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Kinetics of *toxA* and *regA* mRNA Accumulation in *Pseudomonas aeruginosa*

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DNA probes specific for an internal portion of the *toxA* and *regA* genes were used to examine the synthesis of mRNA during the growth cycle of *P. aeruginosa* PA103. RNA dot blot analysis revealed that in a low-iron growth medium, the synthesis of *regA* and *toxA* mRNA followed a biphasic expression pattern. Analysis of ADP-ribosyltransferase activity also indicated that an early and late phase of exotoxin A synthesis occurred. Utilizing an internal *SalI* probe, examination of the size distribution of the *regA* mRNA during the cell cycle indicated that a large transcript (T1) was present at early time points, followed by the appearance of a smaller transcript (T2) during late exponential to early stationary phase. An upstream *AvaI* *regA* probe was found to hybridize to the T1 transcript but not to the T2 transcript. The data indicate that at least two separate functional *regA* mRNA species were produced. Analysis of mRNA accumulation for the *regA* gene when cells were grown in high-iron medium provided additional evidence for two separately controlled transcripts being produced from the *regA* chromosomal locus. Both *regA* transcripts were correlated with exotoxin A transcription and production.

Exotoxin A from *Pseudomonas aeruginosa* represents one constituent of a family of bacterial toxins with ADP-ribosyltransferase activity. Sequence analysis of the cloned structural gene indicates that the exotoxin A locus encodes a monocistronic mRNA leading to the synthesis of a 638-amino-acid precursor molecule (12). A 25-amino-acid hydrophobic leader peptide is presumably removed during secretion to produce the mature form of exotoxin A (12, 21). This form of exotoxin A consists of several domains responsible for binding to a susceptible mammalian cell (16), translocating across the membrane (16), and catalyzing the transfer of an ADP-ribose moiety onto host elongation factor 2 (EF2) (12, 16, 27). The covalent modification of EF2 inhibits eucaryotic protein synthesis, resulting in the death of the cell (17).

Although the composition of exotoxin A and the mechanism by which it acts to stop protein synthesis have been described, the regulation of exotoxin A synthesis by the bacterium has not been characterized. Toxin yields have been shown to be strain dependent (4). However, recent evidence suggests that the structural gene for exotoxin A (*toxA*) is present as a single copy on the chromosomes of all the strains examined (33). Therefore, the yield of exotoxin A from a particular strain probably does not involve a structural gene duplication, as has been shown for cholera toxin genes (25). Exotoxin A synthesis is not constitutive. Early work showed that several environmental and nutritional factors influence the yield of exotoxin A (20). These factors include temperature of incubation, aeration rate, amino acid composition, and most notably the concentration of iron in the medium (3, 4, 20). Iron concentration has been shown to affect the synthesis of several bacterial toxins (32), and investigation of this phenomenon may elucidate a general regulatory pathway.

A second gene involved in exotoxin A synthesis is a regulatory gene which we call *regA* (9, 15; M. S. Hindahl, D. W. Frank, A. Hamood, and B. H. Iglewski, Nucleic Acids Res., in press) previously referred to as *toxR* (37). The cloned *regA* gene restores exotoxin A production (14) in a hypotoxigenic mutant, PA103-29, isolated and characterized by Ohman et al. (28). *regA* and *toxA* transcription are undetectable in this mutant strain (unpublished results). Evidence of the positive regulatory role of *regA* stems from the observation that multiple copies of the *regA* gene in *trans* increase exotoxin A yields by approximately 10-fold (14). In addition, the *regA* gene in a multicopy state can partially overcome the negative effects of iron added to the culture medium (14, 15). Sequence and deletion analysis indicates that the *regA* gene encodes a single polypeptide of 28,824 molecular weight (Hindahl et al., in press). The specific role that *regA* has in controlling exotoxin A transcription has not been defined but may be similar to the role of the *toxR* gene of *Vibrio cholerae* (26) or the *vir* gene of *Bordetella pertussis* (35).

To understand the synthesis of exotoxin A, we have focused on the transcription of the *regA* and *toxA* genes. We have previously shown that *regA* transcription precedes *toxA* transcription and translation, indicating a temporal sequence of events (15). *regA* transcription is repressed when cells are grown in medium where iron is in excess (15). Similarly, *toxA* transcription is repressed under the same growth conditions (5, 9, 15, 22). Thus, the repression of exotoxin A synthesis seen when cells are grown in high-iron medium appears to result from the inhibition of *regA* transcription. In this paper we report an analysis of *regA* and *toxA* mRNA accumulation over the growth cycle of cells cultivated in low- and high-iron media. The techniques we used in measuring transcript accumulation reflect the relative rates of synthesis and degradation of mRNA. These parameters may also vary with the growth cycle.

MATERIALS AND METHODS

Bacterial strains and plasmids. Hypertoxigen-producing *P. aeruginosa* PA103 was used as a host in these experiments (19). Transformation of PA103 with vector plasmid pUC181.8 was accomplished by the $MgCl_2$ method described by Olsen et al. (29). The vector pUC181.8 was constructed by insertion of a 1.85-kilobase (kb) *PstI* frag-

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ment from pRO1614 into the *Pst*I site of pUC18. This approach has been used previously to obtain high-copy-number pUC vectors which are maintained in both *Escherichia coli* and *P. aeruginosa* strains (5). Inclusion of the vector in PA103 does not detectably alter exotoxin A production. The data collected from PA103(pUC181.8) represent the normal combination of one chromosomal *regA* gene (14) and one chromosomal *toxA* gene (33). The analysis of exotoxin A synthesis from PA103 plus vector serves as a baseline for comparison with strains containing multiple copies of the cloned *regA* gene (manuscript in preparation).

Culture conditions. PA103(pUC181.8) cells were grown overnight at 32°C in tryptic soy broth dialysate containing a low (0.05 μg of Fe^{2+} per ml) or a high (10 μg of Fe^{2+} per ml) iron concentration in addition to 400 μg of carbenicillin (Sigma Chemical Co.) per ml (28). The optical density at 540 nm (OD_{540}) was measured with a Beckman DU50 spectrophotometer, and each culture was diluted in the appropriate fresh medium to an OD_{540} of 0.02. Samples consisting of approximately 2×10^{10} cells were removed when the OD_{540} or time reached the following target values: 0.1, 0.3, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0 (12 h), 14 h, and 16 to 17 h (refer to Fig. 3B). Cells grown in high-iron medium tended to reach these OD_{540} values approximately 20 to 30 min sooner than cells grown in low-iron medium. The only other difference in the growth curves appeared after an OD_{540} of approximately 5.0, when high-iron-grown cells continued to increase in density and growth of cells in low-iron medium generally leveled off (Fig. 3B).

Isolation of total RNA. Total RNA was isolated from PA103(pUC181.8) by a hot phenol method outlined by von Gabain et al. (34). After the first precipitation step, the RNA pellet was resuspended in 0.1 M Tris hydrochloride (pH 7.4)–50 mM NaCl–10 mM disodium EDTA–0.2% (wt/vol) sodium dodecyl sulfate (SDS)–200 μg of proteinase K (Sigma Chemical Co.) per ml and incubated at 37°C for 1 h. These preparations were extracted with phenol and chloroform. The RNA was then precipitated with 2.2 volumes of ethanol. Proteinase K treatment was followed by exposure of the RNA pellets to RNase-free DNase (Worthington Biochemicals) as described previously (34). The RNA samples were then reextracted with phenol and chloroform and precipitated in ethanol. RNA was resuspended in sterile distilled water and quantitated spectrophotometrically by A_{260} measurements. Samples were stored at −70°C.

Isolation and labeling of DNA fragments. The location of the probes used to examine the total RNA preparations is shown in Fig. 1. Restriction endonuclease cleavage of plasmid DNA was achieved according to the manufacturer's recommendation (Bethesda Research Laboratories). Fragments were isolated from agarose gels by phenol extraction as described previously (2). The purified fragments were labeled with a primer extension kit purchased from Pharmacia according to the methods of Feinberg and Vogelstein (8). [^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham Corporation.

mRNA analysis with dot blots and glyoxal gels. Dot blot analysis was carried out as follows. RNA was denatured with formaldehyde at 60°C as described by White and Bancroft (36). We have previously found that hybridization of *regA* or *toxA* DNA probes to total RNA isolated from PA103 followed a linear relationship by using RNA concentrations ranging from 0.1 to 6.0 μg per dot. Denatured RNA was applied with suction in 5-μg amounts per 4-mm-diameter dot onto a nitrocellulose sheet (BA45, 0.45 μm) supported on two GB003 blot block sheets (Schleicher & Schuell). The

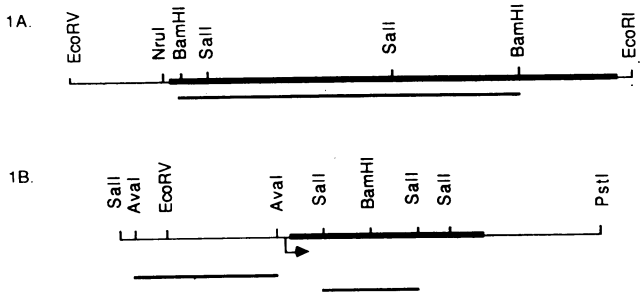

FIG. 1. Map of the *toxA* (A) and *regA* (B) cloned genes. The relevant parts of the genes are shown with restriction site markers for reference. Thick lines within the cloned segment represent the translated portion of the *toxA* gene (1,914 nucleotides or 638 amino acids [12]) and the *regA* gene (777 nucleotides or 259 amino acids [16]). The *Bam*HI 1.530-bp fragment served as a *toxA*-specific probe (12). Two fragments were used to examine *regA* mRNA accumulation, a 449-bp upstream *Ava*I (−489 to −40 bp) fragment and a 363-bp internal *Sal*I (157 to 520 bp) fragment. The transcriptional start site has been previously reported to be 20 bp upstream of the ATG start codon (37) and is denoted by a rightward arrow.

block sheets and the nitrocellulose sheet were held in place by a Schleicher & Schuell Minifold apparatus. After filtration, each well was washed three times with 20× SSC (200 μl per wash) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the nitrocellulose sheet was baked at 80°C for 2 h in vacuo.

For glyoxal gel analysis, total RNA (10 μg per lane) was denatured with glyoxal and electrophoresed in 1.2% agarose gels as described previously (24). RNA size markers were purchased from Bethesda Research Laboratories and visualized on autoradiograms by using lambda DNA as a probe. RNA was fixed to the nitrocellulose sheets by baking at 80°C for 3 h.

All nitrocellulose blots were hybridized to labeled DNA probes, washed under high-stringency conditions, dried, and exposed to Kodak XAR film (9). Dots were cut from nitrocellulose sheets and counted in 4 ml of toluene (EM Science) plus Omnifluor (4 g/liter; New England Nuclear-DuPont) in a Beckman LS1801 liquid scintillation counter.

Analysis of exotoxin A activity. At each of the indicated time points, samples consisting of 2×10^{10} cells were centrifuged at 10,000 × *g* for 10 min at 5°C, and 1 ml of the supernatant was removed and frozen at −70°C until assay. Cell pellets were washed two times with 10 ml of a sterile 50 mM Tris hydrochloride pH 7.4, solution. Pellets were frozen at −70°C and suspended in 2.0 ml of the wash solution containing 2.5 μg of DNase I (Sigma Chemical Co.) and 7,000 U of RNase T_1 (Bethesda Research Laboratories). This suspension of cells was passed through an Aminco French pressure cell (3/8-in. [ca. 1 cm] diameter piston) twice, at maximal pressure. The lysate was collected in an Eppendorf tube and centrifuged for 12 min at 14,000 × *g* at 5°C to remove unbroken cells and debris. Samples of supernatants and cell lysates were assayed for ADP-ribosyltransferase activity with wheat germ EF2 prepared as described by Chung and Collier (6). The standard assay for activated exotoxin A consisted of 10 μl of test material preincubated for 15 min at 25°C with an equal volume of 8 M urea (Boehringer Manneheim) and 2% (wt/vol) dithiolthreitol (DTT) (Sigma Chemical Co.). Unactivated exotoxin A activity was assessed after preincubating test material in an equal volume of distilled water instead of the urea-DTT mixture. Each assay was conducted with 25 μl of wheat germ EF2, 25

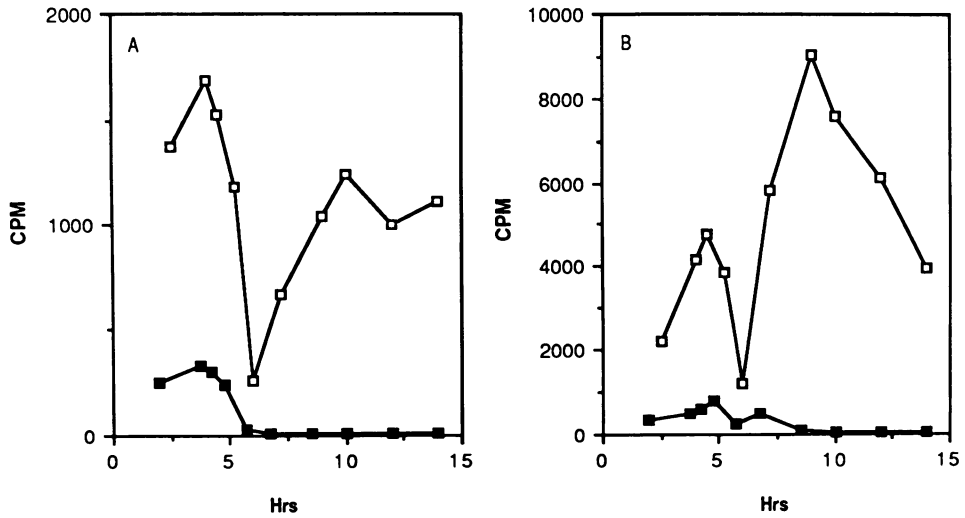

FIG. 2. *regA* mRNA accumulation from cells grown in low- and high-iron medium. Total RNA was isolated over the entire growth curve. The RNA was dot blotted onto nitrocellulose and probed with an internal *regA SalI* fragment (A) or an internal *toxA BamHI* fragment (B). Symbols: □, RNA isolated from PA103(pUC181.8) grown under low-iron conditions; ■, PA103(pUC181.8) grown under high-iron conditions.

μl of 125 mM Tris hydrochloride (pH 7.0) plus 100 mM DTT, and 5 μl of [^{14}C]NAD (530 mCi/mmol; New England Nuclear) for 10 min at 25°C. The assay was stopped with 200 μl of a 10% (wt/vol) solution of trichloroacetic acid (TCA) (Sigma Chemical Co.) and filtered onto nitrocellulose circles. The filters were washed with 5% (wt/vol) cold TCA, dried, and counted in 6 ml of scintillation fluid. Protein content was determined by the method of Lowry et al. (24). Data are expressed per unit of protein for the culture supernatant and lysate samples. Samples were diluted and reassayed to ensure that the exotoxin A activity fell within the linear range of the ADP-ribosyltransferase assay system as well as the linear range of the scintillation counter.

RESULTS

Analysis of *regA* and *toxA* mRNA with internal DNA probes. mRNA accumulation studies for the *toxA* and *regA* structural genes previously established that transcription of both genes was suppressed by addition of iron to the growth medium (15). To define the kinetics of exotoxin A and *regA* production, we analyzed mRNA synthesis as well as exotoxin A production over the entire bacterial growth cycle. Total RNA probed with an internal 363-base-pair (bp) *SalI* fragment of the *regA* gene (Fig. 1B) revealed two distinct phases of transcript accumulation (Fig. 2A). The early phase of transcription occurred between 2.0 and 4.5 h after subculture (OD_{540}, 0.1 to 0.6). A consistent low point in transcript accumulation appeared between 5.5 and 7.0 h after subculture (OD_{540}, 1.0 to 2.0, late exponential phase). After this point the *regA* transcript began to accumulate again, but only if the cells were growing under low-iron conditions. Cells grown in high-iron medium showed a decrease in the accumulation of the initial *regA* transcript. In addition, the second phase of transcript accumulation was undetectable.

A similar pattern of transcript accumulation (Fig. 2B) was seen when the same total RNA preparations were examined with a 1,530-bp *BamHI* internal *toxA* gene probe (Fig. 1A). The early phase of *toxA* transcription peaked between 4.0 and 5.25 h (OD_{540}, 0.3 to 1.0) and declined to a low level as the cells reached late exponential phase (OD_{540}, 2.0). The second phase of *toxA* transcription began if the cells were growing in a low-iron environment. Under these conditions, PA103(pUC181.8) rapidly accumulated *toxA* mRNA until the second peak was reached between 8 and 10 h after subculture. In contrast, if cells were grown in a high-iron medium, low amounts of the early *toxA* mRNA were detected, followed by suppression of the late phase of transcript accumulation.

Analysis of exotoxin A activity. ADP-ribosyltransferase activity was measured to determine the pattern of *toxA* mRNA translation. When low-iron-grown cell supernatants were examined, detectable transferase activity began at 5.0 h (OD_{540}, 2.0) and continued to rise throughout the late exponential and stationary phases. Extracellular exotoxin A product did not accumulate when the cells were grown in high-iron medium (Fig. 3A).

Cells grown in low-iron medium and examined for cell-associated exotoxin A activity showed a biphasic pattern of expression. An early activity peak appeared at the 6.0-h time point. A decline in exotoxin A activity occurred in the next time points taken, h 7.0 and 9.0, followed by an eventual rise which paralleled the rise observed in culture supernatant activity. Transferase measured in lysates of cells grown in high iron showed that cell-associated exotoxin A also failed to accumulate under these growth conditions. Examination of cell lysates by Western blot (immunoblot) analysis with anti-exotoxin A antibody revealed the same pattern of early and late synthesis (data not shown). No change in the molecular weight of the exotoxin A was observed in the Western blot of the cell lysate samples throughout the growth cycle.

When the data for expression of *toxA* mRNA and exotoxin A activity were compared in the same plot, we noted that the initial phase of transcription correlated with the initial peak of cell-associated exotoxin A (Fig. 4A) but was not associated with a peak of extracellular exotoxin A (Fig. 4B). The initial *toxA* mRNA peak slightly preceded the cell-associated exotoxin A peak with respect to time. The second phase of *toxA* transcription correlated with the expression of extracellular exotoxin A (Fig. 4B) and preceded a second peak of cell-associated exotoxin A (Fig. 4A). These results were obtained with exotoxin A samples preincubated in urea-DTT

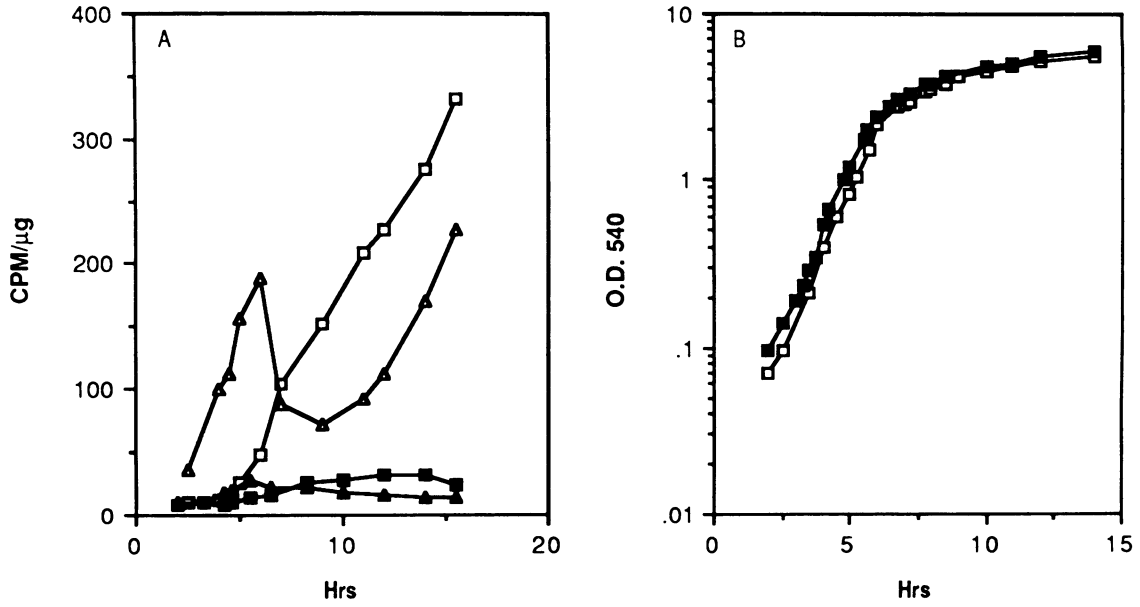

FIG. 3. ADP-ribosyltransferase activity in cell lysates and supernatants of PA103(pUC181.8) (A). Cells were grown in low- or high-iron medium under identical culture conditions and sample volumes as in RNA extraction experiments. ADP-ribosyltransferase activity was measured in counts per minute of [^{14}C]ADP-ribose incorporated into TCA-precipitable material and standardized to the amount of protein in each preparation. Symbols: □, exotoxin A activity from culture supernatants (extracellular) of PA103(pUC181.8) grown in low-iron medium; ■, exotoxin A activity from culture supernatants of cells growing in high-iron medium; △, cell-associated exotoxin A activity of cells grown in low iron; ▲, cell-associated exotoxin A activity of cells grown in high-iron medium. (B) Optical density measurements of PA103(pUC181.8) cells grown in low-iron (□) or high-iron (■) medium.

prior to assay. Different results were obtained if exotoxin A activity was quantitated without prior incubation with urea-DTT. Measurement of ADP-ribosyltransferase activity without activation indicated that lysates contained a constant amount of this form of exotoxin A throughout the time course. The average value was 47.06 cpm/μg, with a range of 40.59 to 51.39 cpm/μg. Culture supernatants never had significant transferase activity unless they were preactivated by the urea-DTT treatment.

Size distribution of *regA* and *toxA* mRNAs. Examination of *toxA* mRNA on glyoxal gels revealed that the exotoxin A mRNA consisted of one band which migrated in the 2,000-bp area of the gel (Fig. 5A). This value is supported by previous S1 mapping determinations of the *toxA* mRNA length (5, 11). Detectable changes in the size of the *toxA* mRNA did not occur over the time course of the experiment (Fig. 5A).

The size of the *regA* mRNA proved to be more difficult to determine on either glyoxal or formaldehyde gels. However,

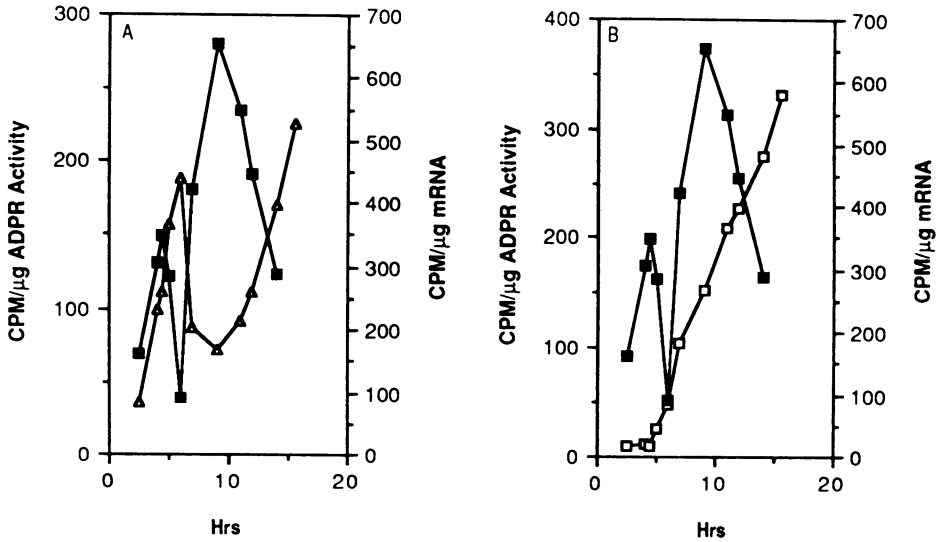

FIG. 4. Correlation of *toxA* transcription and translation. *toxA* transcriptional data (Fig. 2B) were examined for correlation to cell-associated exotoxin A activity (Fig. 4A) or extracellular exotoxin A activity (Fig. 4B). All values represent data obtained from cells grown in low-iron medium. ADP-ribosyltransferase (ADPR) activities are the same data as reported in Fig. 3A. Symbols: △, cell-associated transferase activity; □, extracellular transferase activity; ■, *toxA* mRNA measured with the *Bam*HI internal probe.

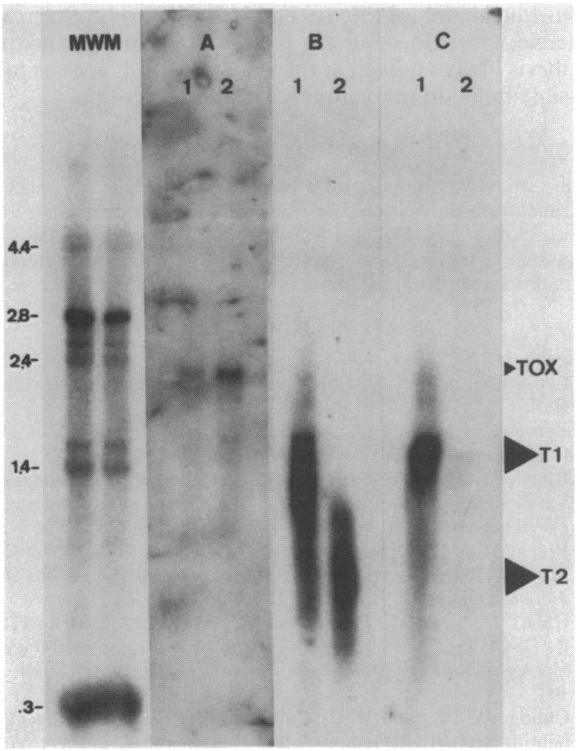

FIG. 5. Northern blot analysis of early and late *regA* and *toxA* mRNAs. Total RNA from low-iron cultures harvested at an OD_{540} of 0.3 (lanes 1) or 4.0 (lanes 2) was denatured with glyoxal and dimethyl sulfoxide. The samples (10 μg per lane) were separated on a 1.2% agarose gel and blotted onto nitrocellulose paper. (A) Probed with the internal *Bam*HI fragment of *toxA*; (B) probed with the internal *Sal*I fragment of *regA*; (C) probed with the upstream *Ava*I fragment of the *regA* gene. Molecular weight markers (MWM) consisted of 3 μg of an RNA ladder probed with lambda DNA *Hin*dIII fragments. Molecular weights are reported in kilobases.

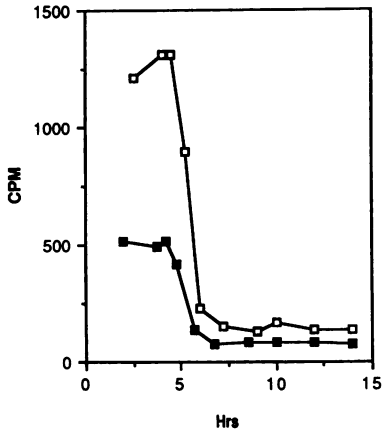

FIG. 6. *regA* mRNA synthesis analyzed with an upstream probe. The total RNA preparations used in Fig. 2A and B were probed with an upstream *Ava*I fragment of *regA*. This fragment lies upstream from the previously mapped start site for *regA* transcription (37). Symbols: □, RNA isolated from PA103(pUC181.8) cells grown in low-iron medium; ■, RNA isolated from cells grown in high-iron medium.

at various time points two size classes of transcripts were detected with an internal *Sal*I probe (Fig. 5B). The larger transcript (1,200 to 1,500 bp) was more prominent in the early RNA samples, whereas a smaller transcript (700 to 800 bp) became more abundant at later time points (Fig. 5B). These results, coupled with the biphasic distribution in the amount of *regA* mRNA, *toxA* mRNA, and exotoxin A, suggested that at least two functional *regA* mRNAs were expressed.

We used an upstream *Ava*I fragment from cloned *regA* sequences (−489 to −40 bp relative to the ATG start codon) to analyze whether the different classes of *regA* transcripts contained material which hybridized to this region. Northern (RNA) blot analysis revealed that the *Ava*I probe hybridized to the larger transcript also recognized by the internal *Sal*I probe (Fig. 5C). The intensity of the *Ava*I probe hybridization to the larger mRNA species was more pronounced than that observed with the *Sal*I probe. However, the smaller transcript produced at late time points was not recognized by the *Ava*I probe (Fig. 5C). We concluded that the *Ava*I probe would be useful in the analysis of the larger *regA* transcript during the growth of PA103(pUC181.8).

RNA dot blot analysis with the *Ava*I upstream probe indicated that we were able to detect the class of larger *regA* mRNA only in samples taken before an OD_{540} of 2.0 (2.0 to 6.0 h) (Fig. 6). The accumulation of this larger transcript recognized by the *Ava*I probe followed a pattern identical to that of the initial phase of transcript accumulation detected with the internal *Sal*I fragment. This initial transcript was present in the first few time points but its accumulation fell off abruptly. The time period during which the larger transcript began to disappear was correlated with the stage of cell growth rather than the presence or absence of iron in the medium. Cells grown in high-iron medium exhibited a reduced amount of the larger transcript throughout the period during which it was expressed. The pattern of transcript accumulation from cells grown in high-iron medium detected with the *Ava*I upstream probe matched that detected with the internal *Sal*I probe. The second phase of *regA* transcription demonstrated with the *Sal*I internal probe was not detectable with the *Ava*I upstream fragment. We concluded that the second phase of *regA* transcription must have an independent start site outside of the *Ava*I probe.

DISCUSSION

Our results indicate that at least two *regA* mRNAs, T1 and T2, are expressed from the chromosome of PA103 cells grown in low-iron medium. Dot blot hybridization studies determined that two phases of transcript accumulation occurred during the growth cycle. Glyoxal gel analysis revealed that each phase of *regA* transcript accumulation correlated with a distinct size class of *regA* mRNA. In addition, we found that an *Ava*I probe, located upstream of a previously mapped transcriptional start site (37), recognized only the larger, early transcript (T1). This indicates that the T1 start site is independent of the T2 start site and lies upstream of that point. The smaller, late transcript T2 probably originates near the start site located by Wozniak and Galloway (37). Our data do not rule out the possibility that the T2 transcript is a processing product of the T1 transcript. However, if T1 were processed to T2, the transcript accumulation pattern generated by the *Sal*I internal DNA probe would not show the extremely low point consistently observed between 5.5 and 7.0 h.

Differences in the iron regulation and temporal expression of the two *regA* transcripts provide additional evidence for

independent start sites, which suggests that each transcript may be under the control of a separate promoter region, P1 and P2. T1 transcript accumulation is expressed only during exponential phase and is partially reduced when cells are grown in high iron. T2 transcript accumulation is detectable during late exponential to early stationary phase and is not detectable in cells grown in high iron. Several models for differential control by postulated inducer or repressor molecules or differential degradation of mRNA molecules could account for our observations. We are in the process of separating and analyzing each promoter region.

The T1 transcript was detectable by using either an upstream *Ava*I fragment or a *Sal*I DNA fragment which lies within the coding region of the *regA* gene. This indicates that the T1 transcript reads through the *regA* structural gene to produce a functional *regA* mRNA. Further evidence that indicates that the T1 transcript results in functional RegA protein includes the observation of cell-associated exotoxin A activity early in the cell cycle. The T2 transcript correlates with the late phase of *toxA* transcription. Comparisons of the transcript accumulation pattern of T1 and T2 with the early and late phases of *toxA* transcript accumulation show unexpected differences. T1 accumulation occurs over a short period of the cell cycle but appears to be greater in intensity than T2 accumulation. The opposite pattern of transcript accumulation appears with *toxA*, where a small initial peak is followed by a larger, more prolonged phase. Differential degradation of the T1 and T2 transcripts could account for the observed pattern. Alternatively, translation of the longer T1 mRNA may be less efficient due to a more complicated RNA secondary structure. In this case the more efficient translation of the shorter T2 mRNA could result in relatively more RegA protein during the second phase of *toxA* transcription. Since specific probes for the RegA protein are unavailable, we cannot eliminate the possibility that slightly different proteins are made from the two transcripts, resulting in their difference in specific activity.

In an attempt to correlate *toxA* transcription with the expression of exotoxin A, we noted that the early and late transcripts of *toxA* resulted in two different cellular locations of the exotoxin A. Transcription of *toxA* mRNA early in the cell cycle resulted only in cell-associated exotoxin A. Part of this activity could be attributed to a precursor form of exotoxin A described by Lory et al. (21). This precursor form was found to be associated with the outer membrane and possessed ADP-ribosyltransferase activity without prior exposure to urea-DTT (21). Transcription of *toxA* at later times resulted in a reduction of cell-associated transferase activity and a concomitant rise of extracellular activity. The pattern of the second phase of exotoxin A synthesis may represent the activation of the cotranslation excretion pathway described previously (21). Our detection of cell-associated exotoxin A during early culture times may indicate that the synthesis of additional factors is needed for this coupled translation-excretion pathway to function. The rise of cell-associated exotoxin A during the last four time points may represent a breakdown or a saturation of that pathway during the stationary phase.

Several regulatory systems exist for single genes or an operon where multiple or tandem promoters are controlled differently. The determination of which promoter is used can depend on a variety of environmental stimuli, including heat shock (10), ppGpp (18), UV light (31), cyclic AMP-CRP (1), nitrogen limitation (30), and iron limitation (7, 13). Our results indicate that the *regA* gene may provide a model for the examination of multiple promoter control in *P. aeruginosa*. Elucidation of the mechanism of control may provide valuable information on the factors that affect exotoxin A synthesis in vivo as well as provide important clues as to the possible function of exotoxin A in the bacterium.

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