JOURNAL OF BACTERIOLOGY, May 1989, p. 2599–2604
0021-9193/89/052599-06$02.00/0

Vol. 171, No. 5

Localization of the Control Region for Expression of Exotoxin A in *Pseudomonas aeruginosa*

MEEI-LING TSAUR† AND ROYSTON C. CLOWES*

Molecular and Cell Biology Program, The University of Texas at Dallas, Richardson, Texas 75083-0688

Received 26 August 1988/Accepted 1 February 1989

The 2,760-base-pair (bp) *Pst*I-*Eco*RI segment of the chromosome of *Pseudomonas aeruginosa* PA103 which carries the exotoxin A structural gene was expressed from an internal promoter when cloned in a pUC9 derivative and transformed into a nontoxigenic mutant of *P. aeruginosa* PAO1. The unique terminal *Eco*RI site was deleted, and a new *Eco*RI site was substituted for a *Pvu*I site located 107 bp 5′ to the transcription initiation site. Following *Eco*RI cleavage, *Bal*31 deletions were generated from this site, and an *Eco*RI linker sequence, GGAATTCC, was inserted in place of the deleted DNA. Mutants with deletions located 73 bp or more upstream of the transcription initiation site retained normal expression, whereas in mutants with deletions extending into the region 69 bp or less upstream of this site, exotoxin synthesis was greatly reduced. From a *Kpn*I site located 473 bp 3′ to the transcription initiation site, a similar series of *Bal*31 deletion mutants were generated in which the inserted *Eco*RI linker sequence was located within the same 72-bp region. Pairs of mutants from the two deletion series were identified in which the *Eco*RI linker was located at the same sequence, and these mutant pairs were ligated to derive a series of constructs in which the *Eco*RI linker sequence GGAATTCC was substituted for an 8-bp sequence within the 72-bp region. Some of these linker-substituted mutants showed greatly reduced exotoxin A synthesis. The results are consistent with a binding site for a positive activator contiguous with the binding site for an RNA polymerase.

We and others have reported that expression of the *Pseudomonas aeruginosa* exotoxin A (*toxA*) structural gene in *Escherichia coli* requires the presence of an external upstream promoter (9, 20). In contrast, when the cloned gene was transformed into MAM4, a *recA*, nontoxigenic mutant of *P. aeruginosa* PAO1, we found that exotoxin was synthesized and secreted into the culture supernatant (8). This expression was shown to be due to a single transcript initiated 88 base pairs (bp) upstream of the initiating codon (Fig. 1). Moreover, when iron was present in excess of 0.1 mM, which inhibits the expression of exotoxin A in *P. aeruginosa* (4), we detected no transcription (8). In the sequence of 80 or more nucleotides upstream of the initiation site, no homology of the *E. coli* σ^{70} promoter consensus sequence (11) could be found (Fig. 2), even though it is known that other *Pseudomonas* genes, such as that for β-lactamase, are transcribed from promoters whose sequences closely resemble the *E. coli* consensus sequence (7).

Other experiments (12, 23) have established that the product of a second gene, termed *toxR*, is required for expression of *toxA* and that, like *toxA*, transcription of *toxR* is inhibited by iron (13). Thus, a simple model for the regulation of *toxA* would be that its transcription requires the binding of the positive activator *toxR* product, and it is therefore indirectly regulated by iron through the repression of *toxR* transcription.

One of the goals of this research is to identify the nucleotide sequences required for exotoxin A expression in *P. aeruginosa* and to further clarify its regulation by iron by the construction and characterization of "linker-scanning" mutants (19).

(A preliminary report of this work has appeared [M.-L. Tsaur and R. C. Clowes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H-87, p. 159].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strains HB101 (*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^- F^- [6]) and AA102 [*recA pro thi supE endA hsdR*$_k$ Δ(*gal-chlD-pgl-att*λ)] (1) and MAM4, a *recA102 met-28 toxA* mutant of *Pseudomonas aeruginosa* PAO1 (8, 20) were used as hosts for the construction and expression of the cloned exotoxin A gene segment. MAM4 was isolated by nitrosoguanidine treatment from PAO286, a *met trp* derivative of the genetic progenitor strain PAO1. It was concluded to have a mutation in or near the *toxA* structural gene by linkage mapping and its ability to be complemented by pRC362 (8).

The vector pRC357 was constructed from pUC9 (22) by insertion of a 1.85-kilobase (kb) *Pst*I segment from pRO1614, which permits replication in *Pseudomonas* hosts (21), and subsequent deletion of the *Pst*I site distal to the multiple cloning site of pUC9 (8). pRC360 was constructed by inserting into pUC9 the 2,760-bp *Pst*I-*Eco*RI chromosomal segment of *P. aeruginosa* PA103 (15) that carries the exotoxin A gene, and pRC362 carries the same *Pst*I-*Eco*RI segment inserted into pRC357 (8). pUC4-KISS (Pharmacia, Inc.) was the source of a 1.3-kb kanamycin resistance (Km^r) cassette (2), excised by *Eco*RI digestion. The level of expression of exotoxin A by pRC362 in MAM4 (about 6 μg per OD_{600} unit) was very similar to that found when pRC362 was expressed in PAO1 when the presence of an active chromosomal *toxA* gene in PAO1 and not in MAM4 is taken into account.

Media and biologicals. The media used were Oxoid No. 2 nutrient broth (NB) and nutrient agar (NA) and tryptic soy broth dialysate (TSBD) as described previously (8), except that TSBD was deferrated to about 1 μM by using Chelex 100 (Bio-Rad Laboratories) (14). Solid TSBD was prepared by the addition of 0.8% agarose (SeaKem). Where appropri-

* Corresponding author.
† Present address: Howard Hughes Medical Institute, University of California, San Francisco, CA 94143.

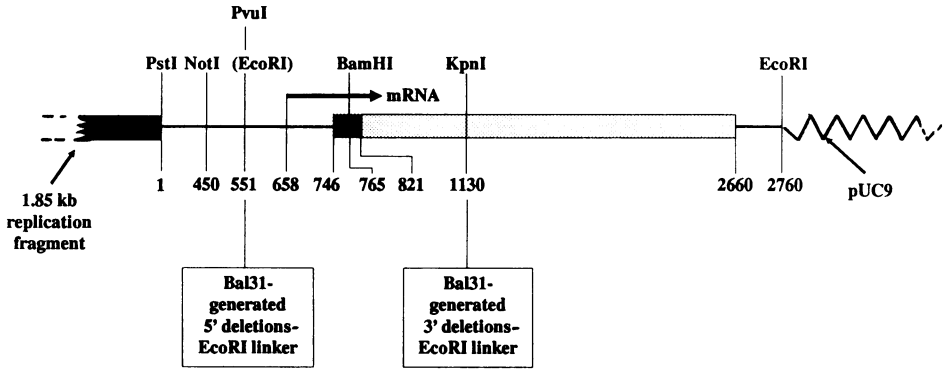

FIG. 1. Details of the exotoxin A gene fragment cloned in pRC362. The 2,760-bp *Pst*I-*Eco*RI chromosomal fragment of *P. aeruginosa* PA103 was cloned in a pUC9 plasmid (wavy line), into which was inserted a 1.85-kb *Pst*I fragment (solid bar) permitting replication in *Pseudomonas* spp. (8). The open bar represents the structural gene sequence, with hatching on a cleaved signal sequence. The transcriptional start site is located at 658 bp, 88 bp upstream of the initiating codon.

ate, disodium carbenicillin, kanamycin (Sigma Chemical Co.), or $FeSO_4 \cdot 7H_2O$ was added to concentrations of 300 μg/ml, 50 μg/ml, or 0.1 mM, respectively. L broth (16) was used for some growth cultures. Restriction enzymes and *Bal*31 nuclease were obtained from Bethesda Research Laboratories, Inc.

Methods. Large-scale preparation of plasmid DNA was done as described by Birnboim and Doly (3). Isolation of plasmid DNA from transformant clones for screening procedures was done by the alkaline method (17). Restriction enzyme cleavage, ligation, and transformation of *E. coli* HB101 and *P. aeruginosa* MAM4 were carried out as described previously (20). *E. coli* AA102 was used for transformation of ligated DNA, essentially by the method of Hanahan (10). The recessed 3′ ends of double-stranded DNA were filled in by the Klenow fragment of *E. coli* DNA polymerase I as described before (17). *Pvu*I sites were converted to *Eco*RI sites with the hexamer TCGAAT (Pharmacia), based on the technique of Barany (2). *Bal*31 digestion followed the procedure described before (17), with 1 U of *Bal*31 nuclease per 16 μg of DNA in 100 μl at 30°C. *Eco*RI linker ligation was carried out with 1 μg of blunt-ended DNA, 3 μg of *Eco*RI linker (GGAATTCC; New England BioLabs), and 5 U of T4 DNA ligase in 10 μl and incubating at 7°C for 1 h and overnight at 12°C. The Km^r cassette was excised as a 1.3-kb *Eco*RI fragment from pUC4-KISS and was incorporated by ligating approximately equal weights of cassette and *Eco*RI-cleaved plasmid DNA.

Exotoxin A synthesis and secretion were detected by using the Elek immunodiffusion test (5) for the rapid screening of clones grown on TSBD agar. A radioimmunoassay (12) was used for more quantitative measurements of exotoxin A in supernatants of MAM4 cultures after growth at 32°C in deferrated TSBD for 20 h. Dilutions of a purified exotoxin A preparation used as a standard showed a linear response from a minimum detection level of 4 ng/ml up to 500 ng/ml. In both methods, radiolabeled anti-exotoxin A immunoglobulin G prepared from rabbits was used (8, 20). Since exotoxin A is normally secreted by MAM4 strains which carry plasmids incorporating the structural *toxA* gene (8), we assumed that lack of exotoxin in the supernatants or growth medium of this host strain was due to its lack of synthesis. Western-immunoblot analysis of supernatants and cell-associated fractions showed that at least 90% of the immunologically cross-reacting material was found in the supernatants of MAM4 cultures and was not detected in cell-associated fractions (R. B. Wilson, Ph.D. thesis, University of Texas at Dallas, Richardson, Tex., 1988).

DNA sequencing. After initial digestion by *Eco*RI and *Kpn*I of 5′ deletion mutants or by *Pst*I and *Eco*RI of 3′ deletion mutants, the digests were incubated with RNase I and then separated by polyacrylamide gel electrophoresis (PAGE) on 5% polyacrylamide gels. The fragments were then electroeluted, dephosphorylated, [γ-^{32}P]ATP end-labeled, and sequenced as described by Maxam and Gilbert (18).

Plasmid constructions. pRC362ΔE was constructed from pRC362 (Fig. 1) by removing the *Eco*RI site (nucleotide 2760) at the 3′ terminus of the cloned exotoxin A gene (8) by *Eco*RI cleavage, followed by fill-in of recessed ends by DNA polymerase I (large fragment) and ligation of resulting blunt-end DNA by T4 DNA ligase. The ligation products were transformed into *E. coli* HB101, selecting carbenicillin-resistant (Cb^r) clones. The plasmid DNA from a clone that was shown to be insensitive to cleavage by *Eco*RI was designated pRC362ΔE.

pRC362(551E) was derived by subjecting pRC362ΔE DNA to partial cleavage by *Pvu*I (sites at nucleotides 551 and 1135 in the exotoxin segment and two sites in the vector). TCGAAT linker DNA (Pharmacia) was added, and the mixture was ligated. It was then cleaved with *Eco*RI, and a 1.3-kb Km^r cassette excised by *Eco*RI from pUC4-KISS was added and ligated. Following transformation into *E. coli* AA102 and selection for Km^r colonies, the plasmid DNA from several clones was isolated and cleaved with *Pst*I and *Eco*RI. One clone that produced two fragments, one of approximately 550 bp and the other of 1.3 kb, was selected, and the Km^r cassette was removed following *Eco*RI digestion, dilution, and religation. The result of these procedures was to insert an *Eco*RI recognition sequence, TCGAATTC GAAT, between nucleotides 551 and 552, to produce the plasmid designated pRC362(551E).

Deletion by *Bal*31. *Eco*RI-cleaved plasmid DNA was digested by *Bal*31 for appropriate times. The recessed ends of the deleted DNAs were filled in and ligated to the 8-bp *Eco*RI linker sequence GGAATTCC. Plasmids with an attached *Eco*RI linker sequence were then selected by *Eco*RI digestion, followed by ligation of a Km^r cassette with *Eco*RI termini, which permitted growth of transformed AA102 hosts on NA plus kanamycin. The plasmid DNA of Km^r transformants was digested with *Eco*RI, diluted, and religated to remove the Km^r cassette.

Determination of deletion sizes of end-labeled fragments. As

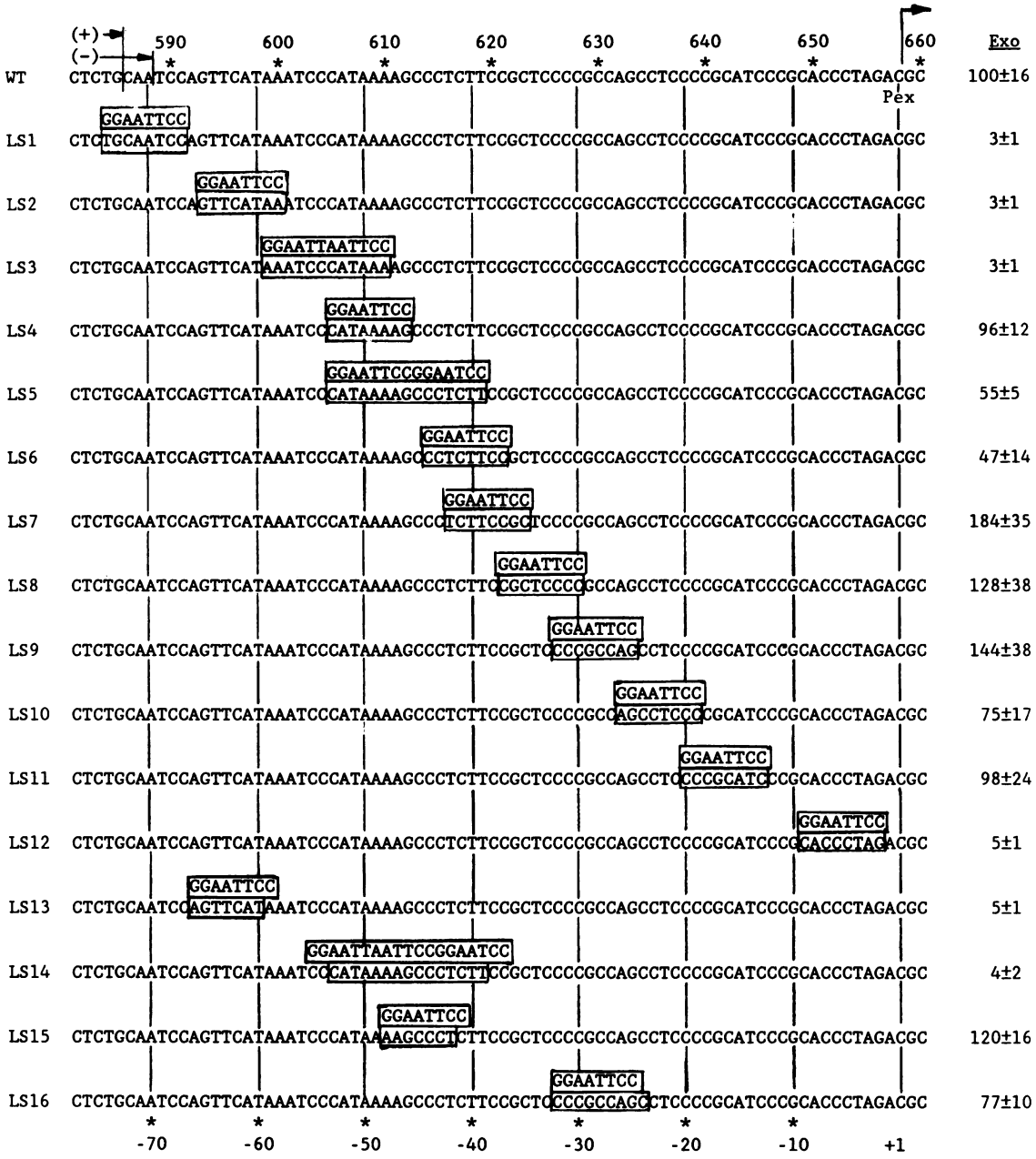

FIG. 2. The top line (WT, wild type) shows the nucleotide sequence as established 5′ to the *toxA* gene of *P. aeruginosa* PA103 (8, 9) when cloned in the plasmid pRC362 (8). The numbers above are nucleotides distant from the *Pst*I site (at nucleotide +1). The transcription initiation site (Pex) is at nucleotide 658 (horizontal arrow) (8), and the initiating codon is at nucleotide 746 (9). The numbers under the bottom line show distances in nucleotides from Pex. 5′ deletions of this sequence terminating 73 bp or distal to Pex (shown by horizontal arrow and +) expressed exotoxin A at the level of pRC362 transformed into the nontoxigenic *P. aeruginosa* recipient MAM4. 5′ deletions terminating at 69 bp or proximal to Pex (shown as horizontal arrow and −) expressed exotoxin A at a level of 5% or less of the pRC362 level. The lines below (LS-1 to LS-16) show the corresponding nucleotide sequences in linker-scanning mutants in which the boxed sequence shown above the line has been substituted for the sequence of pRC362 shown boxed. In LS-1 to LS-12, an equal number of nucleotides are substituted. In LS-13 to LS-16, the numbers of nucleotides substituted in the mutant are unequal to those of pRC362 which were replaced. The numbers to the right under Exo show the level of expression determined by a radioimmunoassay of each mutant when transformed into MAM4 and grown in TSBD medium with about 1 μM Fe^{2+} compared with that of the parental plasmid pRC362 in the same host and the same medium. The values shown are the mean and standard deviation for at least three separate assays from separate cultures or, in the case of pRC362, for six separate cultures assayed. (In TSBD medium with 0.1 mM Fe^{2+}, expression of exotoxin A by all mutants and by pRC362 was between 1 and 3% of the level of pRC362 with 1 μM Fe^{2+}.)

a rapid method for more precise measurement of deletion sizes than allowed by electrophoretic mobility of DNA fragments in polyacrylamide gels, the following technique was used. The size of each deletion was initially roughly estimated by the mobility of restriction fragments in 8% polyacrylamide gels. In mutants carrying deletions from a site 5′ to the transcription initiation site, *Eco*RI-*Bam*HI (nucleotide 765) cleavage fragments were used, or, in the

case of mutants carrying deletions from a site 3′ to the transcription initiation site, *Eco*RI-*Not*I (nucleotide 450) fragments were measured (Fig. 1). Two other deletion mutants were then chosen in which the deletions had been previously defined by DNA sequence analysis (18), one of which had a longer (ΔL) and the other a smaller (ΔS) deletion than the deletion (ΔU) under study. For 5′ deletions, the DNA of all three deletion mutants was cleaved with *Mae*I (unique site at nucleotide 653 in the exotoxin A sequence), end-labeled with [α-^{32}P]dTTP by Klenow fragment, filled-in with [α-^{32}P]dTTP, and cleaved with *Eco*RI. The DNAs of ΔL, ΔS, and ΔU were then separated on a 10% polyacrylamide–urea gel in parallel with the G+A and T+C sequencing reactions of ΔL or ΔS to permit measurement (±1 bp) of the size of ΔU from the 3′ label to the 5′-cleaved *Eco*RI terminus. For 3′ deletions, a similar procedure used cleavage and end-labeling of the *Eco*RI site with [α-^{32}P]dATP, followed by *Pvu*I cleavage at nucleotide 551.

RESULTS

Construction of 5′ deletions. Deletions of nucleotide sequences located 5′ to the exotoxin A transcription initiation site (Pex) at nucleotide 658 were derived by *Bal*31 digestion of pRC362(551E) from nucleotide 551 of the 2,760-bp exotoxin sequence (Fig. 1). pRC362(551E) was first cleaved by *Eco*RI and digested by *Bal*31 for 0, 30, 60, and 90 s, followed by *Eco*RI linker ligation and selection by use of a Km^r cassette. Following removal of the Km^r cassette, the plasmid DNA of several clones transformed with each timed *Bal*31 digest was assayed for the approximate size of the deletion by cleavage with *Eco*RI and *Bam*HI (nucleotide 765), and then the transformed MAM4 hosts were screened for expression of exotoxin A by an Elek test (5). From these tests, it was established that 18 mutants carrying deletions which terminated approximately 70 bp or more upstream of Pex expressed exotoxin A (by production of a precipitin line in the agar), whereas nine mutants carrying deletions which terminated within about 70 bp or less of Pex did not express exotoxin A (data not shown). The 60-s *Bal*31 digest produced some deletions which expressed the toxin and others which did not and was used for further analysis. The size and location of deletions which terminated about 70 bp or less upstream of Pex were more accurately determined (to ±1 bp) in the 60-s digest by the end-labeling method (data not shown). All clones in which toxin was not expressed in an Elek test were found to have deletions terminating within 69 bp from Pex, and all clones which expressed toxin had deletions terminating 73 bp or more from Pex. All hosts that expressed exotoxin A showed no expression when 0.1 mM iron was added to the medium. From these deletion experiments, we conclude that a sequence 72 bp upstream of Pex is necessary and sufficient for the expression and regulation of exotoxin A.

Construction of 3′ deletions. A series of *Bal*31-generated 3′ deletions with *Eco*RI linker insertions were similarly constructed following *Kpn*I cleavage (unique site at nucleotide 1130) of pRC362ΔE. The plasmid DNA of several Km^r transformant clones from each digestion time of 0, 55, 65, and 75 min was screened for size as described for 5′ deletion mutants, except that *Eco*RI and *Not*I (nucleotide 450) were used to roughly determine the location of the 3′ end of the deletion. The 65-min digest, which produced a number of deletions with 3′ termini within the 72-bp region upstream of Pex, was chosen for more accurate (±1 bp) determinations of the deletion sizes.

Construction and analysis of linker-scanning mutants. Following precise determination by nucleotide sequencing of the termini of a number of 5′ and 3′ deletion mutants which had *Eco*RI linkers inserted within a 75-bp region upstream of Pex, 16 derivatives of pRC362 were constructed in which we attempted to substitute the *Eco*RI linker sequence GGAAT TCC for an 8-bp sequence within the region 75 bp upstream of Pex. This was achieved by cleavage and ligation between one of each type of deletion mutant in which the 5′ end of one and the 3′ end of another were found to be terminated by an *Eco*RI linker sequence at the identical location. The *Pst*I-*Eco*RI segment of the 3′ deletion and the *Eco*RI-*Kpn*I segment of the 5′ deletion were separated by PAGE on a 5% polyacrylamide gel, excised, and electroeluted (Fig. 1). In each construct, the large (6.2-kb) *Pst*I-*Kpn*I vector segment of pRC362ΔE, similarly separated from a 0.6% ultrapure agarose gel, was added to the two segments and ligated, and the ligation mixture was transformed directly into *E. coli* HB101. The plasmid DNA from several Cb^r clones was isolated and screened for size by *Pst*I-*Bam*HI cleavage (765 bp) and by *Pvu*I-*Bam*HI cleavage (214 bp). The nucleotide sequence of about 40 bp in each direction from the introduced *Eco*RI site was then established by the Maxam-Gilbert technique for each construct to accurately locate the linker substitutions, as shown in Fig. 2.

In 10 of the constructs, the GGAATTCC sequence was substituted for an equivalent 8-bp sequence in the 72-bp control region. In three mutants it replaced seven bases (in LS-13 and LS-15) or nine bases (in LS-16), and in three other mutants, reiterations of part of the GGAATTCC sequence were substituted for an equivalent number of bases (in LS-3 and LS-5) or for a smaller number of bases (in LS-14) (Fig. 2). Plasmid DNA from *E. coli* clones carrying these mutants was then transformed into *P. aeruginosa* MAM4 to assay for exotoxin A. Toxin expression of the 16 linker-substitution mutants was measured by radioimmunoassays of the supernatant of the MAM4 host carrying the plasmid, grown either in the presence or absence of iron, and were quantitated by using a pure exotoxin A standard. In low-iron medium, the supernatants from MAM4(pRC362), assayed following a 1:200 dilution, were found to contain 6.0 ± 1.0 μg of exotoxin A per OD_{600} unit, whereas those from poorly expressing mutants, e.g., MAM4(pRC362LS-1), contained only 0.16 ± 0.05 μg/OD_{600} unit. The amounts of exotoxin A relative to that synthesized by pRC362 in low-iron medium are shown in Fig. 2. The nucleotide sequences of the region 5′ to the transcription initiation site of the mutants constructed and the level of their expression in the *Pseudomonas* host MAM4 shown in Fig. 2 are summarized in Fig. 3. In only four regions (in LS-1, -2, -3, and -12) of the sequence upstream of the transcriptional initiation site did the substitution of a linker for an equivalent number of nucleotides result in more than a twofold change of expression, and in all of these, a decrease to 5% or less of the control level resulted.

DISCUSSION

Comparisons between the initial and substituted nucleotide sequences upstream of the *toxA* gene (Fig. 2) show that one or more nucleotides in the −74 to −54 sequence are essential for normal expression (LS-1, -2, and -3), as are other nucleotides in the −9 to −2 sequence (LS-12). However, most nucleotides located at −53 to −14 can be substituted, and those located upstream of −72 can be deleted, as observed in the original 5′ deletion *Bal*31 mutants, without

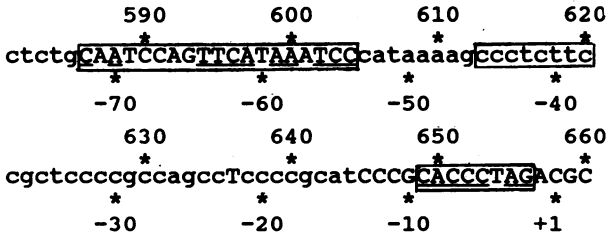

FIG. 3. Nucleotide sequence 5′ to the *toxA* gene as shown in Fig. 2. Nucleotides in capital letters without underlines were not substituted, and those in lowercase letters could be substituted with little (or no more than a twofold) effect on expression, whereas substitution of those in capital letters with underlines, which are located within a single box as shown in Fig. 2, resulted in a decrease in expression to 5% or less. Boxed regions represent approximate locations of putative binding sites for RNA polymerase (centered at −6 and −41) and for a transcriptional activator molecule (centered at −63).

having any more than a twofold influence on expression. This limits the essential distal upstream sequence to nucleotides between −72 and −54. Moreover, the insertion of a single nucleotide within the −66 to −60 sequence (LS-13), or the insertion of four nucleotides in the −53 to −39 sequence (LS-14), also reduced expression to less than 5%. However, the insertion of an extra nucleotide between −48 and −42 (LS-15) or the loss of one nucleotide between −32 and −24 (LS-16) had little effect (Fig. 3).

We earlier concluded that the 72-bp sequence upstream of Pex is adequate and sufficient to define the expression of exotoxin A. It seems reasonable to propose that the sequences centered at nucleotides −41 and −6 are involved in the binding of an RNA polymerase, in which case the nucleotides between −72 and −54 appear to be necessary for the binding of an accessory transcriptional factor. That a single accessory-binding site is present in this region may be inferred from the fact that insertion of a single nucleotide within this region (in LS-13) severely reduced expression. Moreover, the distance between the −72 to −54 region and the −41 region is also critical, as deduced from the effects of inserting four nucleotides (compare LS-5 with LS-14), from which it may be proposed that the binding of the accessory factor at the −72 to −54 region may permit its interaction with the RNA polymerase molecule which binds at the −45 to −2 region.

Substitution of nucleotides between −45 and −38 (in LS-5, -6, or -7) produced smaller (limited to approximately twofold) differences in expression, in contrast to the changes of nucleotides −37 to −10 (in LS-8, -9, -10, and -11), where differences in expression from that produced by the wild-type sequence did not exceed the standard error. It is proposed that the binding of an activator molecule at the −72 to −54 region may be required for the subsequent binding of an RNA polymerase at the −45 to −38 and the −9 to −2 regions. It might then be suggested that the sequence at the −45 to −38 region proximal to the activator-binding site would be of less importance to the stability of RNA polymerase binding (or open complex formation) and can lead only to twofold up or down differences, whereas binding at the −9 to −2 site distal to the activator-binding site is critical, so that 30-fold reductions in expression result from substitutions in this region.

The identification of a region necessary for binding a factor in addition to RNA polymerase which is required for transcription is consistent with the findings of Galloway and colleagues (12, 23), who concluded that a positive activator gene, *toxR*, was required for the efficient expression of exotoxin A. The −72 to −54 region identified here may be the binding site for this activator gene product. It may be noteworthy that this site includes a direct repeat of the sequence AATCC at −71 to −67 and −58 to −54. However, the direct repeat, CTCCCCGC, located at −35 to −28 and −23 to −16, suggested by Wozniak et al. (23) to be the binding site for the *toxR* product, does not in fact appear to be involved in the regulation of exotoxin A expression, since any of the nucleotides in the direct repeat (except for that at −22, which was not changed) can be substituted with little effect (less than twofold) on expression.

ACKNOWLEDGMENTS

We are grateful to Stephen Leppla for a gift of purified exotoxin A and to Rockford Draper and Priscilla Holmans for helpful discussions. We thank Elzora Jordan for developing the radioimmunoassay assay, and we are indebted to Joyce Lee and C. Bailey for the manuscript and figures.

This work is submitted in partial fulfillment of the degree of Ph.D. for M.-L.T. and was accomplished by financial support from the Robert A. Welch foundation and a Public Health Service Biomedical Research Support grant from the National Institutes of Health.

LITERATURE CITED

1. **Ahmed, A.** 1985. A rapid procedure for DNA sequencing using transposon-promoted deletions in *Escherichia coli*. Gene **39:** 305–310.
2. **Barany, F.** 1985. Single-stranded hexameric linkers: a system for in-phase insertion mutagenesis and protein engineering. Gene **37:**111–123.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Bjorn, M. J., B. H. Iglewski, S. K. Ives, J. C. Sadoff, and M. L. Vasil.** 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA103. Infect. Immun. **19:**785–791.
5. **Bjorn, M. J., M. L. Vasil, J. C. Sadoff, and B. H. Iglewski.** 1977. Incidence of exotoxin production by *Pseudomonas* species. Infect. Immun. **16:**362–366.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
7. **Chen, S.-T., and R. C. Clowes.** 1987. Variations between the nucleotide sequences of Tn*1*, Tn*2*, and Tn*3* and the expression of β-lactamase in *Pseudomonas aeruginosa* and *Escherichia coli*. J. Bacteriol. **169:**913–916.
8. **Chen, S.-T., E. M. Jordan, R. B. Wilson, R. K. Draper, and R. C. Clowes.** 1987. Transcription and expression of the exotoxin A gene of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **133:**3081–3091.
9. **Gray, G. L., D. H. Smith, J. S. Baldridge, R. N. Harkins, M. L. Vasil, M. L. Chen, and H. L. Heyneker.** 1984. Cloning, nucleotide sequence, and exression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **81:**2645–2649.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
11. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
12. **Hedstrom, R. C., C. R. Funk, J. B. Kaper, G. R. Pavlovskis, and D. R. Galloway.** 1986. Cloning of gene involved in regulation of exotoxin A expression in *Pseudomonas aeruginosa*. Infect. Immun. **51:**37–42.
13. **Hindahl, M. S., D. W. Frank, and B. H. Iglewski.** 1987. Molecular studies of a positive regulator of toxin A synthesis in *Pseudomonas aeruginosa*. Antibiot. Chemother. **39:**279–289.
14. **Iglewski, B. H., and J. C. Sadoff.** 1979. Toxin inhibitors of protein synthesis: production, purification and assay of *Pseudo-*

monas aeruginosa toxin A. Methods Enzymol. **60**:780–793.
15. **Liu, P. V.** 1966. The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481–489.
16. **Luria, S. E., and M. L. Human.** 1952. A non-hereditary host-induced variation of bacterial viruses. J. Bacteriol. **64**:557–569.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 113–114, 135–139. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
19. **McKnight, S. L., and R. Kingsbury.** 1982. Transcription control signals of a eukaryotic protein-coding gene. Science **217**:316–324.
20. **Mozola, M. A., R. B. Wilson, E. M. Jordan, R. K. Draper, and R. C. Clowes.** 1984. Cloning and expression of a gene segment encoding the enzymatic moiety of *Pseudomonas aeruginosa* exotoxin A. J. Bacteriol. **159**:683–687.
21. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. A. J. Bacteriol. **150**:60–69.
22. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
23. **Wosniak, D. J., D. C. Cram, C. J. Daniels, and D. R. Galloway.** 1987. Nucleotide sequence and characterization of *toxR*: a gene involved in exotoxin A regulation in *Pseudomonas aeruginosa*. Nucleic Acids Res. **15**:2123–2135.