaturing polyacrylamide gel next to the ^{35}S -labeled products of T-, G-, C-, and A-specific DNA sequencing reactions initiated with the same oligonucleotide. DNA templates used were as follows: wild type (lane 1), $P_{RM}^+/P_R 7C$ (lane 2), and P_{RM} KM11/ $P_R x3$ (lane 3). The start sites of cI -specific transcript (representing the sequence complementary to the DNA sequence indicated in panel b) are indicated by arrows, and their positions are numbered relative to the P_{RM} start site (32).

FIG. 4. P_α activity can be eliminated either by mutations in the proposed -10 sequence or by up mutations in P_{RM} . In vitro transcription reactions were performed as described in Materials and Methods. DNA (2 nM) and RNAP (20 nM) were preincubated for 30 min (lanes 1 to 4), 10 min (lanes 5 and 6), or 20 min (lane 7 to 10) prior to the addition of ^{32}P -labeled substrates and heparin. Lanes 1 to 6, effects of mutations in the proposed -10 and -35 sequences of P_α . DNA templates were $P_{RX3}/P_{RM116}/P_\alpha^+$ (lane 1), $P_{RX3}/P_{RM116}/P_\alpha^{7C}$ (lane 2), $P_{RX3}/P_{RM116}/P_\alpha^{11T}$ (lane 3), $P_{RX3}/P_{RM116}/P_\alpha^{35C}$ (lane 4), $P_{RX3,7C}/P_{RM}^+/P_\alpha^+$ (lane 5), and $P_{RX3,7C}/P_{RM}^+/P_\alpha^{7C}$ (lane 6). Lanes 7 to 10, effect of P_{RM} promoter strength on P_α activity. Open complex formation at P_α was assayed on P_{RX3} templates containing the following P_{RM} alleles: 116, wild type, X34, and X34, $up-1$ (lanes 7 to 10, respectively). The P_{RM} -specific transcript from the $P_{RM}X34,up-1$ template is 3 nucleotides shorter than the others because of a difference in cloning strategy (33).

5'-ATGCAA-3', which agrees at three of six positions with the consensus -35 sequence (5'-TTGACA-3').

The validity of the assignment of these sequences to the -10 and -35 regions was tested by assaying presumptive P_α^- templates for P_α activity in vitro. The mutations $\alpha 7C$ and $\alpha 11T$ (Fig. 1) alter highly conserved nucleotides in the putative -10 region of P_α . As expected, these changes abolish P_α activity (Fig. 4, lanes 2, 3, and 6). However, $\alpha 35C$, a substitution for the consensus G in the putative -35 region, does not cause the expected down-promoter phenotype. Therefore, it is not clear whether this sequence is used by RNAP to recognize P_α .

P_α is likely to compete with both P_R and P_{RM} . Regardless of the precise location of the -35 region, it is clear that sequences required for P_α activity are interspersed with the sequences that compose P_R . This overlap should result in competition between the two promoters and would account for the fact that initiation at P_α is evident only when P_R is weakened by mutation. If it is assumed that the P_R^- mutations do not directly affect P_α , the magnitude of the change in fractional occupancy of P_α should be an inverse function of the P_R on-rate ($K_B k_f$) (17). However, as mentioned previously, P_α activity does not increase as P_R activity on the 10G, x3, 7C, and x3,7C templates decreases (Fig. 2). We propose that this is because of the complex interplay among P_{RM} , P_α , and P_R . An increased rate of open complex formation at P_{RM}^+ is observed when P_R activity is reduced because of relief of the interference between P_{RM} and P_R (7, 14). In Fig. 2, the increase is 1.5- to 2-fold, measured densitometrically. However, if P_{RM} and P_α compete for RNAP, each may limit the extent to which the other promoter can be stimulated by progressively stronger mutations in P_R .

Assays of transcription on templates containing up mutations in P_{RM} support the idea that P_{RM} and P_α compete for

RNAP. In a P_{RX3} background, the up mutation $P_{RM}X34$ (Fig. 1) significantly reduces initiation at P_α (Fig. 4, lanes 8 and 9). Furthermore, complete inhibition of P_α is observed when P_{RM} activity is increased further by combining X34 with the $P_{RM}up-1$ mutation (11, 20) to create a perfect match to the consensus -35 sequence (Fig. 4, lane 10). Although the inverse relationship between the activities of P_α and P_{RM} is consistent with the hypothesis that the promoters compete for RNAP, the possibility that the results in Fig. 4 are due to interference with a step in open complex formation subsequent to binding of RNAP cannot be excluded (7, 34).

(Note that the mutation P_{RM116} causes a decrease in P_α activity [Fig. 4, compare lanes 7 and 8]. This is due primarily to the fact that 116 is an up mutation in P_R [6a]. In addition, the activity of P_{RX3} is inversely correlated with the activity of P_{RM} [Fig. 4, lanes 8 to 10]. This appears to be a manifestation of the fact that interference between P_{RM} and P_R [7] is reciprocal [6a]. However, we haven't rigorously ruled out the possibility that the P_{RM} mutations X34 and $up-1$ [Fig. 1] are also intrinsic down mutations in P_R .)

The ability of P_α to inhibit open complex formation at wild-type P_{RM} is not evident in the in vitro transcription experiments. On a $P_{RX3,7C}$ template, the $\alpha 7C$ mutation does not affect the extent of open complex formation at P_{RM}^+ (Fig. 4, lanes 5 and 6). However, P_α^- mutations may increase slightly the number of open complexes formed at the P_{RM116} promoter. On a P_{RX3}/P_α^+ template, few open complexes form at P_{RM116} , even after long (30-min) preincubation times (Fig. 4, lane 1), but on P_α^- templates, the intensity of the P_{RM116} -specific band increases slightly (Fig. 4, lanes 1 to 3). This may reflect relief of competition for RNAP. However, we are unable to explain the fact that the intensity of the P_{RM116} band is increased by the $\alpha 35C$ mutation, which does not decrease the activity of P_α (Fig. 4, lane 4).

Kinetic analysis of the effects of P_α on open complex formation at P_{RM} . To examine the effect of P_α on transcription initiation at P_{RM} more carefully, we used abortive initiation assays (19) to analyze the kinetics of open complex formation at P_{RM}^+ on P_α^+ and P_α^- templates. For these experiments, P_R activity was eliminated by mutation. Lag-time assays were used to determine τ_{obs} , the average time required for open complex formation, at several RNAP concentrations. These values were used to construct tau plots (7), and calculated values of the kinetic parameters are summarized in Table 1.

There are two key results of these experiments. First, the $\alpha 7C$ mutation did not cause a significant change in the occupancy of P_{RM} , which is reflected in the final steady-state rate of abortive synthesis by open complexes at P_{RM} (53 ± 5 and 50 ± 5 mol of product per mol of promoter per min on P_α^+ and $\alpha 7C$ templates, respectively). Second, inactivation of P_α caused a small decrease in τ_{obs} for P_{RM} at all RNAP concentrations tested (7). This results in tau plots for P_{RM} for which the slopes ($1/\text{app}K_B \text{app}k_f$) are the same in a P_α^+ and a P_α^- background but in which the intercepts ($1/\text{app}k_f$) are different (Table 1).

If open complexes at P_{RM} and P_α are mutually exclusive, the failure to observe a significant difference in the final steady-state rates of P_{RM} -directed abortive synthesis implies that P_α is much weaker than P_{RM} . This is consistent with the finding that the slope of the P_{RM} tau plot ($1/\text{app}K_B \text{app}k_f$) is the same in P_α^+ and P_α^- backgrounds, since for competing promoters, the apparent $K_B k_f$ is the sum of the values that would be obtained for each promoter assayed separately (equation 3, Materials and Methods) (17).

TABLE 1. Summary of kinetic parameters derived for P_{RM}^+ in P_{α}^+ and $P_{\alpha}7C$ backgrounds^a

P_{α} allele	τ (min)	k_f (10^{-2} s $^{-1}$)	K_B (10^6 M $^{-1}$)	$K_B k_f$ (10^4 s $^{-1}$)
Wild type	12.9 $\pm$ 0.8	0.13 $\pm$ 0.006	17.0 $\pm$ 1.7	2.2 $\pm$ 0.10
$\alpha 7C$	8.1 $\pm$ 2.7	0.21 $\pm$ 0.07	12.0 $\pm$ 5.8	2.4 $\pm$ 0.42

^a Values were calculated from tau plots (7) as outlined in Materials and Methods. DNA templates were present at 1 nM (P_{α}^+) or 0.2 nM ($P_{\alpha}7C$); each template was also P_{R^-} ($x3,7C$). The parameters τ and $K_B k_f$ were determined by specifying τ ($1/k_f$) and $K_B k_f$ as independent variables, while K_B and k_f were determined in separate calculations by partitioning the slope to yield direct determination of these values. Standard errors were determined from the residuals of a least-squares fit to equation 2. These data, which were published previously (7), are included with permission of the publisher.

An alternative estimate of $K_B k_f$ of P_{α} can be obtained from single-round transcription experiments. Quantification of the intensities of P_{α} and P_{RM} bands in several experiments indicates that P_{α} is approximately 1/3 as strong as P_{RM}^+ on a $P_{R^-}x3,7C$ template. Thus, the $\alpha 7C$ mutation would be expected to cause approximately a 33% increase in the steady-state rate of abortive synthesis at P_{RM} and a 25% decrease in $appK_B k_f$. Although the magnitude of these effects is comparable to experimental error (Table 1), data outlined in the next section provide an alternative explanation for the negligible effect of the $\alpha 7C$ mutation on calculated kinetic parameters for P_{RM} .

Open complexes formed at P_{α} are unstable. When open complex formation on a $P_{RM}^+/P_{\alpha}^+/P_{R^-}x3$ template is assayed as a function of preincubation time prior to addition of substrates, increasing numbers of open complexes are formed at P_{RM}^+ and $P_{R^-}x3$ throughout the time course of the experiment (Fig. 5a). However, P_{α} activity reaches a maximum at preincubation times of 15 to 30 min and declines thereafter. These results are due to the intrinsic instability of open complexes formed at P_{α} , which is demonstrated directly by heparin challenge experiments (Fig. 5b). Open complexes were allowed to form for 15 min, and then heparin was added to inactivate free RNAP but not RNAP present in open complexes (5). Aliquots were removed at various times thereafter and assayed for the number of open

complexes present. Only a very slight decay in transcriptional activity at P_{RM}^+ or $P_{R^-}x3$ was observed during 2 h in the presence of heparin, but P_{α} activity decreased rapidly, with a half-time of 20 to 25 min (Fig. 5b and c).

These results can explain both the unusual time course of open complex formation at P_{α} (Fig. 5a) and the inability to detect an effect of P_{α} on open complex formation at P_{RM} (Table 1). Apparently, RNAP initially forms open complexes at the P_{RM} , P_{α} , and $P_{R^-}x3$ promoters according to their respective on-rates ($K_B k_f$). At later times, open complexes at P_{α} dissociate, allowing further competition among the three promoters. Repetition of this cycle leads to the eventual depletion of template molecules that have RNAP bound in open complexes at P_{α} , resulting in a steady-state rate of P_{RM} -directed abortive synthesis that is the same as that observed in a P_{α}^- background.

DISCUSSION

Data presented here identify a weak λ promoter, P_{α} , located between P_{RM} and P_R . Open complexes formed at P_{α} initiate transcription at either of two A residues 46 and 48 bp upstream from P_{RM} . Open complexes form at P_{α} at about 1/3 the rate at which they form at P_{RM} when assayed by using single-round transcription assays and short (15-min) preincubation times. However, this is likely to be an underestimate of relative promoter strength, since open complexes formed at P_{α} are unstable (half-life = 20 to 25 min); since P_{α} must compete with P_{RM} and/or P_R , its instability reduces its occupancy by RNAP following longer preincubation times.

These properties of P_{α} explain why its presence had previously gone unnoticed. First, its transcript is only detectable in vitro when P_R activity is reduced by mutation. Assays of the effects of the $x3$ mutation on cI transcription in vivo (13) did not identify transcription start sites, and assays in vitro (10) were confined to an analysis of P_R -specific abortive initiation. In other experiments, mutations that inactivated P_R would also have inactivated P_{α} (20). A transcript that may initiate at P_{α} can be seen in the autoradiographs of Hersherberger and deHaseth (14), but the P_{α} band was so faint that it was ignored.

FIG. 5. Open complexes formed at P_{α} are unstable. (a) The extent of open complex formation on $P_{RM}^+/P_{\alpha}^+/P_{R^-}x3$ templates was assayed at the indicated times after combination of DNA (2 nM) and RNAP (20 nM). Aliquots removed from the bulk reaction mixture were combined with substrates and heparin as described in Materials and Methods. (b) For heparin challenge experiments, a reaction mixture containing the $P_{R^-}x3/P_{RM}^+$ template was combined with RNAP as in the experiment outlined in panel a. After 15 min at 37°C, heparin was added to a final concentration of 50 μ g/ml. Aliquots were removed at the indicated times thereafter and added to 32 P-labeled substrates (without additional heparin) to monitor activity of the remaining open complexes. (c) The fractional activities (F/F_0) of P_{α} ($\blacklozenge$), P_{RM} ($\blacksquare$), and $P_{R^-}x3$ ($\bullet$) are plotted as a function of time after addition of heparin. F_0 is the activity 0.5 min after addition of heparin.

The inverse correlation between P_α activity and the strength of P_{RM} or P_R is the principle evidence that open complexes at P_α preclude RNAP binding to either of the flanking promoters. Because of the interdigitation of P_α and P_R consensus sequences and the alterations in promoter structure that accompany open complex formation, it is highly unlikely that open complexes could form at these promoters on the same template molecule. That is, the -35 region of P_α is located near the P_R transcription start site, a region in which the DNA strands should be separated in P_R open complexes (27, 30). It is less clear whether open complexes at P_{RM} and P_α are mutually exclusive. DNase I protection assays have demonstrated that RNAP in open complexes at the *lacUV5*, T7A3, and P_{RM} promoters protects sequences extending from approximately $+20$ to -55 relative to the corresponding transcription start sites (28, 31, 33). If RNAP interacts with P_α as it does with these other promoters, open complexes formed at P_α should extend to approximately -20 with respect to P_{RM} and result in an overlapping footprint that includes the -35 region of P_{RM} and the -10 region of P_α , a total of approximately 25 to 30 bp. Simultaneous occupancy of P_{RM} and P_α therefore seems unlikely, but the possibility that it might occur cannot be excluded. Although the DNase I footprints of RNAP at the $P_{RM}434$ and P_R promoters overlap by 10 to 15 bp, open complex formation at the two promoters is mutually inhibitory but not mutually exclusive (33).

The possibility of simultaneous competition between P_α and P_R and between P_α and P_{RM} has interesting consequences. RNAP bound at P_R inhibits open complex formation at P_{RM} (7, 14); this interference is reciprocal (6a). However, even though interference with open complex formation at P_{RM} may be relieved by P_R^- mutations, this effect should be counteracted to some extent by the concomitant increase in P_α activity. P_α therefore acts to buffer the otherwise activating effects of P_R^- mutations on P_{RM} . Furthermore, if open complexes at P_{RM} and P_α are mutually exclusive, repression of P_α by RNAP bound at P_R frees P_{RM} from competition with P_α .

The complexity of these interactions is increased because open complexes formed at P_α are unstable. The half-life of P_α open complexes is approximately 2 orders of magnitude lower than that of open complexes at P_{RM} or P_R (Fig. 5b) (10, 11). Instability does not result from displacement of P_α open complexes by RNAP at P_R or P_{RM} , since dissociation rates were measured in the presence of heparin, which precluded further open complex formation. The instability explains the unusual time course of open complex formation at P_α (Fig. 5a) and accounts, in part, for the fact that mutations in P_α have very little effect on occupancy or the apparent value of $K_B k_f$ for P_{RM} .

The results of β -galactosidase assays of single-copy $P_{RM}::lacZ$ transcriptional fusions (33) indicate that P_α is a very weak promoter in vivo. On a $P_{RM}116::lacZ/p_{R\lambda}x3$ template, *lacZ* transcription should initiate primarily at P_α . However, the level of β -galactosidase was at most 15 to 20% of the amount produced by a $P_{RM}^+::lacZ/p_{R\lambda}x3$ fusion in the absence of repressor.

In spite of its low transcriptional activity, P_α may contribute disproportionately to repressor levels in vivo. Since transcription from P_{RM} initiates with the *cI* AUG start codon, the mRNA lacks a Shine-Dalgarno sequence (23, 32). A suitable ribosome-binding site (5'-GGUGAU-3') separated by 11 nucleotides from the *cI* AUG (23) may facilitate translation of mRNAs which initiate farther upstream, including the λP_{RE} (34) or P_α transcripts. Data presented by

Shean and Gottesman (29) indicate that *cI* may be translated up to six times as efficiently on these transcripts as on the P_{RM} -specific transcript.

However, even if the *cI* message transcribed from P_α is translated efficiently, the amount of repressor attributable to P_α should be much less than the amount produced either by P_{RE} early in infection or by P_{RM} in a lysogen (in the presence of repressor). Thus, *cI* expression directed by P_α can contribute significantly to repressor levels only at times immediately after infection before P_{RM} or P_{RE} are turned on or after induction of a prophage. Consequently, although P_α^- mutations might slightly decrease the frequency of lysogenization, such mutations would not produce a detectable phenotype in a plaque assay.

Regulation of the *E. coli gal* and *lac* promoters by cyclic AMP-catabolic activator protein also takes place in the context of upstream overlapping promoters (1, 17, 24). In all three cases (*galP*, *lacP*, and P_{RM}), a weak promoter overlaps the activatable promoter, the recognition site for the activator overlaps the upstream promoter, and the upstream promoter is repressed by the activator. Furthermore, at *lacP* and at P_{RM} , the upstream promoters have unusual properties. The *lacP2* promoter appears to bind RNAP but is unable to initiate transcription efficiently (17, 21), while P_α forms unstable open complexes. It may be that, in general, multiple weak promoters originally directed expression of essential sequences prior to the superposition of additional regulatory sites and selection of a single, primary transcription initiation site.

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