Activation of the *trpBA* Promoter of *Pseudomonas aeruginosa* by TrpI Protein In Vitro†

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We have developed an in vitro transcription system in which purified TrpI protein and indoleglycerol phosphate (InGP) activate transcription initiation at the *trpBA* promoter ($trpP_B$) and repress initiation at the *trpI* promoter ($trpP_I$) of *Pseudomonas aeruginosa*. The phenotypes resulting from mutations in the −10 region of both promoters indicate that the −10 region consensus sequence in *P. aeruginosa* is probably the same as that in *Escherichia coli*. Furthermore, in the absence of TrpI and InGP, the activities of the two promoters are inversely correlated: down mutations in $trpP_I$ lead to increased activity of $trpP_B$, and up mutations in $trpP_B$ cause a decrease in $trpP_I$ activity. These results are a consequence of the fact that the two promoters overlap, so that RNA polymerase cannot form open complexes with both promoters simultaneously. Thus, in theory, by preventing RNA polymerase from binding at $trpP_I$, TrpI protein could indirectly activate $trpP_B$. However, oligonucleotide-induced mutations that completely inactivate $trpP_I$ do not relieve the requirement for TrpI and InGP to activate $trpP_B$. Therefore, activation of $trpP_B$ is mediated by a direct effect of TrpI on transcription initiation at $trpP_B$. In addition, the oligonucleotide-induced mutations in $trpP_I$ alter site II, the weaker of two TrpI binding sites identified in DNase I and hydroxyl radical footprinting studies (M. Chang and I. P. Crawford, Nucleic Acids Res. 18:979–988, 1990). Since these mutations prevent full activation of $trpP_B$, we conclude that specific base pairs in site II are required for activation.

In *Escherichia coli* and most other eubacteria (6), *trpB* and *trpA*, which encode the subunits of tryptophan synthase, are directly repressed in the presence of tryptophan. However, in *Pseudomonas aeruginosa* the corresponding genes are positively regulated by the product of the *trpI* gene and indoleglycerol phosphate (InGP) (20). Thus, derepression of *trpE*, *trpG*, *trpD*, and *trpC* leads to increased synthesis of InGP in a reaction catalyzed by the *trpC* gene product, InGP synthase. As InGP accumulates, it facilitates activation of the *trpBA* promoter by TrpI protein (7, 20).

The *trpI* gene and the *trpBA* operon of *P. aeruginosa* are transcribed divergently from overlapping promoters (see Fig. 1), with a separation of only 51 bp between the two transcription start sites (5, 9). TrpI is a member of a family containing at least nine evolutionarily related, DNA-binding regulatory proteins from diverse bacterial species (5, 12). All of these proteins are encoded by genes that are transcribed divergently from the genes they regulate. Of the proteins in this family, all but one act primarily as activators; most require small molecules as coactivators. In addition, because of the location of their DNA target sites, many are autoregulatory.

Gel retardation and footprinting studies identified two adjacent TrpI binding sites (I and II), which were proposed to be required for activation of the *trpBA* promoter (3). Although a potential protein-binding sequence possessing twofold symmetry could be identified in site I, no such sequence is readily apparent in site II. Since these sites overlap the *trpI* promoter, it was suggested that TrpI simultaneously represses expression of the *trpI* gene and activates expression of the *trpBA* operon (3). In fact, because the two divergent promoters overlap, by repressing $trpP_I$, TrpI protein could activate $trpP_B$ indirectly merely by blocking access of RNA polymerase (RNAP) to $trpP_I$.

We have used an in vitro transcription system to investigate the roles of sites I and II in the regulation of expression of $trpP_B$ and $trpP_I$. In this system, we find that formation of open complexes of RNAP and $trpP_I$ (in the absence of TrpI protein) is inversely correlated with open-complex formation at $trpP_B$, indicating that the two overlapping promoters cannot be simultaneously occupied by RNAP. In the presence of TrpI (and InGP), $trpP_B$ is activated and $trpP_I$ is repressed. Deletion of site I abolishes both effects of TrpI, which is consistent with the fact that this deletion abolishes binding of TrpI both to site I and to site II (3). We also demonstrate that mutations in site II prevent full activation of $trpP_B$. This is the first indication that particular nucleotide sequences in site II are required for activation.

Finally, the mutations in site II coincidentally fall in the −10 region of $trpP_I$. In spite of the fact that the mutations completely inactivate $trpP_I$, TrpI is still required for activation of $trpP_B$; therefore, we conclude that TrpI must exert a direct effect on transcription initiation at $trpP_B$. Indirect effects of TrpI through repression of $trpP_I$ should not contribute significantly to activation of $trpP_B$ in vivo.

MATERIALS AND METHODS

Plasmids and bacteriophage. pZAZ167 (20) was the source of a 479-bp *Bgl*II fragment from *P. aeruginosa* PAC174 that contains both $trpP_I$ and $trpP_B$; this fragment was cloned into the *Bam*HI site of M13mp19 (31) to yield phage M2100, in which $trpP_I$ directs expression of an in-frame TrpI-LacZα fusion protein. M2110, M2101, and M2111 are mutagenized derivatives of M2100 that contain the mutations −10C and −11C, and the double mutation −10C/11C in $trpP_I$ (see Fig. 1).

Derivatives of pVDX18 (16) are described in the accompanying paper (9): in pHH1, pHH2, pHH3, and pHH4, the
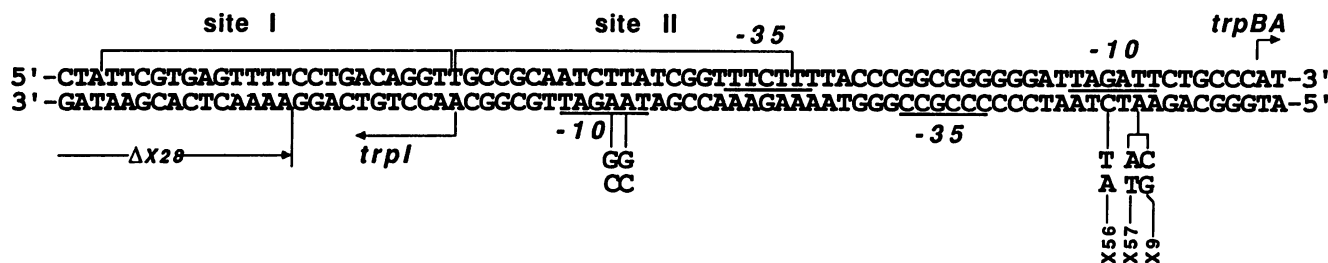

FIG. 1. Nucleotide sequence of the *trpI-trpBA* control region of *P. aeruginosa* PAO1. The sequence extending from +26 with respect to *trpP*$_I$ to +2 with respect to *trpP*$_B$ was determined by Chang et al. (5). The corresponding sequence in strain PAC174 differs at four positions: at −2, −3, −26, and −31 with respect to *trpP*$_I$, A · T, A · T, G · C, and T · A are substituted for C · G, C · G, A · T, and G · C, respectively. These changes did not detectably affect transcription from either promoter. Transcripts initiated at each promoter are indicated by arrows (3, 5); the start site for *trpP*$_B$ was determined by Han et al. (9). TrpI binding sites I and II, identified by DNase I and hydroxyl radical footprinting studies (3), are indicated by brackets. The −10 and −35 regions for each promoter are underlined. The site I deletion ΔX28 removes sequences extending leftward from the position indicated by the arrow (4). Point mutations used in this study are indicated below the wild-type sequence; X56, X57, and X9 are described by Han et al. (9).

trpP$_B$ region is from *P. aeruginosa* PAO1. The plasmids contain *trpP*$_B$$^+$ and the point mutations X9, X56, and X57, respectively (Fig. 1) (9). The site I deletion mutation ΔX28 is contained in pMIX28, which was constructed by M. Chang as a derivative of pMI603; pMI603 contains the *trpP*$_B$ region from *P. aeruginosa* PAO1 (3).

Transcription in vitro. Transcription conditions were the same as those described previously (8); the KCl concentration was 0.05 M. RNAP (active concentration, 25 nM) from *P. aeruginosa* was incubated with the appropriate DNA fragment (2.5 nM) for 10 min prior to the addition of substrate nucleoside triphosphates (NTPs) and heparin (50 μg/ml); the final concentrations of RNAP and DNA were 20 and 2 nM, respectively. Where indicated, purified TrpI protein (3), InGP, and the DNA template (3.33 nM) were incubated for 10 min before the addition of RNAP. In each experiment, the indicated final concentrations of TrpI and InGP are 0.6 times the concentrations during the initial 10-min incubation period. After electrophoresis, urea-acrylamide gels were analyzed autoradiographically. Bands on autoradiograms were quantified on a Bio-Rad video densitometer.

Oligonucleotide-directed mutagenesis. A 21-nucleotide ambiguous deoxynucleotide primer was used to generate mutations in TrpI binding site II at positions corresponding to −10 and −11 of *trpP*$_I$ by the protocol outlined in the Mutagene kit (Bio-Rad, Richmond, Calif.). Putative mutants were detected as white-plaque formers on strain MV1190, and their DNA sequences were determined by using the procedure of Sanger et al. (25) as modified for the Sequenase enzyme system (U.S. Biochemical, Inc., Cleveland, Ohio).

Enzymes. Restriction enzymes were purchased from New England BioLabs (Beverly, Mass.) or Promega Corp. (Madison, Wis.); T4 DNA ligase was purchased from Bethesda Research Laboratories (Gaithersburg, Md.). *P. aeruginosa* RNAP was purified by the procedure of Burgess and Jendrisak (2) from *P. aeruginosa* PAO1 (8). Its activity was determined by titration against a DNA fragment containing only one promoter, the *p*$_R$ promoter of bacteriophage λ. Purified TrpI protein was provided by M. Chang (4).

Materials. [α-^{32}P]UTP was obtained from Amersham/Searle (Chicago, Ill.). We obtained crystallized InGP from M. Chang and I. P. Crawford; it was originally prepared by K. Kirschner. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used as a chromogenic indicator for *lacZ* expression.

RESULTS

Activation and repression by TrpI protein. The effects of TrpI on transcription initiation were studied using runoff transcription assays (8). In the absence of TrpI, a 479-bp *Bgl*II DNA fragment containing the entire *trpI-trpBA* control region (Fig. 1) and *P. aeruginosa* RNAP (final concentration, 20 nM) were incubated for 10 min prior to the addition of substrates. Under these conditions, even fairly weak promoters should have time to form open complexes with RNAP (21). After the 10-min incubation period, heparin (50 μg/ml) was added along with NTPs to preclude initiation from closed complexes and to limit each open complex to one round of transcription. In the experiment in Fig. 2 (lane 1), there was almost no detectable transcription from *trpP*$_B$, but transcription from *trpP*$_I$ was appreciable. When the template DNA was incubated with InGP and increasing concentrations of TrpI for 10 min prior to the addition of RNAP, the activity of *trpP*$_B$ progressively increased and the activity of *trpP*$_I$ progressively decreased (Fig. 2). Repression of *trpP*$_I$ by TrpI is expected because TrpI binding sites I and II overlap *trpP*$_I$ (Fig. 1) (3).

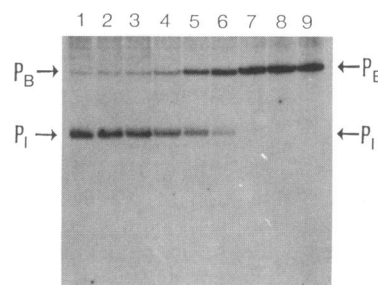The amount of product in each band in Fig. 2 was determined by using a video densitometer. Data from this

FIG. 2. Effect of TrpI on formation of open complexes at *trpP*$_I$ and *trpP*$_B$. Runoff transcription assays (see Materials and Methods) were performed in the presence of 4 × 10^{-5} M InGP and final TrpI concentrations of 0, 0.6, 1.3, 2.5, 5.0, 7.5, 10, 12.5, and 15 μg/ml in lanes 1 to 9, respectively. TrpI and InGP were incubated with template DNA for 10 min, RNAP was added, and incubation was continued for an additional 10 min prior to the addition of heparin and NTPs. Transcripts 256 and 169 nucleotides in length originate from *trpP*$_B$ and *trpP*$_I$, respectively. The template was a 479-bp *Bgl*II fragment from pZAZ167 (5).
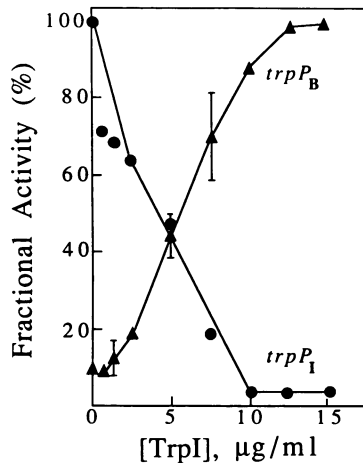
FIG. 3. Activity of *trpP*$_B$ and *trpP*$_I$ as a function of TrpI concentration. Data from Fig. 2 and from a similar experiment (data not shown) were quantified by using a Bio-Rad video densitometer. Data points are averages of the percent activity in two experiments; error bars indicate ranges that exceed the size of the symbols. The lines connecting the points are arbitrary.

FIG. 4. Effects of mutations in *trpP*$_B$ on open-complex formation. Runoff transcription assays were performed in the presence (+) or absence (−) of TrpI InGP (final concentrations, 15 μg/ml and 4 × 10^{-5} M, respectively). Template DNAs were 215-bp *Hind*III-*Sma*I fragments isolated from pHH1, pHH2, pHH3, and pHH4, which contain *trpP*$_B$$^+$ or the *trpP*$_B$ mutations X57, X56, and X9 (9). Arrows indicate transcripts 71 and 93 nucleotides in length originating from *trpP*$_B$ and *trpP*$_I$, respectively. Relative mobilities of transcripts originating from the two promoters are reversed in comparison with mobilities of transcripts in Fig. 3, 5, and 6. wt, wild type.

and a similar experiment are averaged in Fig. 3. If repression of *trpP*$_I$ and activation of *trpP*$_B$ were mediated by TrpI binding to identical sites, the two titration curves should intersect at 50% activity for both promoters. The difference between the observed intersection point (44%) and the predicted value of 50% is within experimental error. Most importantly, these data indicate that if TrpI binding to site I were sufficient for repression of *trpP*$_I$ and if binding of a second TrpI tetramer to site II were required for activation of *trpP*$_B$ (3, 4), then binding to the two sites must be highly cooperative. In subsequent experiments, we used a TrpI concentration of 15 μg/ml to ensure maximal activation of *trpP*$_B$.

Mutations that increase activity of the *trpBA* promoter. In the accompanying paper (9), three mutations that lead to increased expression of the *trpBA* operon in the absence of InGP are described. All three mutations are single base pair substitutions in the −10 region of the *trpP*$_B$ promoter (Fig. 1), and all three increase transcription initiated at *trpP*$_B$ in vivo both in the presence and absence of InGP. Figure 4 illustrates the results of runoff transcription experiments using wild-type and mutant *trpP*$_I$-*trpP*$_B$-containing DNAs as templates in the presence and absence of TrpI and InGP. As expected, in the absence of TrpI, all three −10 region mutations increased the activity of *trpP*$_B$; in addition, in each case, the activity of *trpP*$_I$ was diminished (Fig. 4, "−" lanes).

The most dramatic effect (on both promoters) was obtained with mutation X57, which is a change from T to A at nucleotide −8 in *trpP*$_B$. On the assumption that consensus sequences for RNAPs from *E. coli* and *P. aeruginosa* are the same, this mutation defines the nucleotide sequence of the −10 region of *trpP*$_B$ as TAGATT (Fig. 1); the corresponding sequence of X57 is TAGAAT, a closer match than wild-type *trpP*$_B$ to the presumed consensus sequence, TATAAT. The effects of X9, X56, and X57 correlate with their phenotypes in vivo (9) and with the relative effects in vivo of mutations in the −10 region of the phage P22 *ant* promoter (22, 32) (Table 1). On the basis of the frequencies of the mutant and wild-type nucleotides at −8 in *E. coli* promoters (Table 1),
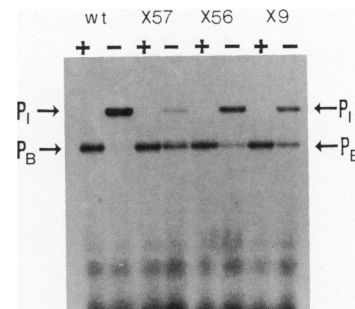
we would not have expected mutation X9 to have a significant effect on *trpP*$_B$. However, in a P22 *P*$_{ant}$ derivative, the same nucleotide change is an up mutation (22) (Table 1).

Because the two promoters overlap (Fig. 1), we were not surprised to find that mutations that increase the activity of *trpP*$_B$ decrease the activity of *trpP*$_I$. These results support the idea that RNAP should be unable to form open complexes at both promoters simultaneously.

Mutations in TrpI binding site I. A partial deletion of site I has been shown previously to prevent the formation of complexes between *trpI-trpBA* DNA and TrpI protein at

TABLE 1. Summary of effects of *trp* promoter mutations

Gene and mutation	Activity in vitro[a]		Nucleotide frequency[b] (%)		Relative activity in vivo	
	trpP$_B$	*trpP*$_I$	Wild type	Mutant	Reference 22[c]	Reference 9[d]
trpP$_B$						
None (wild type)	−	+++			1.0	1.0
X57 (−8 T → A)[e]	++	+/−	19	49	4.4	4.3
X9 (−8 T → C)	+	++	19	21	3.2	2.5
X56 (−10 G → T)	+/−	++	12	52	3.4	2.0
trpP$_I$						
None (wild type)	−	+++			1.0	1.0
−11C (−11 A → C)	+	−	89	3	0.007	NT[f]
−10C (−10 A → C)	+/−	++	26	10	1.2	NT

[a] Based on assays illustrated in Fig. 4 and 6 in absence of TrpI. Relative promoter strength estimated visually: +++ (highest activity) > ++ > + > +/− > − (no detectable activity).

[b] Frequency of occurrence of indicated base in *E. coli* promoters (data from reference 10).

[c] Effect of corresponding mutation in the P22 *ant* promoter fused to *lacZ*; the values presented for mutations at −8 were obtained with a variant of the P22 *ant* promoter in which the −35 region deviated by 1 bp from consensus (22).

[d] Data for *trpP*$_B$ fused to *xylE* from Table 4 of Han et al. (9). Assays were performed in *E. coli* in the absence of InGP (without anthranilate); however, TrpI was present and may have had partial activity even in the absence of InGP.

[e] A change from T to A at nucleotide −8.
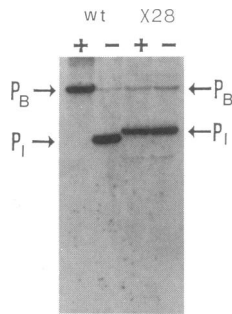
[f] NT, not tested.

FIG. 5. Effect of a site I deletion on activation of $trpP_I$ and repression of $trpP_B$. Runoff transcription assays were performed with ΔX28 DNA in the presence (+) or absence (−) of TrpI protein (15 μg/ml) and InGP (4 × 10⁻⁵ M). The wild-type (wt) template was a 471-bp XhoI-BglII fragment from pMI603 (3, 4), which yields transcripts 161 ($trpP_I$) and 256 ($trpP_B$) nucleotides long; the mutant template was a PvuI-BglII fragment from pMIX28 (3, 4), which yields transcripts 167 ($trpP_I$) and 256 ($trpP_B$) nucleotides long.

both site I and site II (3). When DNA containing this deletion (ΔX28) was used as the template (Fig. 5), there was no response of either promoter to the addition of TrpI (X28 lanes). Furthermore, comparison of the levels of transcription of ΔX28 DNA with the corresponding levels obtained with wild-type DNA (Fig. 5, wt lanes) indicates that deleting site I has virtually the same effect as incubating wild-type DNA in the absence of TrpI protein. Thus, binding of TrpI to site I is required both for activation of $trpP_B$ and for repression of $trpP_I$.

Mutations in TrpI binding site II. In gel retardation studies, deletion of site II and replacement of the deleted sequences by vector DNA had no detectable effect on TrpI binding. In particular, there was no effect on formation of a more slowly migrating complex thought to have TrpI tetramers bound to both site I and site II (3, 4). This raised the possibility that the nucleotide sequence of site II was not important and that protection of site II against attack by DNase I was a consequence of a strong cooperative interaction between tetramers bound at the two adjacent sites (Fig. 1). The effect of the deletion on activation of $trpP_B$ could not be studied because the deletion removed the $trpP_B$ promoter.

To investigate the role of site II in activation, we therefore used oligonucleotide mutagenesis (33) to mutate two adjacent base pairs in site II; in the presence of InGP, TrpI protein strongly protected both base pairs against attack by hydroxyl radicals (3). Both mutations are also in the −10 region of the $trpP_I$ promoter (Fig. 1). At either or both sites (−10 and −11 with respect to the $trpP_I$ transcription start site) a G · C base pair was substituted for a T · A base pair. Assays of transcription from these mutant templates (Fig. 6) revealed the following.

(i) The double mutation (−10C/11C) severely reduced the activity of the $trpP_I$ promoter (Fig. 6, "−" lanes). In the absence of TrpI (and InGP), there was no detectable $trpP_I$ transcript whether RNAP was preincubated with the DNA template for 1 or 10 min prior to the addition of substrates and heparin. The defect in $trpP_I$ was primarily due to the mutation at −11, since this mutation alone also made transcription from $trpP_I$ undetectable in the absence of TrpI. However, the mutation at −10 also had an effect. With a 1-min preincubation, the −10C mutant promoter was only about 10 to 20% as active as wild-type $trpP_I$ (Fig. 6a). When the time allowed for open-complex formation was 10 min,
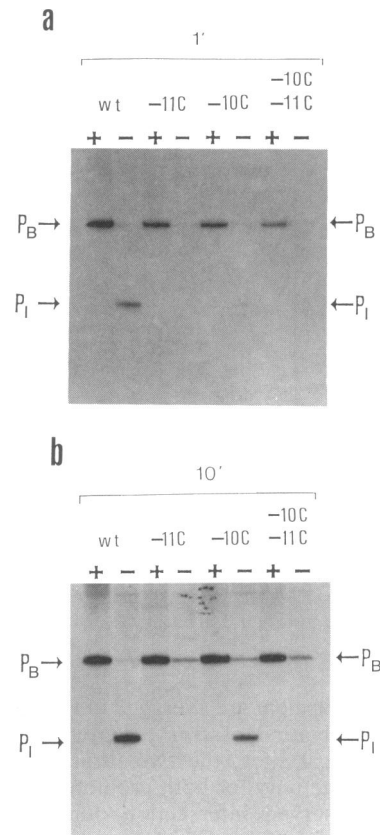

FIG. 6. Effect of mutations in site II on activation of $trpP_B$. Transcription assays were performed in the presence (+) or absence (−) of TrpI (15 μg/ml) and InGP (4 × 10⁻⁵ M). The 479-bp BglII fragment (from pZAZ167) containing $trpP_B$ and $trpP_I$ (5) was cloned into the BamHI site of M13mp19 prior to oligonucleotide-directed mutagenesis. In each case, the DNA template was a 528-bp EcoRI-HindIII fragment obtained by cleaving the M13 recombinant phage at sites in the polylinker. Nucleotide changes associated with the indicated mutations are shown in Fig. 1. Indicated transcripts are 190 ($trpP_I$) and 286 ($trpP_B$) nucleotides long. The experiments shown in panels a and b were performed at the same time, and autoradiograms were subjected to identical exposure times; RNAP was incubated with the DNA template for 1 min (a) or 10 min (b) prior to the addition of heparin and NTPs.

more open complexes formed at the mutant (−10C) promoter and its activity relative to wild-type was higher, 40 to 60% (Fig. 6b). The effects of the mutations on $trpP_I$ were exactly what would be predicted if the −10 consensus region of P. aeruginosa was identical to that of other well-studied eubacteria, including E. coli, Salmonella typhimurium, and Bacillus subtilis. For example, mutation −11C is a change in a base pair that is very highly conserved in E. coli (10), while mutation −10C affects a base pair which, in the wild-type promoter, is nonconsensus (A · T). In the latter case, a modest reduction in promoter activity might be expected because a C · G pair is less likely to occur at −10 than is an A · T pair (Table 1).

(ii) There was an increase in the activity of $trpP_B$ in the absence of TrpI protein when the mutant DNAs were used as templates. This effect was marginal, however, and could only be observed when the preincubation time was 10 min (Fig. 6b, "−" lanes). Under these conditions, the number of open complexes at $trpP_B$ increased about two- to threefold

relative to the wild-type level, regardless of the mutant template used. Because the bands in the autoradiogram are relatively faint, there is considerable variability in their intensities; this makes it impossible to distinguish among the three mutant templates. Nevertheless, the observed effects are consistent with the idea that, because of competition between the two promoters, a reduction in activity of $trpP_I$ should be correlated with an increase in the activity of $trpP_B$.

(iii) After either a 1- or 10-min incubation of RNAP with −11C or −10C/11C DNA, there was much less $trpP_B$ activity in the absence of TrpI than in its presence. Thus, even though there was no detectable activity of $trpP_I$, TrpI protein was still required for full activation of the *trpBA* promoter. This indicates that activation of $trpP_B$ cannot be effected merely by preventing open-complex formation at $trpP_I$. Rather, TrpI must exert a direct effect on open-complex formation at the *trpBA* promoter. The degree of stimulation by TrpI with a 10-min incubation period for open-complex formation was roughly sixfold for all three mutants; with a 1-min preincubation period, the degree of stimulation was greater than 10-fold (these factors are only rough estimates because the level of activity in the absence of TrpI was low in all cases).

(iv) The double mutation causes a defect in activation of $trpP_B$ by TrpI and InGP. The effect of the mutation can be observed by comparing the results of experiments performed with the mutant and wild-type templates in which RNAP and DNA were allowed to incubate for only 1 min prior to the addition of NTPs and heparin. In three different experiments similar to the one shown in Fig. 6a, in the presence of TrpI and InGP, the level of $trpP_B$ activity on the −10C/11C template was only one-fourth to one-third as great as that on the wild-type template. When DNA containing either the −11C or −10C substitution was used as the template, the level of activation of $trpP_B$ by TrpI was also reduced, but to a lesser extent than was observed for the −10C/11C double mutant. Apparently, both substitutions contribute to the activation defect of the double mutant. Thus, specific nucleotide sequences in site II are required for TrpI-mediated activation of $trpP_B$.

Note that the activation defect of these mutations could not be observed following a 10-min preincubation period (Fig. 6b, "+" lanes); the mutants were nearly as active as wild-type $trpP_B$ in the presence of TrpI and InGP. Apparently, there was sufficient time for open complexes to form on all templates to mitigate the effects of the mutations.

DISCUSSION

Our working model for regulation of *trpBA* gene expression in *P. aeruginosa* is based on in vivo genetic studies (20), DNA sequence determination (5), and footprinting studies (3), which revealed the spatial relationship between the *trpI* and *trpBA* promoters and the TrpI binding sites. Data presented here provide direct proof of several major features of this model. In particular we have demonstrated the following. (i) Purified TrpI protein activates $trpP_B$ and represses $trpP_I$ in vitro. (ii) RNAP is unable to form open complexes at $trpP_B$ and $trpP_I$ simultaneously. (iii) TrpI binding site I is essential both for activation of $trpP_B$ and for repression of $trpP_I$. (iv) Specific nucleotide sequences in site II are necessary for full activation of $trpP_B$. (v) Binding of TrpI to sites I and II is not sufficient for full activation of $trpP_B$; activation requires in addition a direct effect (presumably through an interaction with RNAP) on transcription initiation from $trpP_B$.

Mechanism of activation by TrpI. Very little is known about the mechanism by which transcriptional activators influence transcription initiation by RNAP. Genetic (14), biochemical (13, 15, 24, 27), and electron microscopic (28) evidence suggests that activation involves direct contact between the bound activator and RNAP. On the basis of footprinting data, site II and the −35 region of $trpP_B$ overlap by 4 bp (3); if the two proteins were bound to opposite faces of the DNA helix in the region of overlap, direct contact between the two proteins would certainly be possible. Our results with the site II double mutant (Fig. 6a) demonstrate that site II is required for full activation of $trpP_B$. Thus, it is likely that activation is in fact mediated by an interaction between TrpI and RNAP.

The activation defect of the −10C/11C mutant promoter was not readily apparent following a 10-min incubation of RNAP and template DNA (Fig. 6b). This result suggests that the mutations do not merely affect binding of TrpI to site II. By comparison with results obtained with wild-type DNA (Fig. 2 and 6b), a binding defect would be expected to produce two populations of templates: one population to which TrpI had bound and activated $trpP_B$ and a second population, free of TrpI, which should have yielded almost no detectable activity even after a 10-min incubation (Fig. 2, lane 1). Therefore, the relative activity of the double mutant would be approximately the same whether RNAP and DNA were incubated for 1 or 10 min. The fact that this was not the case suggested that the mutations do not affect binding of TrpI to site II but rather cause a defect in a step subsequent to binding. Preliminary DNase I protection experiments confirm this interpretation since the footprint of TrpI on −10C/11C DNA is substantially the same as the wild-type footprint (8a).

One possibility is that the conformation of TrpI bound to the mutant site II is altered in such a way that the efficiency of the interaction between TrpI and RNAP is no longer optimal. In this case, TrpI might occupy site II on all mutant DNA molecules but only partially activate $trpP_B$. That is, the rate of open-complex formation could be stimulated by TrpI, but not to the extent that it is stimulated on a wild-type template. Thus, the relative effects of the mutations could be dependent on the time of incubation of RNAP and DNA.

To test the effect of the mutations on TrpI binding to site II, we lowered the concentration of TrpI by a factor of 4. Under these conditions, we observed no difference in the efficiency of activation during a 10-min incubation (data not shown). Thus, even with the mutations in site II, the level of TrpI used in the experiment in Fig. 6 was saturating. This could be a further indication that the mutations do not affect TrpI binding. However, an alternative explanation is that the level of binding of TrpI protein to wild-type DNA is determined by competition with RNAP binding to $trpP_I$. Mutations that inactivate $trpP_I$ should increase the apparent affinity of TrpI for sites I and II in the presence of RNAP. This could compensate for a defect in TrpI binding to site II.

Role of InGP. In principle, InGP could act by facilitating binding of TrpI to site I or II, by facilitating a cooperative interaction between TrpI tetramers bound to the two sites or by facilitating an interaction between bound TrpI protein and RNAP. Comparison of site I sequences in the *trpI-trpBA* control regions of *P. aeruginosa* and *Pseudomonas putida* suggest that the TrpI recognition sequence in site I is 5'-TGT(G/C)AG-N5-CT(C/G)ACA, which in *P. aeruginosa* is located between +21 and +5 with respect to the *trpI* transcription start site (3, 5). There is no reasonable approximation to this sequence in site II, although there are

candidates for half-sites. In assays of TrpI binding to sites I and II in the absence of RNAP (3, 4), binding to site I was enhanced 20-fold by InGP and efficient binding to site II occurred only in the presence of InGP. In preliminary experiments, we find unexpectedly that, in the presence of RNAP, InGP has only a modest effect on TrpI binding to site I (8a). Thus, in vivo InGP may act primarily by facilitating the cooperativity of binding of TrpI to sites I and II. Such cooperativity is indicated in our experiments by the fact that repression of $trpP_I$ and activation of $trpP_B$ in the presence of InGP require roughly the same concentrations of TrpI (Fig. 3). Furthermore, deletion of site I abolishes activation of $trpP_B$ (Fig. 5) and prevents binding to site II even in the presence of InGP (3, 4).

Numerous transcriptional activators whose activity is regulated by a small effector molecule have been described. Among the best studied are AraC, MerR, and catabolite activator protein (CAP). For AraC and MerR, the effector molecules, arabinose and Hg^+, respectively, are not required for binding of the activators to DNA but are required to allow bound AraC (17, 18) or MerR (23, 29) to stimulate transcription initiation. For CAP, cyclic AMP is required for CAP to bind DNA, but the question of whether it is also required for activation once CAP is bound is still open (1).

There are several activation systems that appear to be related evolutionarily to the $trpI$-$trpBA$ system (5, 12). For E. coli $ilvY$-$ilvC$, it has been proposed that acetohydroxybutyrate (AHB) enables IlvY to activate the $ilvC$ operon by altering the conformation of the bound activator (30). As is the case with $trpI$-$trpBA$, in the ilv system, there are two adjacent binding sites for the activator, and the affinity for both sites is increased by AHB. However, in contrast with the TrpI system, site II can bind IlvY with reasonable affinity even in the absence of site I and AHB (30).

Indirect versus direct activation. It was possible that the ability of TrpI to activate $trpP_B$ was entirely due to the fact that by binding to site I it represses $trpP_I$, thereby relieving the competition between the two promoters for formation of open complexes. Such a mechanism has been shown to be partially responsible for activation of the lac and gal operon promoters by cyclic AMP-CAP (1, 19). However, this cannot be the primary mechanism of activation of $trpP_B$ because mutations that inactivate $trpP_I$ do not result in the high level of activity of $trpP_B$ attained in the presence of TrpI (Fig. 6).

There are two possible objections to this interpretation. First, it could be argued that the $-10C/11C$ double mutation does not inactivate $trpP_I$ completely. However, when we preincubated RNAP and template DNA for 60 min to allow open complexes to form prior to the addition of substrates, we were unable to detect activity from the $trpP_I$ double mutant (data not shown). Under the same conditions, open complexes formed at $trpP_B$ in the absence of TrpI. Thus, the mutant derivative of $trpP_I$ is essentially a nonfunctional promoter and, regardless of preincubation time, is significantly weaker than $trpP_B$. Second, we have not ruled out the possibility that the double mutant can form closed complexes at $trpP_I$ or that closed complexes might in turn prevent open complex formation at $trpP_B$. However, several wild-type promoters subject to activation have been assayed kinetically and shown to form highly unstable closed complexes (11, 19, 26). Therefore, it is unlikely that closed complexes at the doubly mutant $trpP_I$ could interfere with open-complex formation at $trpP_B$.

Promoter sequences. The activities of mutant derivatives of $trpP_I$ and $trpP_B$ in vitro agree well with the phenotypes one would predict for identical changes in E. coli promoters (Table 1). The one exception is the $trpP_B$ mutation X56, which is a weaker up mutation than one would predict based on nucleotide frequency in E. coli promoters but is clearly an up mutation both in vitro and in vivo. (Note that nucleotide frequencies are expected to correlate well with the rate of open-complex formation in vitro, but not necessarily with promoter activity in vivo [21, 22].) Altogether, these data suggest that the -10 consensus regions in E. coli and P. aeruginosa are the same even though the absolute levels of promoter activity may differ (8, 9).

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