The EMBO Journal vol.10 no.13 pp.4137-4144, 1991

Mutations in TrpI binding site II that differentially affect activation of the *trpBA* promoter of *Pseudomonas aeruginosa*

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Communicated by H.Eisen

In vitro, Pseudomonas aeruginosa **TrpI protein activates transcription initiation at the *trpBA* promoter ($trpP_B$) and represses initiation at its own promoter ($trpP_I$), which diverges from, and overlaps, $trpP_B$. Indoleglycerol phosphate (InGP) reduces the TrpI concentration required for binding to its strong binding site (site I), as measured by repression of $trpP_I$; it also facilitates activation of $trpP_B$, presumably because it enables TrpI to bind to a weaker binding site (site II) and thereby interact with RNA polymerase. The role of site II and InGP in regulation of the two promoters was investigated by constructing site II mutants. A 2 bp substitution affected the ability of TrpI to activate $trpP_B$, but did not significantly affect TrpI binding to site II. A more extensive (8 bp) substitution inhibited TrpI-mediated activation of $trpP_B$ and TrpI-mediated protection of site II in a DNase I footprinting assay. However, the mutation did not alter the pattern of TrpI binding observed in gel retardation experiments. In particular, a more slowly-migrating complex (Complex 2) whose appearance was correlated with TrpI binding to site II was formed equally well on a wild-type or substituted DNA fragment. Based on the mutant phenotypes, we propose that a particular sequence of protein-protein and protein-DNA interactions is required for activation of $trpP_B$ by TrpI and InGP.**

Introduction

In most eubacteria (Crawford, 1989), genes encoding the subunits of tryptophan synthase are directly repressed in the presence of tryptophan. However, in *Pseudomonas aeruginosa,* the corresponding genes are not subject to repression. Rather, they are positively regulated by the product of the *trpI* gene and indoleglycerol phosphate (InGP) (Manch and Crawford, 1982). In the absence of TrpR-mediated repression, expression of *trpE, trpF, trpG, trpD* and *trpC* leads to increased synthesis of InGP, a substrate for tryptophan synthase. As InGP accumulates, it facilitates activation of transcription of the tryptophan synthase genes, *trpB* and *trpA*, by TrpI protein (Crawford and Gunsalus, 1966; Manch and Crawford, 1982). The *trpI* gene and the *trpBA* operon of *P.aeruginosa* are transcribed divergently from two transcription start sites separated by only 51 bp (Chang *et al.*, 1989; Han *et al.*, 1991). TrpI is a member of the LysR family which includes at least nine evolutionary related, DNA-binding regulatory proteins from diverse bacterial species (Chang *et al.*, 1989; Henikoff *et al.*, 1988). All members of the family are encoded by genes that are transcribed divergently from the genes they regulate. All but one act primarily as activators and most require small molecules as co-activators. In addition, because of the location of their DNA recognition sites, many repress their own synthesis.

Gel retardation and footprinting studies (Chang and Crawford, 1990, 1991) identified two adjacent TrpI binding sites (I and II), which were proposed to be required for activation of the *trpBA* promoter (Figure 1). Since these sites overlap the *trpI* promoter, it was not surprising to find that TrpI simultaneously repressed *trpI* and activated the *trpBA* operon (Gao and Gussin, 1991b). We also demonstrated that TrpI binding to site I is necessary for activation of $trpP_B$ and repression of $trpP_I$ and that activation also depends on the presence of site II (Figure 1).

Numerous studies of activatable promoters have identified two (or more) binding sites for the activator (e.g. Johnson *et al.*, 1979; Wek and Hatfield, 1988). In most such systems, binding of the activator to the weaker site can be demonstrated in the absence of the stronger site. In contrast, TrpI binding to site II cannot be detected in the absence of site I (Chang and Crawford, 1990, 1991). Although InGP is also required for TrpI binding to site II, its precise mechanism of action has not been determined. It has been suggested that InGP is essential for a cooperative interaction between TrpI tetramers bound at the two sites (Chang and Crawford, 1990).

The experiments reported here demonstrate that InGP is required for maximal activation of $trpP_B$ even at saturating TrpI concentrations. Thus, InGP has an effect on activation subsequent to binding of the activator. In addition, the phenotypes of two site II mutants constructed *in vitro* suggest that activation of $trpP_B$ requires four steps: (i) a TrpI tetramer binds to site I; (ii) a second TrpI tetramer interacts with the first; (iii) nucleotide-specific interactions between the second tetramer and site II are established; and (iv) the second tetramer interacts with RNA polymerase (RNAP) to stimulate transcription of $trpP_B$. An 8 bp substitution in site II blocks step (iii), while a 2 bp substitution appears primarily to inhibit step (iv).

Results

Activation and repression by TrpI protein

The effects of TrpI on transcription initiation were studied using run-off transcription assays (Gao and Gussin, 1991b). In these assays, RNAP was incubated with a 479 bp *Bgl*II DNA fragment containing the entire *trpI/trpBA* control region (Figure 1) for 10 min prior to the addition of substrates and heparin (50 μg/ml). Thus, transcription levels reflect the number of open complexes formed during the

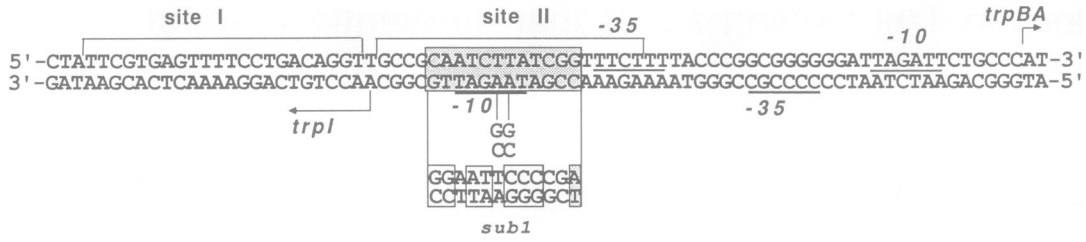

Fig. 1. Nucleotide sequence of the *trpI–trpBA* control region of *P.aeruginosa* PA01. The sequence extends from +26 with respect to $trpP_I$ to +2 with respect to $trpP_B$ as determined by Chang and Crawford (1990). The corresponding sequence in strain PAC174 differs at −2, −3, −26, and −31 with respect to $trpP_I$ (Chang *et al.*, 1989); these changes do not affect initiation at either promoter (Gao and Gussin, 1991b). Transcripts initiated at each promoter are indicated by arrows (Chang *et al.*, 1989; Han *et al.*, 1991). The −10 and −35 regions for each promoter are underlined. The shaded box indicates the region substituted by the *sub1* mutation. Within the *sub1* sequence, boxes enclose nucleotides that differ from wild-type sequence; the shaded box at −16 encloses the mutation −16T. The changes from T:A to G:C at −10 and −11 with respect to $trpP_I$ (denoted −10C and −11C in the text) are also indicated.

10 min preincubation period (see McClure, 1985). When these assays were performed in the absence of TrpI or InGP (Figure 2, lane 1), we observed almost no detectable transcription from $trpP_B$, but transcription from $trpP_I$ was appreciable. As we reported previously (Gao and Gussin, 1991b), when the template DNA was incubated with increasing concentrations of TrpI and InGP for 10 min prior to addition of RNAP, the activity of $trpP_B$ progressively increased and transcription from $trpP_I$ progressively decreased (Figure 2, lanes 10–18). The latter observation was expected because TrpI binding sites I and II overlap $trpP_I$ (Figure 1; Chang and Crawford, 1990).

When the same experiments were performed in the absence of InGP, a similar pattern was observed (Figure 2, lanes 1–9), but the omission of InGP had two effects. First, repression of $trpP_I$ required somewhat more TrpI protein in the absence of InGP than in its presence. Second, partial activation of $trpP_B$ could be observed even in the absence of InGP; however, the maximal level of activation was only ~30–35% the maximal level achieved in the presence of InGP.

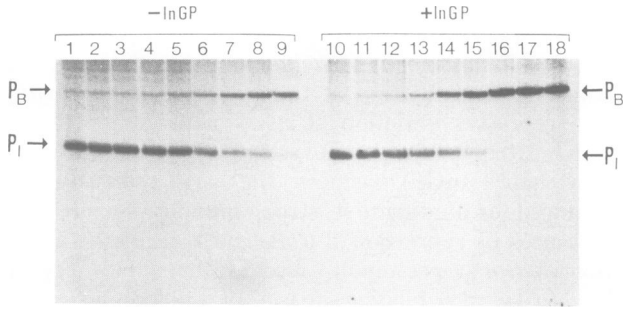

Fig. 2. Effect of InGP and TrpI on activity of $trpP_B$ and $trpP_I$ *in vitro*. Run-off transcription assays were performed in the presence or absence of 4×10^{-5} M InGP and final TrpI concentrations of 0, 0.6, 1.3, 2.5, 5.0, 7.5, 10,12.5 and 15 μg/ml in lanes 1–9 and 10–18, respectively. When present, 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 addition of heparin and NTPs. Transcripts 256 and 169 nucleotides long originate from $trpP_B$ and $trpP_I$, respectively. The template was a 479 bp *Bgl*II fragment from pZAZ167. The right half of the figure has been published previously (Gao and Gussin, 1991b).

Activation in the absence of InGP

Two mechanisms could account for the partial activation of $trpP_B$ by TrpI protein in the absence of InGP. (i) TrpI protein could activate $trpP_B$ indirectly, by repressing $trpP_I$, thereby eliminating competition for open complex formation resulting from the overlap of the two promoters. (ii) The changes in conformation of TrpI necessary for it to interact directly with RNAP might occur at some low frequency even in the absence of InGP. We have demonstrated previously that increased activity of $trpP_B$ due to repression of $trpP_I$ could not account for activation of $trpP_B$ by TrpI in the presence of InGP (Gao and Gussin, 1991b). However, we did not investigate the relative importance of TrpI-mediated repression of $trpP_I$ in the absence of InGP.

Therefore, we examined the double mutation −10C/11C (Figure 1), which severely inactivates $trpP_I$ more closely. In fact, the doubly mutant promoter is unable to form open complexes with RNAP even during a 1 h incubation prior to the addition of substrate NTPs (Gao and Gussin, 1991b). Therefore, competition between $trpP_I$ and $trpP_B$ should be eliminated; thus, if activation of $trpP_B$ were due solely to relief of repression of $trpP_I$, then on the mutant template, the unactivated level of transcription from $trpP_B$ should be quantitatively the same as the level that would be observed on the wild-type template if $trpP_I$ were repressed. The question is whether or not transcription from the wild-type template in the presence of TrpI (but no InGP) exceeds the level obtained on the mutant template in the absence of TrpI.

To address this question, run-off transcription assays were performed on the mutant and wild-type templates in the presence and absence of TrpI protein with or without InGP (Figure 3). Crucial to the experiment is the fact that the mutation completely inactivates $trpP_I$ (lanes 6–10). The data also confirm that on the wild-type template, full activation of $trpP_B$ requires both TrpI and InGP (compare lanes 2 and 4 with lanes 3 and 5). Because 10 min were allowed for open complex formation, the level of activation of $trpP_B$ on the mutant template in the presence of InGP (lanes 8 and 10) is comparable to that obtained on the wild-type template. Due to some inactivation of TrpI over time, 15 μg/ml of TrpI protein was not sufficient in the absence of InGP to repress $trpP_I$ completely; indeed, even at 18.8 μg/ml, we observed only 90–95% repression (lane 4). On the other hand, complete repression of $trpP_I$ was observed at both concentrations of TrpI in the presence of InGP (lanes 3 and 5), which is further indication that InGP stimulates binding of TrpI to site I.

The key point is that the activated level obtained on the wild-type template in the presence of TrpI (lane 2 or 4) is

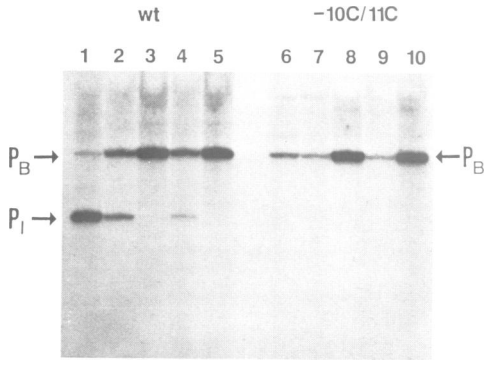

Fig. 3. Effect of a 2 bp substitution on activation of $trpP_B$. Run-off transcription assays were performed as described in Figure 2. The DNA templates were 528 bp *Eco*RI–*Hin*dIII fragments obtained after subcloning the 479 bp *Bgl*II fragment of pZAZ167 into M13mp19 for oligonucleotide mutagenesis (Gao and Gussin, 1991b). Indicated transcripts are 190 ($trpP_I$) and 286 ($trpP_B$) nucleotides long. Additions: lane 1, no TrpI or InGP; lane 2, TrpI (15 μg/ml); lane 3, TrpI (15 μg/ml) plus InGP; lane 4, TrpI (18.8 μg/ml); lane 5 TrpI (18.8 μg/ml) plus InGP. Additions in lanes 6–10 correspond to those in lanes 1–5 respectively. InGP concentration was 4×10^{-5} M.

greater than the level achieved for the mutant template in the absence of TrpI (lane 6). These data suggest that activation of $trpP_B$ in the absence of InGP has two components: an indirect effect leading to a level of transcription comparable to that observed for the mutant template in the absence of TrpI (lane 6) and a direct effect that reflects the ability of TrpI to interact with RNAP at $trpP_B$ even in the absence of InGP (the difference between lanes 4 and 6). In fact, these experiments may slightly underestimate this difference because of failure to achieve saturation with TrpI (see previous paragraph).

The ability of TrpI to activate $trpP_B$ directly in the absence of InGP was confirmed by abortive initiation assays of open complex formation at $trpP_B$ (McClure, 1980). The protocol for these assays was essentially the same as that used for the experiments illustrated in Figure 3 except that the substrates were CpA (corresponding to the nucleotides at −1 and +1 in $trpP_B$ RNA) and UTP (corresponding to the nucleotide at +2); the rate of synthesis of CpApU is proportional to the number of open complexes formed in the 10 min incubation of RNAP and template DNA prior to the addition of substrates. In these experiments, the TrpI concentration was increased to 20 μg/ml to achieve saturation in the absence of InGP.

The results of these assays are presented in Table I. For each template, the data are presented as a fraction of the fully activated level obtained for that template. In the absence of TrpI or InGP (line 1), the fractional $trpP_B$ activity on the mutant templates is higher than that on the wild-type template; the fractional activity is greatest for the double mutant, for which the $trpP_I$ promoter is weakest (Gao and Gussin, 1991b). The increase in $trpP_B$ activity from 8.2% (for wild-type) to 20.8% (for −10/11C) represents indirect activation of $trpP_B$ as a consequence of the inactivation of $trpP_I$ by mutation. A similar increase in $trpP_B$ activity would be expected for the wild-type template if TrpI were added to repress $trpP_I$. However, the fractional occupancy of the wild-type template actually increases to 32.3% of the maximum when only TrpI is present (line 2); thus we

Table I. Effects of InGP and mutations in site II on formation of open complexes at $trpP_B$

	Fractions of open complexes formed (% of maximal level)[a]			
Additions	Wild-type	−11C	−10C	−10C/11C
1. None	8.2 ± 0.9	19.4 ± 1.3	14.1 ± 0.8	20.8 ± 1.4
2. TrpI	32.3 ± 1.1	19.0 ± 1.1	15.2 ± 0.8	16.8 ± 0.7
3. TrpI and InGP	100[b]	100	100	100

[a]Mean per cent ± S.D. for three experiments.
[b]Values in the presence of TrpI (20 μg/ml) and InGP (4×10^{-5} M) were defined as 100% in each experiment for each template separately.
[c]Activity for each template expressed relative to the wild-type activity, which was defined as 100% in each experiment. Actual activity levels (mol CpApU/mol of DNA/min) for line 3 were: wild-type, 40.2 ± 6.6; −11C, 47.6 ± 10.8; −10C, 46.7 ± 10.2; −10C/11C, 46.4 ± 10.2.

conclude that TrpI is able to activate $trpP_B$ directly even in the absence of InGP.

Table I (last line) also indicates (as does Figure 3) that with a 10 min incubation of RNAP and DNA prior to addition of substrates and heparin, the mutant templates produce at least as much CpApU as the wild-type template. On the other hand, we previously reported that with only 1 min incubation of RNAP and DNA, the mutant templates were defective in their response to TrpI and InGP (Gao and Gussin, 1991b). In those experiments, the activity of the double mutant was only about one-quarter of the activity of wild-type. Therefore, we suggested that the mutations do not (only) alter the affinity of TrpI for site II, but must (also) affect the ability of the bound protein to interact with RNAP and thereby activate $trpP_B$.

To test this possibility, we probed the binding of TrpI protein to wild-type and doubly mutant DNA by assaying protection against digestion with DNase I (Figure 4). These footprinting assays indicate that there is very little, if any, difference in binding of TrpI protein to the two templates. In both cases, in the absence of InGP, site I is protected and the degree of protection is maximal at a TrpI concentration of 0.94 μg/ml (lanes 3 and 8). Similarly, in the presence of InGP (lanes 4,5,9 and 10), site II is partially protected in both cases and the degree of protection does not change when the TprI concentration is increased from 0.49 μg/ml (lanes 4 and 9) to 1.9 μg/ml (lanes 5 and 10). Some differences are observed (arrows in Figure 4), which may reflect conformational changes in TrpI–DNA complexes due to the sequence change in site II. Because protection of site II is incomplete, precise quantification of the degree of protection is not possible. However, inspection of the uppermost portion of the site II footprint and the band indicated by the lowest arrow in Figure 4 indicates that the degree of protection is approximately the same on both templates.

An 8 bp substitution in site II

Since the double mutation −10/11C does not prevent the binding of TrpI protein to site II and does not completely inhibit activation of $trpP_B$ in the presence of InGP, we constructed a mutant, *sub1*, in which a more extensive region of site II was altered (Figure 1). The mutant contains a 12 bp substitution, of which four base pairs are the same as in wild-type DNA. In order to construct the mutant, we first introduced a point mutation in site II to create a *Cla*I restric-

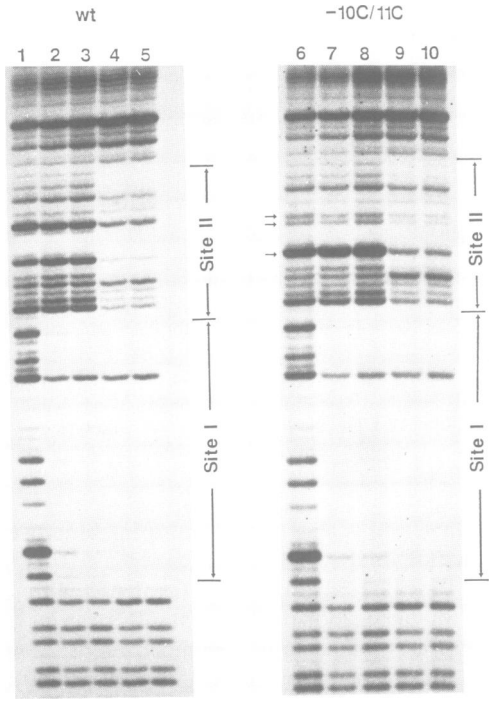

Fig. 4. TrpI-mediated protection of sites I and II on wild-type and −10C/11C DNA. DNase I footprinting assays were carried out as described in Materials and methods. The top strand in Figure 1 is the labeled strand. Arrows indicate some of the major differences between the wild-type and mutant footprints presumably resulting from changes in conformation of the mutated DNA. Additions: lane 1, no TrpI or InGP; lane 2, TrpI (0.94 μg/ml); lane 3, TrpI (1.9 μg/ml); lane 4, TrpI (0.94 μg/ml) plus 4×10^{-5} M InGP; lane 5, TrpI (1.9 μg/ml) plus 4×10^{-5} M InGP. Additions in lanes 6−10 correspond to those in lanes 1−5 respectively.

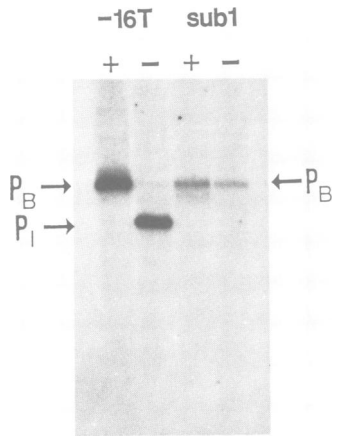

Fig. 5. Run-off transcription on −16T and *sub1* DNA. The DNA templates for the −16T and *sub1* templates were a 437 bp *Eco*RI−*Hin*dIII fragment and a 432 bp *Hin*dIII fragment, respectively, both of which were derived after subcloning as described in Materials and methods. Indicated transcripts are 180 ($trpP_I$) and 203 ($trpP_B$) nucleotides long. Additions: lanes 1 and 3, TrpI (15 μg/ml) plus 4×10^{-5} M InGP; lanes 2 and 4, no TrpI or InGp.

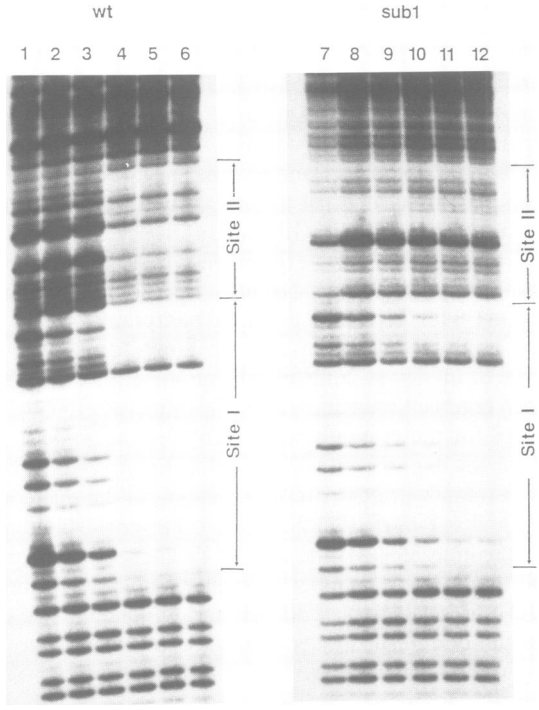

Fig. 6. Effect of the *sub1* mutation on TrpI binding to site II. DNase I footprinting assays were carried out as described in Materials and methods. The top strand in Figure 1 is the labeled strand. Additions: lane 1, no TrpI or InGP; lane 2, TrpI (0.47 μg/ml); lane 3, TrpI (0.94 μg/ml); lane 4, (0.47 μg/ml) plus 4×10^{-5} M InGP; lane 6, TrpI (1.9 μg/ml) plus 4×10^{-5} M InGP. Additions in lanes 7−12 correspond to those in lanes 1−6 respectively.

tion site. This point mutation, −16T (Figure 1), does not alter the activity of either promoter in the presence or absence of TrpI and InGP (see below).

Run-off transcription assays were performed with a 10 min incubation of RNAP and template DNA prior to addition of substrates (Figure 5). The −16T mutant yielded the expected wild-type pattern (lanes 1 and 2): TrpI protein activated $trpP_B$ and repressed $trpP_I$. When the template contained the *sub1* mutation, no $trpP_I$ activity was detectable whether TrpI was present or absent (lanes 3 and 4). As a consequence, the basal activity of $trpP_B$ was increased somewhat (compare lanes 2 and 4), presumably because of the absence of competition from $trpP_I$. Most importantly, the ability of the promoter to respond to TrpI and InGP is nearly eliminated by the *sub1* mutation (lane 3).

We next examined the effect of *sub1* on binding of TrpI to sites I and II. In contrast to the results with the double mutant −10C/11C (Figure 4), footprinting assays reveal that the substitution prevents the binding of TrpI to site II, even at relatively high concentrations and in the presence of InGP (Figure 6). Note that for wild-type DNA (lanes 1−6), site I is protected to some extent even in the absence of InGP (lanes 1−3). However, site II protection is observed only in the presence of InGP (lanes 3−6). (As in Figure 4, even in the presence of InGP, site II is not protected completely and the degree of protection does not increase with increasing TrpI concentrations.)

When binding to *sub1* DNA was assayed (Figure 6, lanes 7−12), the pattern of protection of site I was virtually the same as the pattern observed with wild-type DNA. However, there was no protection of site II on the *sub1* template even at a TrpI concentration of 1.94 μg/ml (lane 12). Thus, the effect of *sub1* on binding of TrpI to site II is qualitatively different from that of the −10C/11C mutation. We conclude that sequences of site II are required both for the binding of TrpI and its subsequent activation of $trpP_B$ in the presence of InGP.

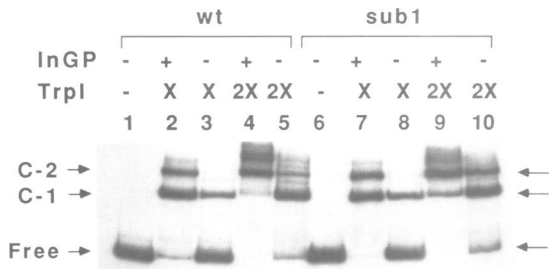

Fig. 7. Formation of Complexes 1 and 2 in gel retardation assays. DNA binding reactions were carried out as described in Materials and methods. Incubations were carried out in the presence (+) or absence (−) of 4×10^{-5} M InGP; TrpI concentrations were 0 (−), 0.75 μg/ml (×), or 1.5 μg/ml (2×), as indicated above the lane numbers. Arrows indicate the positions of bands corresponding to free DNA, Complex 1 (C-1), and Complex 2 (C-2).

We also used gel retardation assays to investigate binding of TrpI to sites I and II (Figure 7). In previous studies, two retarded complexes were observed (Chang and Crawford, 1990, 1991). Complex 1 was obtained primarily at low concentrations of TrpI, or after incubation in the absence of InGP. Complex 2 was detected only at very high concentrations of TrpI or in the presence of InGP. Formation of Complex I was correlated with DNase I protection of site I alone, while formation of Complex 2 was correlated with protection of both sites. Figure 7 (lanes 1−5) repeats these observations. When TrpI (0.75 μg/ml) and InGP are incubated with wild-type DNA (lane 2), substantial quantities of both Complex 1 and Complex 2 are detected, but there are still some free DNA. Doubling the TrpI concentration (lane 4) eliminates the free DNA band and significantly increases the proportion of DNA in Complex 2. When the corresponding experiments were performed without InGP (lanes 3 and 5) the fraction of bound DNA was decreased and very little, if any, of the bound DNA was in Complex 2. Thus, Complex 2 was detected in substantial quantities only in the presence of InGP (lanes 2 and 4). To our surprise, the pattern obtained for *sub1* DNA (lanes 6−10) was indistinguishable from that of the wild-type control. In fact, even under conditions in which no footprint of site II was observed (Figure 6, lanes 11 and 12), formation of Complex 2 was unaffected by the substitution (Figure 7, lanes 7 and 9).

To assure ourselves that, at least on wild-type DNA, Complex 2 contained TrpI molecules bound both to site I and site II, we performed a footprinting analysis of complexes separated on acrylamide gels. In these experiments, complexes were allowed to form *in vitro*, were subjected to treatment with DNase I, and then were separated on gels. The bands corresponding to Complexes 1 and 2 were eluted from the gel and deproteinized, and the eluted DNA was analyzed on a sequencing (footprinting) gel. When this was done with wild-type DNA, Complex 1 (obtained after incubation with 1.5 μg/ml of TrpI in the absence of InGP) produced a footprint only on site I (Figure 8, and lane 2). On the other hand, Complex 2 (obtained after incubation with 5 μg/ml of TrpI in the presence of InGP) produced a footprint at both sites (lane 3). With *sub1* DNA, the results with Complex 1 (lane 5) were similar to the results obtained with wild-type DNA. However, Complex 2, as expected, failed to yield a footprint on site II (lane 6).

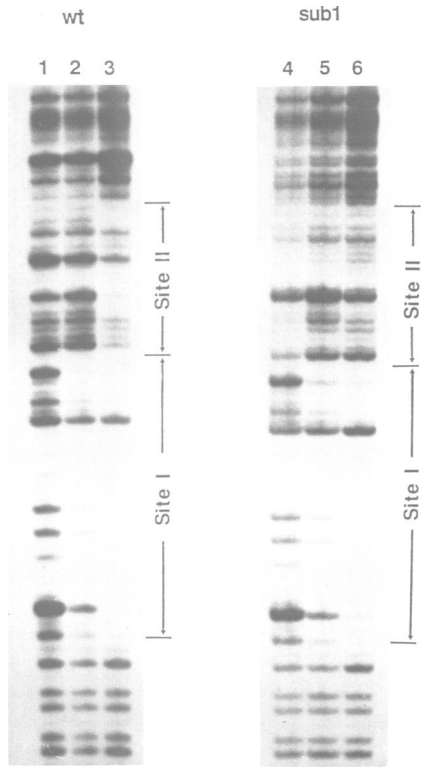

Fig. 8. Footprints of sites I and II on Complexes 1 and 2 formed on wild-type and *sub1* DNA. DNase I footprinting reactions were followed by gel retardation to separate Complexes 1 and 2, which were then analyzed on a sequencing gel as described in Materials and methods. The top strand in Figure 1 is the labeled strand. Lane 1, unprotected (free) DNA; lane 2, Complex 1 isolated after incubation with TrpI (1.5 μg/ml) alone; lane 3, Complex 3, isolated after incubation with TrpI (5 μg/ml) plus 8.3×10^{-4} M InGP. Lanes 4−6 correspond to lanes 1−3 respectively, except that *sub1* DNA was used instead of wild-type DNA. To improve recovery of complexes, 5−10 ng of DNA (100 000 c.p.m.) were used in each experiment.

If Complex 2 forms by the same mechanism on the wild-type and mutant DNAs, then formation of Complex 2 may be essentially independent of the nucleotide sequence of site II.

Discussion

Role of InGP in activation of $trpP_B$

Activation of $TrpP_I$ *in vivo* is dependent on InGP (Manch and Crawford, 1982; Han *et al.*, 1991); furthermore, footprinting and gel retardation studies (Chang and Crawford, 1990, 1991) indicate that the binding of TrpI to both site I and site II is stimulated by InGP. Our studies of transcription *in vitro* differ quantitatively from the earlier work. First, in the absence of InGP, the maximal level of activity of $trpP_B$ was unexpectedly high, ~30−35% of that obtained in the presence of InGP (Figures 2 and 3). This result is somewhat of a paradox. If TrpI binding to site II is required for activation and if binding to site II only occurs in the presence of InGP (or very high TrpI concentration), how can $trpP_B$ be activated by TrpI in the absence of InGP? A partial answer is that much of the activity of $trpP_B$ is due to the fact that, by binding to site I and repressing $trpP_I$, TrpI protein relieves the competition between $trpP_I$ and $trpP_B$. Furthermore, this effect was enhanced in our experiments because RNAP and DNA were incubated for

10 min prior to addition of substrates, thereby increasing the number of open complexes formed at $trpP_B$.

Nevertheless, there is a fraction of the activity of $trpP_B$ (Table I, line 3) that cannot be explained by the ability of TrpI to repress $trpP_I$. Thus, we propose that the conformational change induced in TrpI by InGP occurs, albeit infrequently, in the absence of the ligand. *In vitro*, this accounts for ~10% (32.3 − 20.8%) of the fully activated level of $trpP_B$ in the presence of InGP. However, the corresponding effect on the activity of $trpP_B$ *in vivo* may not be so great. For reasons just mentioned, by allowing 10 min for open complexes to form, we may have exaggerated the activity of $trpP_B$ in the absence of InGP; that is, we do not know whether the time required for open complex formation at $trpP_B$ is reduced to the same extent by TrpI in the presence as in the absence of InGP. The limited activation seen in the absence of InGP also requires that the nucleotide sequence of site II be wild-type (Table I, line 2). Binding to this sequence may either facilitate or stabilize the presumed conformational change in TrpI that permits the protein to interact with RNAP and activate $trpP_B$.

The amount of TrpI necessary for half-maximal activation of $TrpP_I$ transcription (Figure 2) is not affected so dramatically by InGP as binding of TrpI to site II in the absence of RNAP (Chang and Crawford, 1990, 1991). The unexpectedly small difference (Figure 2) is due in part to the fact that much of the activation in the absence of InGP is indirect, and therefore depends only on TrpI binding to site I.

Role of InGP in repression of $TrpP_I$

InGP reduces the amount of TrpI protein required for 50% repression of $trpP_B$ by less than a factor of two (Figure 2). In gel retardation assays, InGP reduced the amount of TrpI required for 50% occupancy of site I by a factor of three to four (see Figure 6c, Chang and Crawford, 1991). It is not clear whether this difference in the degree of dependence on InGP is significant. Note also that the presence of RNAP substantially increases the amount of TrpI needed for binding to site I (and consequent repression of $TrpP_I$) (compare Figures. 2,3,4 and 7). This is presumably due to competition between binding of RNAP to $TrpP_I$ and binding of TrpI to site I (see Hawley *et al.*, 1985), which appears to be relieved by mutations that inactivate $TrpP_I$ (Gao and Gussin, 1991b).

Effects of site II mutations

The site II mutations at −10 and −11 with respect to $TrpP_I$ have two phenotypes: (i) they prevent TrpI protein from stimulating $trpP_B$ in the absence of InGP (Table I) and (ii) they cause a defect in activation of $trpP_B$ in the presence of InGP (Gao and Gussin, 1991b). Thus, both InGP and the nucleotide sequence of site II play a role first in the binding of TrpI protein to site II and then in the activation of $trpP_B$. Comparison of site I sequences in *P.aeruginosa* and *P.putida* (Chang and Crawford, 1991) suggests that the TrpI recognition sequence in site I is 5′-TGT(G/C)AG-N_5-CT(C/G)ACA, which in *P.aeruginosa* is located between +21 and +5 with respect to the *trpI* transcription startsite. Although there is no comparable sequence in site II, TrpI protects several nucleotides in site II, including the wild-type nucleotides at −10 and −11, against attack by hydroxl radicals (Chang and Crawford, 1990). Furthermore, the *sub1* mutation inhibits both binding of TrpI to site II and activation of $trpP_B$.

However, the site II sequence does not strongly influence binding of TrpI protein *per se*. For example, the effect of the double mutation (−10C/11C) on activation by TrpI plus InGP can be observed followed a 1 min incubation of RNAP and DNA (Gao and Gussin, 1991b), but cannot be observed following a 10 min incubation (Table I, last line). The differential effect of incubation time on transcription from the mutant and wild-type templates indicates that the mutant phenotype cannot be due simply to a defect in binding to site II (see Gao and Gussin, 1991b). The mutations must (also) alter the activity of bound protein. In fact, DNase I footprinting experiments indicate that the double mutation does not significantly affect TrpI binding to site II (Figure 4). These results could be explained by a DNA sequence-induced conformational change in the bound activator which prevents it from fully stimulating open complex formation at $trpP_B$. On the mutant template, the rate of open complex formation might be stimulated by TrpI and InGP to a lesser extent than it is on the wild-type template. Thus, the relative number of open complexes formed on the mutant and wild-type templates would depend on the RNAP−DNA incubation time. A more detailed analysis of Trp1 binding is required to determine whether in fact the activation defect of the double mutant is independent of any quantitative effect on Trp1 binding to site II.

Changing 8 bp in site II prevented activation of $trpP_B$ by Trp1, an effect that appeared on the basis of DNase I footprinting analyses to be due to a defect in binding of Trp1 to site II. However, the substitution (*sub1*) did not block the formation of Complex 2, which in the case of the wild-type template was shown to involve binding of Trp1 to both site I and site II. The question is whether formation of Complex 2 on the mutant template is functionally related to its formation on wild-type DNA. For example, with the mutant template, Complex 2 might arise simply through aggregation of Trp1. However, we believe that Complex 2 forms in the same way on both templates, but in the case of *sub1* DNA Trp1 cannot interact tightly enough with the template to protect it against DNase I digestion. The gel retardation data provide indirect support for this suggestion. The extent of formation of Complex 2 on *sub1* and wild-type DNA is virtually identical at two different Trp1 concentrations, and in the presence or absence of InGP. Chang and Crawford (1991) also found that an extensive substitution of site II (similar to *sub1*) did not prevent formation of Complex 2. However, in those experiments the effects of the mutation on protection of site II and activation of $trpP_B$ were not investigated.

Steps in activation

Together, the gel retardation data, footprinting and activation studies suggest the following sequence of events: (i) a Trp1 tetramer (Chang and Crawford, 1990) binds to site I to form Complex 1; (ii) through a cooperative interaction, a second Trp1 molecule binds to the first to form Complex 2; (iii) the second Trp1 molecule then establishes contacts with specific nucleotides in site II; these contacts are required for Trp1 to form a DNase I footprint on site II and thus may stabilize Complex 2; and (iv) Trp1 then interacts with RNAP to stimulate initiation at $trpP_B$. Thus, *sub1* appears to be defective in step (iii), while −10C/11C appears to be defective in step (iv). Conceivably, InGP plays a role at each of

these steps. Although the data in Table I demonstrate that a small amount of activation is possible in the absence of InGP, the largest effects of InGP for which we have direct evidence are manifest at steps (i) and (ii) (Chang and Crawford, 1990; 1991). The conformation of the bound protein that is required for activation is established properly only when site II is wild-type and in the presence of InGP (Table I, line 2; Figure 2).

Many transcriptional activators are regulated by interaction with a small molecule. Among the best studied are AraC, MerR and catabolite activator protein (CAP). In the case of AraC and MerR, the co-activators (arabinose and Hg^+, respectively) are not required for DNA binding, but are required to allow bound AraC or MerR to stimulate transcription initiation (Lobell and Schleif, 1990; O'Halloran *et al.*, 1989; Summers, 1986). In the case of CAP, cAMP is required for CAP to bind DNA, but the question of whether or not it is also required for activation once CAP is bound is still open (Adhya and Garges, 1990). In general, the way that transcriptional activators influence transcription initiation is not well understood. Genetic (Hochschild *et al.*, 1982), biochemical (Ho and Rosenberg, 1985; Ren *et al.*, 1988; Hwang and Gussin, 1988) and electron microscopic (Su *et al.*, 1990) data suggest that activation involves direct contact between the bound activator and RNAP. Based on footprinting analysis (Chang and Crawford, 1990), site II and the −35 region of $trpP_B$ overlap by 4 bp; if TrpI and RNAP interact with opposite faces of the DNA helix in the region of overlap, direct contact between the two proteins would be possible.

Materials and methods

Phage, bacteria and plasmids

pMI603 (Chang and Crawford, 1990) contains a 1427 bp *Kpn*I fragment from *P.aeruginosa* strain PA01 cloned into pUC18; the boundaries of the *Kpn*I fragment are +844 with respect to the $trpP_I$ transcription startpoint and +532 with respect to the $trpP_B$ transcription startpoint. pZAZ167 (Manch and Crawford, 1982) is the source of a 479 bp *Bgl*II fragment that contains both $trpP_I$ and $trpP_B$ (from *P.aeruginosa* strain PAC174); this fragment was cloned into the *Bam*HI site of M13mp19 (Yanisch-Perron *et al.*, 1985) to yield phage M2100, in which $trpP_I$ directs expression of an in-frame TrpI−LacZα fusion protein. M2100 was mutagenized to produce the $trpP_I$ mutations −10C, −11C and −10C/11C, which are also in site II (Figure 1) (Gao and Gussin, 1991b).

Construction of the mutants sub1 and −16T

The substitution, *sub1* was isolated starting with a 471 bp *Xho*I−*Bgl*II fragment from pMI603. Seven steps (details available on request) were required to produce the recombinant phage M2125 in which the substitution (Figure 1) is contained in a derivative of this *Xho*I−*Bgl*II fragment. In M2125, the fragment was inserted between the *Hinc*II and *Sal*I sites of M13mp19, and the *Bgl*II site was filled in, prior to ligation to the *Hinc*II site of mp19. During the course of this construction, a *Cla*I restriction site was generated using a primer 19 nucleotides long for oligonucleotide-directed mutagenesis (Zoller and Smith, 1981) according to the protocol outlined in the Mutagene kit (Bio-Rad, Richmond, CA). In the corresponding mutant phage (M2122), an A:T bp is substituted for a G:C bp at −16 relative to the $trpP_I$ transcription startsite. DNA sequence analysis (Sanger *et al.*, 1977) revealed a 69 bp deletion of *trp*B sequences extending from +210 with respect to the $trpP_B$ transcription start site to the original *Bgl*II site. The deletion, which is present both in −16T and in *sub1*, is irrelevant to our results; we do not know how it arose.

Transcription in vitro

Transcription conditions have been described previously (Gao and Gussin, 1991b); the KCl concentration was 0.05 M. RNAP (25 nM active enzyme) from *P.aeruginosa* was incubated with the appropriate linear DNA fragment for 10 min prior to addition of substrate NTPs and heparin (50 μg/ml); final concentrations of RNAP and DNA were 20 and 2 nM, respectively. Where indicated, purified TrpI (Chang and Crawford, 1991) and InGP were added to the DNA for 10 min prior to the addition of RNAP. For each experiment, indicated concentrations of TrpI are final concentrations. Concentrations of RNAP, TrpI and DNA during the initial 10 min incubation were 1.67 times the final concentrations. After electrophoresis through 7 M urea−5% acrylamide gels, the products were analyzed autoradiographically.

Abortive initiation

The transcription assay was used, except that the substrates (added with heparin) were 0.2 mM cytidylyl (3′−5′)-adenosine (CpA) and 0.04 mM [α-^{32}P]UTP (2 Ci/mmol). Synthesis of CpApU was allowed to proceed for 10 min at 37°C, at which time 0.2 volumes of stop solution (7 M urea, 0.1% SDS, 80 mM Tris, pH 8, 25 mM EDTA, 1% bromophenol blue, 1% xylene cyanol) was added. The product (CpApU) was separated on a denaturing 20% polyacrylamide gel (Carpousis and Gralla, 1980). After location of the product by autoradiography, the band was cut out of the gel and assayed in a liquid scintillation counter.

Gel retardation

Gel retardation assays were performed as described by Chang and Crawford (1990, 19991); pMI603 or M13 M2125 DNA was digested with *Bss*HII and *Eco*0109 to yield a 172 bp fragment, which was end-labeled with DNA polymerase I (Klenow fragment) in the presence of 20 μCi [α-^{32}P]dCTP (>400 Ci/mmol) and 4 nM dGTP, and then isolated from a 6% polyacrylamide gel. The 20 μl binding mixture contained 1−2 ng DNA fragment (~7000 c.p.m.) in binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 50 μg/ml BSA, 5% glycerol) along with 0, 0.75 or 1.5 μg/ml TrpI protein, and 0 or 4×10^{-5} M InGP. After 20 min at 37°C, 2 μl of 0.1% xylene cyanol in 50% glycerol was added to the mixture and the sample was loaded onto a polyacrylamide gel in 10 mM Tris, pH 7.4, 1 mM EDTA and 0.38 M glycine. The gels were subjected to electrophoresis for 2.5 h at 1.8 mA/cm at room temperature; autoradiography of the dried gel was carried out at room temperature for 12−14 h.

DNase I footprinting

pMI603 DNA and mutant derivatives were cleaved with *Bss*HI and then end-labeled as outlined above for gel retardation studies. The DNA Pol I reaction was stopped by incubation at 70°C for 10 min, followed by *Eco*0109 cleavage at 37°C. The 172 bp fragment was isolated as described in the gel retardation section and then incubated in 20 μl of binding buffer with TrpI protein for 20 min at 37°C. DNase I digestion was begun by adding 2.5 μl of a solution containing 25 mM $CaCl_2$, 25 mM $MgCl_2$ and 1 μl of DNase I (1.2 ng/μl). After 20 s at room temperature, 4.5 μl of 0.2 M EDTA was added to stop the reaction; the DNA was precipitated by adding 10.5 μl $NH_4$0Ac, 8 μl calf thymus DNA (1 μg/μl) and 200 μl 95% ethanol. The precipitate was resuspended in water and then diluted into loading buffer (final concentrations, 47.5% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and loaded onto an 8% polyacrylamide sequencing gel.

For footprinting of Complexes 1 and 2, TrpI binding was carried out as described above. Then 1 μl of a DNase I solution (60 mM $CaCl_2$, 60 mM $MgCl_2$, 1.2 ng/μl DNase I) was added; after 20 s at room temperature, 4 μl of stop solution [0.18 M EDTA, 0.34 μg/ml poly(dI:dG), 30% glycerol] was added and the sample was immediately loaded onto a 6% polyacrylamide gel. Free DNA, Complex 1 or Complex 2 were cut out and eluted from the gel and then analyzed on an 8% polyacrylamide footprinting gel.

Enzymes and chemicals

Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) or Promega Corp. (Madison, WI); T4 DNA ligase was purchased from Bethesda Research Laboratories (Gaithersburg, MD). *P.aeruginosa* RNA polymerase was purified according to the procedure of Burgess and Jendrisak (1975) from *P.aeruginosa* strain PA01 (Gao and Gussin, 1991a). Purified TrpI protein was provided by Dr M.Chang (Chang and Crawford, 1991). Sequenase (Bio-Rad, Richmond, CA) was used for DNA sequence analysis.

[α-^{32}P]UTP was obtained from Amersham/Searle (Chicago, IL). We obtained crystallized InGP from Drs M.Chang and I.P.Crawford; it was originally prepared by Dr K.Kirschner. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used as a chromogenic indicator for *lacZ* expression. CpA (purchased from ICN Biochemical Corp.) was generously provided by W.McClure. Poly(dI:dG) was purchased from Sigma Chemical Co. (St Louis, MO).

Acknowledgements

We are grateful to Dr M.Chang for purified TrpI protein and InGP, to Dr W.McClure for CpA, and Dr M.Feiss for a helpful suggestion that led to the experiment shown in Figure 8. This work was supported by NIH grant AI17508.

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Received on August 2, 1991