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Differential Regulation by Iron of *regA* and *toxA* Transcript Accumulation in *Pseudomonas aeruginosa*

DARA W. FRANK,† DOUGLAS G. STOREY,‡ MICHAEL S. HINDAHL,§ AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642

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Iron regulation of *toxA* and *regA* transcript accumulation was examined in *Pseudomonas aeruginosa* PA103 containing the *regA* gene on a multicopy plasmid. The patterns of transcript accumulation for *toxA* and *regA* were found to be positively correlated. Dot blot and Northern (RNA) blot analysis of total RNA isolated throughout the bacterial growth cycle indicated that multiple copies of the *regA* gene uncoupled iron repression of the first phase of transcript accumulation for both *regA* and *toxA* genes. However, regulation by iron of the second phase of transcript accumulation for each gene was unaffected by several *regA* gene copies. Total toxin production was increased in cells with multiple copies of *regA* grown in either low- or high-iron medium. Primer extension analysis of *regA* mRNA extracted from cells grown in high- and low-iron medium and examined at different points in the cell growth cycle supported the hypothesis that iron regulation of *regA* transcription occurs at the level of transcriptional initiation. Two start sites were shown for *regA* transcription at −164 and −75 base pairs from the ATG start codon. The differential regulation of *regA* transcript accumulation when *regA* is present in single or multiple copy and the mapping of independent start sites for *regA* mRNA support the evidence that *regA* transcription is directed by independently regulated promoter regions.

Initial studies on the accumulation patterns of *Pseudomonas aeruginosa regA* and *toxA* transcripts indicated that maximal *toxA* transcription depended on the expression of the positive regulatory gene for exotoxin A synthesis, *regA* (8). Evidence to support this hypothesis includes the tight coupling of *regA* and *toxA* transcript accumulation patterns throughout the bacterial growth cycle when RNA is extracted from cells grown for optimal expression of exotoxin A (8). A similar pattern of iron-repressed transcript accumulation was observed for both genes when *P. aeruginosa* cells were grown in high-iron medium, for maximal inhibition of exotoxin A production (8, 15). In studies in which the temporal nature of *toxA* transcription and translation was examined relative to the expression of *regA* transcripts, it appeared that *regA* transcription occurred before *toxA* transcription, especially in early parts of the cell growth cycle (8). In addition, the *regA* mutant strain PA103-29 expressed neither *regA* nor *toxA* transcripts in detectable amounts when RNA was extracted at optimal points for the expression of each transcript (D. Frank and B. Iglewski, unpublished observations). Therefore, alteration of *regA* transcription, whether through mutation (PA103-29) or through iron inhibition, similarly affected the ability of *toxA* transcripts to accumulate. For these reasons, it appeared that in order to understand the regulation of exotoxin A production, an understanding of the regulation of *regA* transcription was necessary.

Two transcripts have been characterized for the *regA* gene, T1 and T2, from the hypertoxigenic *P. aeruginosa* strain PA103 (8). This strain has been shown to contain one chromosomal copy each of the *regA* and *toxA* genes (13, 32). Production of both the *regA* T1 and T2 transcripts correlated with the transcription and translation of *toxA*, an observation which indicated that each *regA* transcript was functional and was affecting *toxA* transcription (8). Differential hybridization patterns with a *regA* upstream probe in dot blot analyses suggested that T1 was expressed exclusively in the early phases of cell growth. T2 accumulation appeared later in the growth cycle. Northern (RNA) blots of RNA samples taken at early and late phases of growth determined that the T1 transcript was significantly larger than the T2 transcript. Differences in transcript size could indicate that T2 was a processing product of T1 or that T1 and T2 had different sites of initiation. The pattern of iron inhibition of T1 and T2 seemed to favor the hypothesis of different start sites, since T1 accumulation was inhibited while T2 accumulation seemed not to initiate when cells were cultivated in high-iron medium. Thus, two independently regulated phases of *regA* transcription appeared to account for the pattern of *regA* and *toxA* expression (8).

The direct correlation of *regA* transcription and *toxA* transcription-translation made it possible to use the production of exotoxin A as a tool to examine the iron regulation of *regA* transcription-translation. Gene dosage experiments have determined that a different pattern of iron regulation of exotoxin A production occurs when multiple copies of the *regA* gene are present in strain PA103 (15). Previous studies have shown that *toxA* expression was inhibited 90 to 95% when PA103 cells (containing a single chromosomal copy of *regA* and *toxA*) were grown in high-iron medium (3). This pattern of iron inhibition did not change when the copy number of the *toxA* structural gene was increased (9, 15). However, when the copy number of the *regA* gene was increased, the inhibition level for exotoxin A production dropped to a range of 40 to 70%, suggesting that the

* Corresponding author.

† Present address: Department of Microbiology, The Medical College of Wisconsin, Milwaukee, WI 53226.

‡ Present address: Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada T2N 4N1.

§ Present address: Central Research, Pfizer Incorporated, Groton, CT 06340.

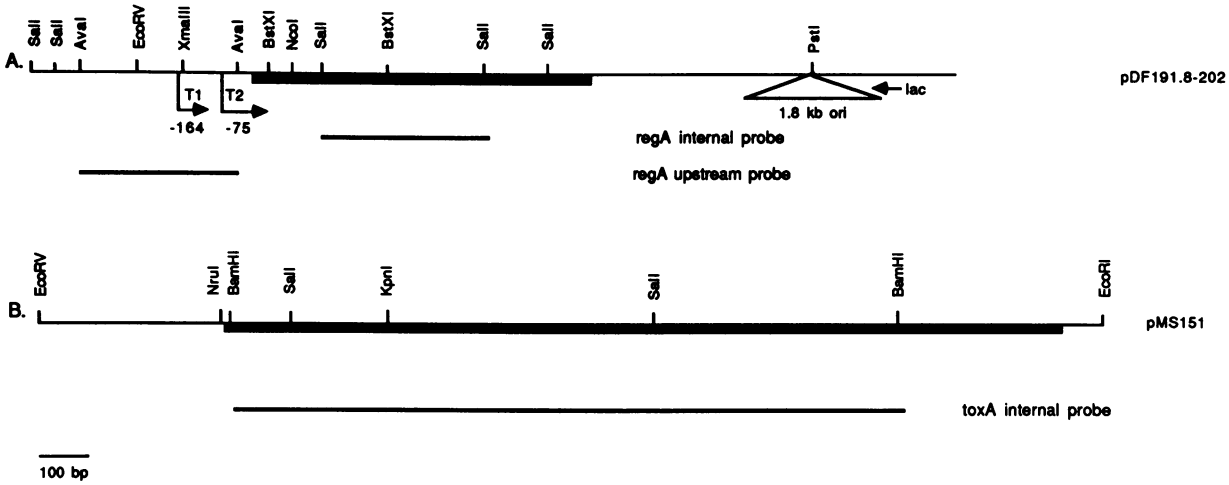

FIG. 1. DNA probes used to quantitate transcripts for the *regA* (A) and *toxA* (B) genes. Each gene is drawn to scale, with relevant restriction endonuclease sites shown. Thin black boxes represent the open reading frames as defined by sequence analysis of the *regA* gene (14; this study) and the *toxA* gene (11). The fragments used as probes for transcript accumulation studies are shown below each map. Two fragments were used to analyze *regA* mRNA expression, a 449-bp upstream *Ava*I (−489 to −40 bp) and a 363-bp internal *Sal*I (157 to 520 bp) fragment. Transcriptional start sites for the *regA* gene are denoted by rightward arrows (this study). A 1,530-bp internal *Bam*HI fragment was used to examine *toxA*-specific transcripts.

mechanism of iron inhibition involves more than the simple sequestration of repressor molecules by a larger number of *regA* upstream regions (13, 15).

In this study, we examined the iron regulation of *toxA* and *regA* transcript accumulation when *regA* was present in multiple copies in strain PA103. In addition, we show by primer extension analysis that *regA* transcripts initiate from two different start sites.

MATERIALS AND METHODS

Bacterial strains and plasmids. The hypertoxigenic *P. aeruginosa* strain PA103 (18), which has one chromosomal copy each of the *regA* gene (13) and *toxA* gene (32), was used as a host in these experiments. PA103 was transformed with plasmid DNA by the $MgCl_2$ technique outlined by Olsen et al. (27). Vector plasmids consisted of pUC18 and pUC19 which contained a 1.8-kilobase (kb) *Pst*I fragment from pRO1614 (27) inserted into the *Pst*I site of the multiple cloning region. Insertion of the *Pst*I fragment allows maintenance of pUC plasmids in *P. aeruginosa* and *Escherichia coli* (6, 8).

The clone of *regA* used in these experiments was a 1.9-kb fragment subcloned and sequenced in this laboratory (14, 15). The 1.9-kb *Xho*I-*Pst*I *regA* fragment was cloned in both pUC18 and pUC19 *Sal*I-*Pst*I sites, resulting in plasmids pDF18-202 and pDF19-202. The addition of the 1.8-kb *Pst*I fragment from pRO1614 into the single *Pst*I site of plasmids pDF18-202 and pDF19-202 enabled these *regA* constructs, pDF181.8-202 and pDF191.8-202, respectively, to be present at 13 to 14 copies in *P. aeruginosa*. To determine the contribution of the *lac* promoter or other vector sequences to the synthesis of *regA*, a 449-base-pair (bp) *Ava*I fragment containing both transcriptional start sites was deleted from each construction. Note that the *regA* ATG and Shine-Dalgarno sequences were maintained in these deletion derivatives. Each deletion was transformed into PA103-29, a *regA* mutant strain (26), and assayed for the ability to complement the *regA* mutation. When the *lac* promoter was located downstream of the *regA* gene (pDF191.8-202 *Ava*I deletion), PA103-29 was unable to produce exotoxin A. In contrast, when the *lac* promoter was located upstream of the *regA* gene (pDF181.8-202 *Ava*I deletion), PA103-29 was able to produce high levels of extracellular exotoxin A. These results indicated that the *lac* promoter was able to initiate *regA* mRNA synthesis in the absence of the *regA* start sites and presumed promoter regions. To eliminate possible readthrough of the *regA* gene by the *lac* promoter, we used a construction of *regA*, pDF191.8-202, in which the *lac* promoter region of the pUC vector was located downstream of the *regA* coding region (Fig. 1A).

Culture conditions. PA103(pDF191.8-202) cells were grown for 14 to 16 h at 32°C in Trypticase soy broth (BBL Microbiology Systems) dialysate containing a low (0.05 μg of Fe^{2+} per ml) or a high (10.0 μg of Fe^{2+} per ml) iron concentration (26). Carbenicillin (Sigma Chemical Co.) at a concentration of 400 μg/ml was included in all cultures to ensure plasmid maintenance. Cells grown overnight in either low- or high-iron medium were subcultured in fresh low- or high-iron medium at a starting OD_{540} of 0.02 (8). Samples, consisting of approximately 2×10^{10} cells, were removed and processed for total RNA or exotoxin A (8).

Isolation of total RNA. Total RNA was isolated at different points during the growth cycle by a hot phenol extraction method (33). The RNA samples were treated with proteinase K (Sigma Chemical Co.) as described previously (8). After proteinase K treatment and further extraction and precipitation, the RNA pellet was exposed to RNase-free DNase (Worthington Biochemicals) (33). DNase was removed by phenol and chloroform extractions. Total RNA was precipitated with 2.2 volumes of absolute ethanol, washed, suspended in sterile distilled water, and quantitated spectrophotometrically by A_{260} measurements with a Beckman DU50 spectrophotometer.

Isolation and labeling of DNA probes. Transcript accumulation for the *regA* gene was monitored with two DNA probes, a 449-bp upstream *Ava*I fragment (−489 to −40 relative to the ATG start codon of *regA*) and an internal *Sal*I

fragment (157 to 520 bp) (Fig. 1A) (14). *toxA* transcript accumulation was examined with a single internal 1,530-bp *Bam*HI probe obtained from plasmid pMS151 (Fig. 1B) (11, 19). Fragments were obtained by restriction endonuclease cleavage of cloned genes and extracted from agarose gel slices as described previously (1). Restriction enzymes were used according to the manufacturer's recommendations (Bethesda Research Laboratories, Inc.). The purified DNA fragments were labeled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.) with a primer extension kit purchased from Pharmacia.

RNA analysis. Dot blot and glyoxal gel analyses of total RNA samples were performed as described previously (8). For dot blots, 5 μg of RNA per 4-mm-diameter well was denatured with formaldehyde (34) and bound to nitrocellulose sheets with a Schleicher & Schuell Minifold apparatus. For glyoxal gel separation of RNA size classes, 10 μg of total RNA per lane was denatured with glyoxal and dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) and electrophoresed in 1.2% phosphate-buffered agarose gels as described before (21). After transfer to nitrocellulose paper, RNA was fixed by being baked for 2 to 3 h in vacuo at 80°C.

Nitrocellulose blots were hybridized with 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 a Beckman LS1801 liquid scintillation counter as described previously (8).

Primer extension mapping of the *regA* mRNA. The oligonucleotide (5'-TCTGTCGCAGTCATAAGTGAT-3') homologous to a region that spanned the AUG start site of the *regA* mRNA (see Fig. 5) was labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) by using T4 polynucleotide kinase (IBI) (5, 7). The labeled primer was purified by Sephadex G-25 column chromatography. Primer (1.5×10^6 cpm) was annealed to one of four total cellular RNA preparations. RNA was extracted, as detailed above, from strain PA103(pDF191.8-202) grown in low-iron medium to an OD_{540} of 0.18, strain PA103(pDF191.8-202) grown in high-iron medium to an OD_{540} of 0.18, strain PA103(pDF191.8-202) grown in low-iron medium to an OD_{540} of 4.0, and strain PA103(pDF191.8-202) grown in high-iron medium to an OD_{540} of 4.0. After annealing, the reaction mix was incubated at 42°C for 30 min in the presence of 100 μM of each of the deoxynucleoside triphosphates, dactinomycin (8 μg/ml), and 15 U of reverse transcriptase (Life Sciences) (31). The nucleic acids were precipitated with ethanol, suspended in formamide buffer, heated to 95°C, and electrophoresed on an 8% polyacrylamide gel. Sizes of the primer-extended transcripts were determined from DNA sequencing ladders run in parallel to the primer extension reactions. The DNA sequencing ladders were generated from DNA complementary to the promoter regions. Size determinations were also carried out for other primer extension reactions and other cDNA ladders. In all cases, the size estimations of the primer-extended transcripts were within ±2 bases of the sizes shown in Fig. 4.

Direct sequencing of the *regA* mRNA was carried out in the same manner as the primer extension with the following modifications. The annealed primer-mRNA was divided into four different reaction mixes. Each mix contained 100 μM of the four deoxynucleoside triphosphates and one of the dideoxynucleoside triphosphates (30).

DNA sequencing of the upstream regions of the *regA* gene. Subclones from pDF18-202 containing the sequences upstream of the *regA* gene were inserted into M13 vectors. Single-stranded template DNA was prepared by the procedures of Messing (22). DNA sequencing was done by the dideoxynucleotide chain termination method of Sanger et al. (29) with the Sequenase Kit supplied by United States Biochemical.

Analysis of exotoxin A activity. ADP-ribosyltransferase activity for each sample point was determined for culture supernatant and cell lysate material as described previously (8). All samples were standardized to contain approximately 2×10^{10} cells per point. Because exotoxin A production was enhanced with multiple copies of the *regA* gene present, each sample was assayed at several dilutions to ensure that the exotoxin A activity fell within the linear range of the ADP-ribosyltransferase assay system. Protein content was measured by the method of Lowry et al. (20). Data for exotoxin A activity were expressed as counts per minute per microgram of protein.

RESULTS

Dot blot analysis of *regA* transcript accumulation. The *regA* gene in multiple copies has been shown to have a significant effect on the iron regulation of exotoxin A production (13, 15). To determine whether *regA* or *toxA* transcript accumulation patterns were altered when the *regA* gene dosage was increased, we constructed a PA103 strain [PA103(pDF191.8-202)] in which the *regA* gene was represented on a multicopy plasmid. Figure 2A shows a growth curve analysis of this strain cultivated in low- or high-iron medium. Multiple copies of the *regA* gene did not seem to alter the general growth properties of the cells. Total RNA was isolated from PA103(pDF191.8-202) grown under low- or high-iron conditions at defined points in the bacterial growth cycle (8). Previous studies had indicated that the first phase of chromosomally encoded *regA* transcript accumulation, which resulted in the production of the T1 transcript, could be selectively studied with an *Ava*I upstream fragment from the *regA* gene (8) (Fig. 1A). Primer extension studies to map the transcriptional initiation sites (see below) determined that the *Ava*I probe overlapped the T2 transcriptional start site by only 40 bp. T2 mRNA was undetectable with the *Ava*I probe when *regA* was present in single copy (8). Since overall *regA* multiple-copy RNA dot blot patterns generated by using the *Ava*I probe matched previous single *regA* copy patterns, we concluded that although the upstream *Ava*I probe was not absolutely specific for T1, it was operationally useful in T1 transcript accumulation studies. When the *Ava*I probe was used in RNA dot blot analyses to examine T1 accumulation from PA103(pDF191.8-202) cells grown in low-iron medium, high levels of T1 *regA* mRNA were detected from the initial time points (Fig. 2B). T1 accumulation fell abruptly until 5.25 h (OD_{540}, 1.1), when an additional peak appeared (Fig. 2B). After this point, the accumulation of T1 generally continued to decline until the end of the growth cycle. When cells were cultivated in high-iron medium, the pattern of *regA* T1 transcript accumulation remained essentially unchanged. This observation contrasts sharply with the inhibitory effect of iron on T1 accumulation when *regA* is represented by a single chromosomal copy (8).

Both phases of *regA* transcript accumulation could be detected with an internal *Sal*I probe (Fig. 1A) (8). When the *Sal*I probe was used to simultaneously monitor T1 and T2 transcripts from PA103(pDF191.8-202) cells grown in low-iron medium, high levels of early transcript were detected beginning with the first time point (Fig. 2C). These levels dropped in the next two time points but rose to form another

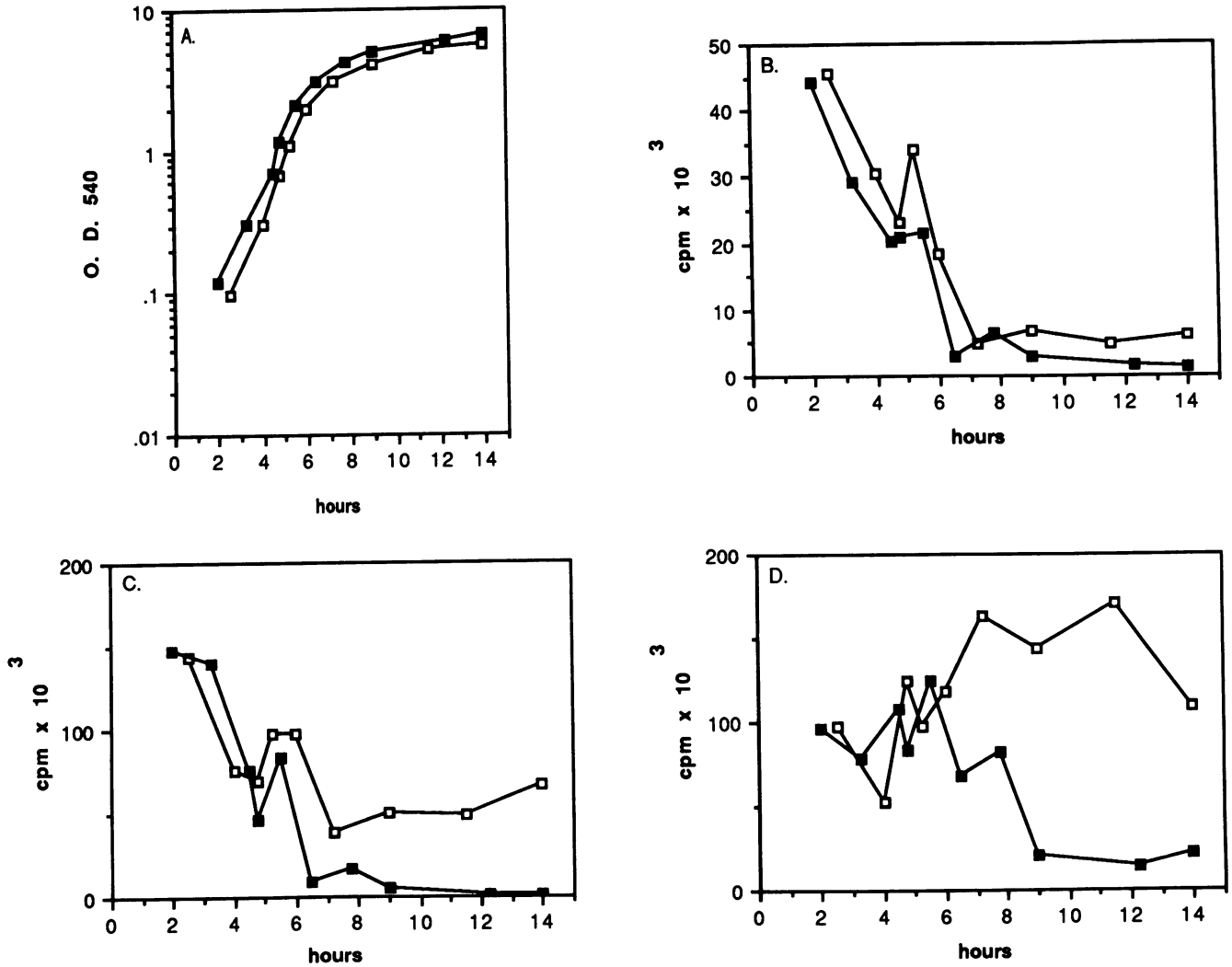

FIG. 2. *regA* and *toxA* mRNA accumulation from cells with multiple copies of the *regA* gene. (A) Growth curve of PA103(pDF191.8-202) cells cultivated in medium containing a low (□) or high (■) iron concentration. Total RNA isolated from PA103(pDF191.8-202) cells at the points shown in panel A were dot blotted onto nitrocellulose and probed with the upstream *Aval* probe for *regA* (B), and internal *Sal*I probe for *regA* (C), or the internal *Bam*HI probe for *toxA* (D) transcripts.

peak in the 5.25- and 6-h samples (OD_{540}, 1.1 to 2.0). After the 7-h time point, late *regA* transcript accumulation appeared to remain constant. No difference was observed between the *regA* mRNA detected from cells grown in low- or high-iron medium before the 6.0-h time point. After the 6.0-h time point, however, RNA from cells grown in high-iron medium failed to accumulate any late transcript (Fig. 2C). Thus, the second phase of *regA* transcript accumulation did not occur after growth in high-iron medium even though *regA* was present in multiple copies in this strain.

Dot blot analysis of *toxA* transcript accumulation. The same pattern of an iron-deregulated initial phase of transcript accumulation followed by an iron-regulated phase was obtained when the RNA samples were examined with the *toxA* probe (Fig. 1B). Multiple copies of the *regA* gene allowed detection of high levels of *toxA* transcript early in the growth cycle whether cells were grown in low-iron or high-iron medium (Fig. 2D). As the cells approached late-log–early-stationary phase (6 to 7 h), *toxA* transcripts continued to accumulate only if the cells were cultivated in low-iron medium. The second phase of *toxA* transcript accumulation was severely inhibited when cells were grown in high-iron medium (Fig. 2D).

Northern blot analysis of *toxA* and *regA* mRNA throughout the bacterial growth cycle. The size distribution of *toxA* mRNA was examined over the bacterial growth cycle by Northern blot analysis after glyoxal gel electrophoresis (Fig. 3A and B). *toxA* mRNA consisted of a single band which migrated in an area of the gel which corresponded to approximately 2,000 bp. The size distribution of *toxA* mRNA was independent of growth conditions or phase of bacterial growth cycle. The pattern of *toxA* transcript accumulation as assessed in autoradiograms of Northern blots reproduced the pattern generated by quantitative dot blot analysis. RNA from cells grown in low-iron medium showed a *toxA* mRNA band in the first sample whose intensity appeared to remain constant or to increase slightly between 2.5 and 6.0 h (Fig. 3A, lanes 1 to 5). The intensity of the *toxA* band increased substantially after the 6-h time point when cells were grown in low-iron medium (Fig. 3A, lanes 5 to 9). The opposite pattern was apparent when RNA from PA103(pDF191.8-202) cells grown in high-iron medium was examined (Fig. 3B).

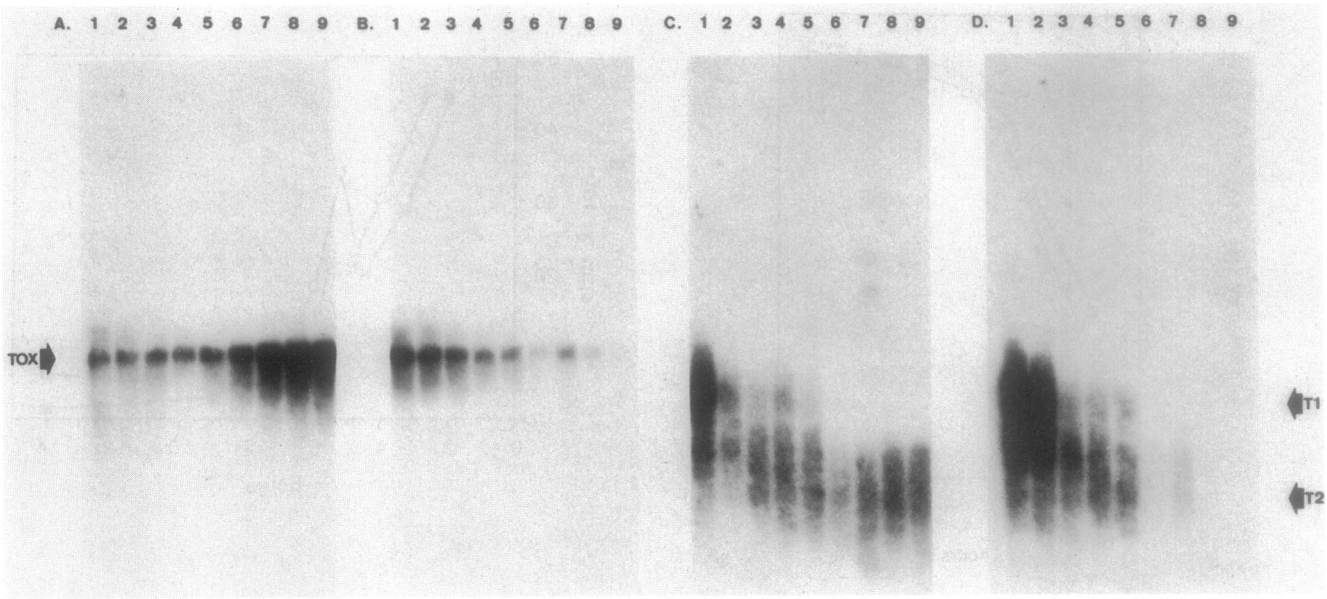

FIG. 3. Northern blot analysis of *toxA* and *regA* mRNA throughout the bacterial growth cycle. Total RNA isolated from cells grown in low- or high-iron medium was denatured with glyoxal and dimethyl sulfoxide. Each lane was standardized to contain 10 μg of total RNA per well. Panels A and C are duplicate RNA samples from cells grown in low-iron medium. Lanes 1 to 9 correspond to samples taken at 2.5, 4.0, 4.75, 5.25, 6.0, 7.25, 9.0, 11.5, and 14.0 h, respectively. Panels B and D also represent duplicate RNA samples, but RNA was extracted from cells grown under high-iron conditions. Lanes 1 to 9 correspond to samples taken at 2.0, 3.25, 4.5, 4.75, 5.5, 6.5, 7.75, 9.0, and 12.25 h, respectively. Panels A and B were probed with the internal *Bam*HI fragment for detection of *toxA*-specific mRNA. Panels C and D were probed with the internal *Sal*I probe to detect *regA* T1 and T2 transcripts. For estimating the size of mRNA species, each gel contained separate lanes loaded with 3 μg of a 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories). After blotting, these lanes were probed separately with ^{32}P-labeled *Hin*dIII-digested bacteriophage lambda DNA.

The most intense signal for *toxA* hybridization occurred in the initial five time points (Fig. 3B, lanes 1 to 5), followed by a marked decline in the signal in the subsequent four samples (Fig. 3B, lanes 6 to 9). It should be noted that the RNA samples did not appear to have been degraded, as no lower-molecular-weight products of *toxA* mRNA were detected at any phase of cell growth.

Identical RNA samples were electrophoresed on a duplicate glyoxal gel and examined for T1 and T2 *regA* mRNA by probing the Northern blot with the *Sal*I internal DNA probe (Fig. 3C and D). Even though the *regA* gene was present in multicopies and the dot blot analysis indicated that *regA* mRNA was overproduced compared with the normal single-copy gene state, it was difficult to assign molecular weights to the *regA* T1 and T2 transcripts. Samples from the first time point (2.5 h) analyzed from cells grown in low-iron medium contained the most intense area of hybridization for the T1 transcript (Fig. 3C, lane 1). The approximate size of the transcript was consistent with the previously reported figure of between 1,200 and 1,500 bp (8). Faint hybridization to the T1 transcript could be seen in the next four time points (4.0 to 6.0 h, Fig. 3C, lanes 2 to 5); however, a smaller smear between the T1 and T2 areas was also detectable. The signal from the 7.25-h sample represented a low point of hybridization (Fig. 3C, lane 6), followed by an increase in the hybridization in the T2 area of the gel (600 to 800 bp) for the last three samples.

When RNA was examined from cells grown in high-iron medium, the T1 transcript showed a strong signal in the initial 2.0- and 3.25-h time points (Fig. 3D, lanes 1 and 2), followed by very weak hybridization in the next three time points (Fig. 3D, lanes 3 to 5). After the 5.5-h time point, virtually no signal was detected with the *Sal*I probe (Fig. 3D, lanes 5 to 9). These results suggested that the T2 transcript was not being expressed in cells grown in high-iron medium after 5.5 h.

Primer extension mapping of the start site for *regA* transcription. Northern blot analysis techniques were useful in

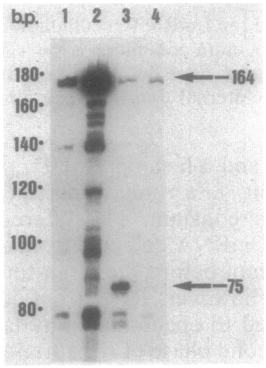

FIG. 4. Primer extension mapping analysis of the *regA* transcriptional start sites. Total RNA was isolated from PA103(pDF191.8-202) cells at an early point in the growth cycle (OD_{540}, 0.18) (lanes 1 and 2) or late in the growth cycle (OD_{540}, 4.0) (lanes 3 and 4). In addition, lanes 1 and 3 represent early and late RNA samples extracted from cells grown in low-iron medium; lanes 2 and 4 represent early and late samples from cells grown in high-iron medium. RNA samples were hybridized with the labeled oligonucleotide probe (5′-TCTGTCGCAGTCATAAGTGAT-3′) and extended with reverse transcriptase. Major stop sites are shown to the right and refer to the number of base pairs from the ATG start codon of *regA*. Numbers on the left, corresponding to transcript size, were generated from a DNA sequencing ladder run in parallel to the primer extension reactions.

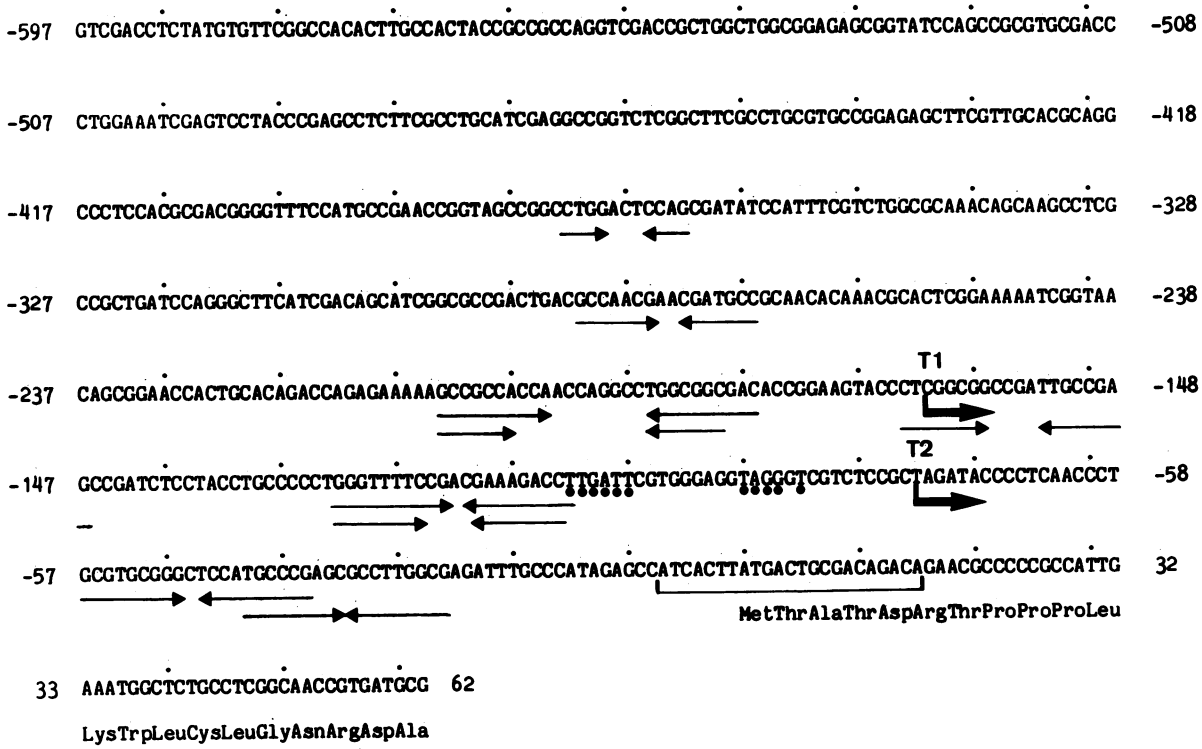

FIG. 5. DNA sequence of the upstream flanking regions of the *regA* gene. Bold arrows at positions −75 and −164 denote the 5′ ends of the T2 and T1 *regA* mRNAs as determined by primer extension analysis. Underlined sequence denotes the primer used for mapping the mRNA initiation sites. Dots under the sequence indicate sequence homology to the −10 and −35 boxes of diphtheria toxin promoter (16, 17). Arrows under the sequence indicate perfect and imperfect areas of dyad symmetry located in the vicinity of the *regA* transcriptional start sites.

determining the approximate size range of mRNA species at a given time, the relative integrity of the mRNA preparation, and the general change in size classes over the time period of the experiment. Although two general size classes of *regA* mRNA, T1 and T2 (8; this study), could be identified by this technique, several other size species of *regA* mRNA were represented in the broad zones of hybridization (Fig. 3C and D). Since Northern blot analysis cannot discern degradation products and initiation sites, we first chose to specifically explore the initiation of *regA* transcription by S1 analysis. Attempts to map start sites by S1 analysis yielded general areas of initiation (D. Storey and B. H. Iglewski, unpublished observations). To accurately determine the location of transcriptional starts and to test our hypothesis that the initiation of transcription of *regA* T1 was independent of the initiation of *regA* T2, we subjected RNA isolated at early and late time points from strain PA103(pDF191.8-202) to primer extension analysis. The pattern of initiation was also examined by including RNA samples from cells grown in either low- or high-iron medium. Reverse transcription of the *regA* mRNA with a specific primer revealed two transcriptional start sites (Fig. 4). RNA extracted early in the low-iron growth cycle (OD_{540}, 0.18) showed only one strong stop at position −164 ±2 bases (Fig. 4, lane 1). Two faint bands located at −67 and −128 were also observed (Fig. 4, lane 1). These bands varied in intensity depending on the primer extension reaction conditions used, but were located in strings of four or five C residues, respectively. Presumably, the bands are caused by the reverse transcriptase pausing at areas of strong secondary structure in the *regA* mRNA. This phenomenon may also account for the numerous faint bands seen in Fig. 4, lane 2.

In cells grown in high-iron medium and harvested early in the growth cycle (Fig. 4, lane 2), the *regA* mRNA also had a start site located at −164 from the ATG start codon. The mapping of this start site predominantly for early RNA samples regardless of the iron growth conditions reinforced earlier S1 data and indicated that this start site represented the beginning of the T1 transcript. The T1 initiation site was also detected in RNA from cells grown to an OD_{540} of 4.0 (Fig. 4, lanes 3 and 4). Again, the presence or absence of iron in the medium did not effect initiation of transcription from this site.

The strongest stop position in RNA samples from cells harvested late in the growth cycle was located at position −75 (Fig. 4, lane 3). In contrast to the pattern of T1 initiation, this site could not be mapped in RNA samples from cells grown in high-iron medium (Fig. 4, lane 4). The rigid iron control of transcriptional initiation as well as the predominance of this site in late RNA samples indicated that the −75 position corresponded to the start of T2.

The labeled primer was used to sequence the *regA* mRNA in order to confirm that the primer was specific for the *regA* message. We showed by direct sequencing of the *regA* mRNA that the 5′ end of the T2 transcript occurred at the thymidine residue at position −75 (Fig. 5). The exact sequence of the 5′ end of the T1 transcript could not be determined when dideoxynucleotides were incorporated into the primer extension reaction mixes. However, direct sequencing from the mRNA confirmed that the T1 transcript contained sequences upstream of the T2 start site. A DNA sequence analysis of the upstream region of the *regA* gene was done to provide information for synthesizing additional primers to examine the possibility that other initiation sites

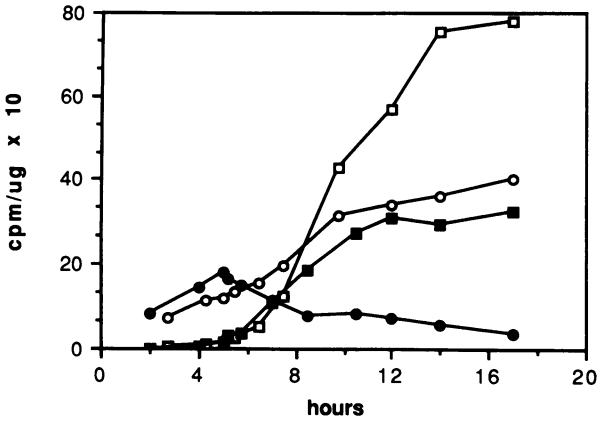

FIG. 6. ADP-ribosyltransferase activity in cell lysates and supernatants of PA103(pDF191.8-202). Cells were cultivated in low- or high-iron medium under identical conditions and sample volumes as for RNA extraction experiments. ADP-ribosyltransferase activity was measured in counts per minute of [^{14}C]ADP-ribose incorporated into trichloroacetic acid-precipitable material and standardized to the amount of protein in each preparation. Open symbols represent exotoxin A activity from cells grown in low-iron medium, and solid symbols indicate activity measured from cells grown in high-iron medium. Activity measured in lysates (cell-associated exotoxin A) is shown by circles, and activity in supernatants (excreted exotoxin A) is shown by squares.

might be present. Several primers that lay upstream of the T1 and T2 sites of initiation were tested. None of these primers were extended with reverse transcriptase, suggesting that in the 600-bp region of the *regA* upstream sequence examined, T1 and T2 were the only detectable initiation sites (Fig. 5).

Analysis of exotoxin A activity. Exotoxin A activity was measured in cell lysate and supernatant samples of PA103(pDF191.8-202) cells grown in low- or high-iron medium to determine the pattern of *toxA* translation throughout the bacterial growth cycle. Multiple copies of the *regA* gene allowed the detection of ADP-ribosyltransferase activity in the initial lysate samples of cells grown in either low- or high-iron medium (Fig. 6). Cells grown in low-iron medium continued to accumulate cell-associated toxin throughout the experiment. Growth in high-iron medium allowed the first phase of exotoxin A synthesis to occur, however, after the 5-h time point, ADP-ribosyltransferase activity declined in subsequent cell lysate samples. Thus, analysis of cell-associated exotoxin A activity reflected the *toxA* and *regA* transcriptional analysis. The first phase of production was found to be iron deregulated, while the second phase of production was iron repressed.

Exotoxin A activity began to accumulate in the supernatant of cells grown in both low- or high-iron medium after the 5-h time point. The production of exotoxin A appeared to be similar in the low- or high-iron cultures until 7 h, after which the rate of exotoxin A accumulation in high-iron supernatants slowed. The end points, assayed at 14 and 17 h, showed 58 and 61% inhibition of exotoxin A production, respectively, when activity was compared in supernatants from cells grown in low- or high-iron medium.

DISCUSSION

The sensing of iron concentrations in the environment by bacterial cells can serve as a stimulus to regulate gene expression (2, 17, 25). In the case of exotoxin A production by *P. aeruginosa*, several investigators have shown that the addition of iron to growth medium severely reduces exotoxin A production at the *toxA* transcriptional level (6, 9, 10, 15, 19). Additional copies of the *toxA* structural gene did not relieve iron inhibition of exotoxin A production (9, 15). These results suggested that the reduction of *toxA* transcription by the action of a repressor protein which binds upstream of *toxA* was unlikely (15). We have further shown that the iron-dependent reduction of *toxA* transcription correlated with the reduction of transcription of the positive regulatory gene *regA* (8, 15). In this case, increasing the gene dosage of the *regA* gene affected the iron regulation of exotoxin A synthesis. These results indicated that the focus of iron regulation of exotoxin A production was at the level of *regA* expression.

To determine whether multiple copies of the *regA* gene disrupted the iron regulation of transcription of either the *toxA* or *regA* gene, we analyzed transcript accumulation patterns throughout the bacterial growth cycle in a strain of *P. aeruginosa* transformed with a *regA* construction on a multicopy plasmid. With the same techniques, previous studies had established that both phases of *regA* transcript accumulation were regulated by growth in high-iron medium when *regA* was encoded on a single chromosomal copy (8). The early phase of transcript accumulation, which resulted in the production of the large T1 *regA* transcript, was reduced. The second, later phase, which resulted in the synthesis of the smaller *regA* transcript, T2, did not initiate under high-iron growth conditions.

Comparison of cultures with multiple *regA* copies grown in both low- or high-iron medium also revealed a two-phase pattern. Dot blot hybridization with the T1 probe showed that the iron content of the growth medium no longer affected the accumulation of T1. Utilization of the *regA SalI* probe, which detects both T1 and T2 transcripts, confirmed that T1 accumulation is deregulated with respect to iron. The T2 transcript, however, remains fully regulated in the presence of multiple copies of *regA* and growth in high-iron medium. T2 accumulation reproduces the same pattern reported previously for a single *regA* gene copy. This late transcript does not initiate unless the cells are entering late log or early stationary phase and are growing in low-iron medium regardless of the copy number of the *regA* gene. Thus, multiple copies of the *regA* gene result in two independently regulated phases of transcript accumulation. The primary or early phase (T1 transcript) is no longer reduced by growth in high-iron medium. The secondary or late phase (T2 transcript) is only detectable after growth in low-iron medium.

An iron-deregulated primary phase followed by an iron-regulated secondary phase was shown when both *toxA* transcript accumulation and cell-associated exotoxin A activity were examined. The iron regulation of extracellular exotoxin A, however, appeared incomplete. These results suggest that the exotoxin A activity measured in supernatants from cells grown in high iron is due to the early phase of *toxA* transcription-translation allowed by the presence of multiple copies of the *regA* gene. Our data indicate that once *toxA* transcripts are synthesized, they are translated, and exotoxin A is properly excreted to the medium even though the cells are growing in a high-iron environment. In addition, the timing of excretion of exotoxin A appears to be identical whether cells are grown in low- or high-iron medium. As shown in Fig. 6, exotoxin A excretion began between 5.0 and 5.5 h (OD_{540}, 0.7 to 1.0) regardless of the iron content of the

medium. The accumulation of exotoxin A activity in supernatants diverged only after the rate of *toxA* transcript accumulation dropped in high-iron cultures. These observations contribute to the notion that the iron-dependent regulation of exotoxin A synthesis lies at the transcriptional level and may not affect the translation or secretion of the *toxA* product.

Comparison of transcript accumulation patterns indicated that the high amount of *regA* mRNA detected early in the growth cycle correlates to relatively low quantities of *toxA* mRNA. Conversely, late in the growth cycle, low quantities of *regA* mRNA result in relatively high levels of *toxA* transcript. This pattern is not the result of high copy number or overproduction of RegA, since the same general pattern was apparent in studies of transcript accumulation when *regA* and *toxA* were expressed from the chromosome of PA103 (8).

Several hypotheses could be formulated to account for these observations. RegA may be interacting with an additional regulatory factor which then has a direct positive effect on *toxA* transcription. In this situation, *regA* transcription would still correlate with *toxA* transcription, but the absolute level of each mRNA would not have to be identical. The T1 transcript for *regA* includes additional upstream sequences which may form secondary structures which inhibit *regA* translation. Thus, although the total accumulation of transcripts appear to be high, the overall rate or efficiency of *regA* translation is low early in the growth cycle. As cells enter the late log and early stationary phase, the switch to the P2 promoter, which is closer to the translational start, may eliminate the inhibitory sequence and increase the rate or efficiency of *regA* translation. Higher concentrations of RegA protein would result in the increase of *toxA* transcription late in the growth cycle. A major theme in several regulatory systems (*NRI-NRII*, *envZ-ompR*, *cheA-cheB*) includes the transduction of an environmental signal from a sensor or modulator to an effector or regulator molecule (24). The transmission of information takes the form of a covalent modification (i.e., phosphorylation and dephosphorylation) of the effector by the modulator (24). Differences in the specific activity of RegA may be connected to covalent modification of the protein. The modification of RegA would depend on other regulatory molecules whose expression or activity may also be temporally regulated. Finally, using probes which hybridize to downstream regions of the *regA* clone, we found that the *regA* mRNA extends past the translational stop codon (data not shown). Additional gene products which are cotranscribed with *regA* may serve to modify the functional properties of the RegA protein or other proteins which may ultimately be involved in *toxA* transcription.

Northern and dot blot analyses of *regA* mRNA in both single (8) and multiple copies indicated that two transcripts were produced from the *regA* gene. In addition, these results indicated that the two transcripts may have unique 5′ ends in strain PA103. However, Wozniak et al., using RNA extracted from PA103-29 containing multiple copies of the *regA* gene, were able to map only a single *regA* start site with S1 nuclease (35). To clarify this discrepancy, we mapped the 5′ ends of the *regA* mRNAs and determined the pattern of transcript initiation by primer extension analysis of RNA isolated at different points in the growth cycle. The *regA* RNA was also analyzed from cells grown in either low- or high-iron medium. T1 transcript initiation occurred at base −164, the majority of which was detected early in the growth cycle, and was not regulated by iron (Fig. 4). The T2 transcript initiated at position −75, occurred late in the growth cycle, and was tightly regulated by iron (Fig. 4). These patterns of initiation are consistent with the accumulation patterns revealed by RNA dot blot and Northern blot analyses. Our analysis reflects the relative rates of synthesis and degradation of RNA, and since these parameters may vary with the growth cycle, these data cannot eliminate the possibility that *regA* T1 is processed to *regA* T2 during growth. The processing of T1 to T2, however, appears unlikely, since one would predict that in a strain with multiple copies of *regA*, in which T1 accumulation has been shown to be deregulated with respect to iron, T2 accumulation would also be deregulated. We have shown that T2 accumulation is fully regulated by growth in high-iron medium even though the *regA* gene is present in multiple copies. The differential regulation of T1 and T2 accumulation from PA103(pDF191.8-202) cells grown in high iron and the mapping of separate transcription start sites support our initial hypothesis that *regA* transcription is controlled by separately regulated promoter regions.

Mapping the T1 transcriptional initiation site was difficult due to the lability of the transcript, its transient expression, and the high G+C content of the mRNA. Our attempts to map the T1 5′ end with S1 nuclease yielded only the general area where the T1 start site might reside, since the length of the transcripts depended on the time of sampling. When primer extension analysis was used to map the 5′ end of the T1 transcript, a number of nonspecific bands were consistently observed (Fig. 4, lanes 1 and 2). However, in all cases the band at position −164 was always the strongest and the other bands varied in intensity (D. Storey and B. H. Iglewski, unpublished observation). It is likely that the faint bands seen in Fig. 4 represent pauses or terminations of the reverse transcriptase due to either strong secondary structure or the high G+C content of the sequence.

Although the patterns of T1 transcript accumulation and initiation essentially match, it must be noted that primer extension analysis detected T1 start sites late in the growth cycle (OD_{540}, 4.0) in both low- or high-iron cultures. These results suggest that a basal level of T1 may be expressed throughout the growth cycle. Higher levels of T1 transcript might then represent an induced state that occurs during early log phase only. Alternatively, degradation of the T1 RNA from the 3′ end, late in the growth cycle, may leave some molecules with intact 5′ ends. In this case, full-length *regA* transcripts would not appear on Northern blots but start sites might be detectable. T1 degradation products may account for the extensive smearing of the *regA* mRNA seen on Northern blots throughout the time course. Thus, the appearance of T1 start sites late in the cell cycle may be indirectly related to the overproduction and incomplete degradation of the transcript in a cell with multiple copies of the *regA* gene.

Analysis of the sequence upstream of each start site reveals little homology between the two regions corresponding to the proposed P1 and P2 promoters (Fig. 5). The lack of homology is not surprising, because a consensus sequence for *Pseudomonas* promoters has yet to be defined. Furthermore, the initiation of transcription is differentially regulated, so one might expect dissimilar promoter sequences. A comparison of the sequences upstream of the T1 start site with the highly conserved *E. coli* −10 and −35 boxes also revealed little homology (12). It is interesting that a region 24 bases upstream of the T2 start site (TTGATT) has limited homology to the −35 box of *E. coli* (TTGACA) (12). This same *Pseudomonas* sequence (TTGATT) has perfect homol-

ogy with the −35 region of the iron-regulated diphtheria toxin promoter (Fig. 5) (16, 17). In addition, we noticed a −10 region upstream of the T2 start site (TAGGGT) which is homologous to the putative −10 box of the diphtheria toxin promoter (TAGGAT) (4, 16, 17). Since both the *regA* P2 and the diphtheria *tox* gene promoters are tightly iron regulated, one might speculate on a common mechanism of regulation. Miller et al. (23) have shown that the sequence TTTTGAT is required in three to eight direct repetitions for *Vibrio cholerae toxR*-mediated activation of *ctx* transcription. It is curious that within the *ctx* tandem repeats of TTTTGAT, the sequence TTGATT, found in the putative P2 promoter of *regA*, would also repeat three to eight times in the *ctx* upstream region. Further work is needed to determine the significance of this observation.

Bacterial operator sequences frequently show twofold symmetry in the vicinity of a promoter (28). Accordingly, we examined the *regA* sequences in the vicinity of the two promoters. A number of dyad repeats are located in the upstream regions of the *regA* gene. Of note are two sites of nested dyad repeats which lie within 50 bp of the T1 and T2 start sites. Potentially, both of these regions could serve as operators for their respective promoters. Work is under way to determine the relevance of these regions.

The differential regulation of *regA* and *toxA* during the bacterial growth cycle may underscore the importance of expression of exotoxin A at specific times and under specific environmental conditions. Further study of the mechanism of RegA action on *toxA* transcription as well as the regulation of *regA* production, modification, and/or degradation may be necessary to clarify the roles of these products in the survival of *P. aeruginosa* in the compromised human host.

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