Detection of *pur* Operon-attenuated mRNA and Accumulated Degradation Intermediates in *Bacillus subtilis*

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Transcription of the *Bacillus subtilis* *pur* operon encodes the 10 enzymes required for de novo synthesis of IMP (1). Transcription of the operon is initiated from a σ43-dependent promoter 242 nucleotides upstream of the first structural gene, *purE*, and terminates approximately 38 nucleotides downstream of *purD*, the last structural gene, thus yielding a polycistronic mRNA leader region. We have identified an apparently intact, terminated transcript of ~200 nucleotides in length, having a half-life of about 0.7 min. The terminated transcript is degraded in a series of discrete steps resulting in the accumulation of stable intermediates in vivo. We have used Northern blot analysis, primer extension, and nuclease S1 mapping to align the degradation intermediates with the nucleotide sequence and assign secondary structures that may contribute to the stability of the intermediates. Degradation is initiated by endonucleolytic cleavage of the ~200-nucleotide leader transcript generating ~93- and ~97-nucleotide 5' and 3' moieties, respectively. The ~93-nucleotide 5' and ~97-nucleotide 3' intermediates are further degraded to ~88 and ~58 nucleotides, respectively. The 5'-end of *pur* mRNA and the attenuated transcript are degraded by different pathways.

The *Bacillus subtilis* *pur* operon encodes the 10 enzymes required for de novo synthesis of IMP (1). Transcription of the operon is initiated from a σ43-dependent promoter 242 nucleotides upstream of the first structural gene, *purE*, and terminates approximately 38 nucleotides downstream of *purD*, the last structural gene, thus yielding a polycistronic mRNA of approximately 13,080 nucleotides. Initial experiments have indicated independent regulation of transcription by the intracellular pool of adenine and guanine nucleotides. Adenine nucleotides regulate transcription initiation, while guanine nucleotides regulate transcription by an attenuation mechanism in the 5'-untranslated leader region. By computer analysis a number of plausible secondary structures were found to accumulate in vivo and several degradation products of the attenuated mRNA were found to accumulate in vivo. Some of these RNA molecules accumulate to an estimated steady state level up to 40-fold greater than the intact attenuated mRNA. They are apparently protected from rapid degradation by secondary structures that form in the attenuated transcript. We propose a model which relates RNA stability to RNA secondary structure and incorporates a series of steps to account for the degradation of the *pur* operon-attenuated mRNA in *B. subtilis*.

**MATERIALS AND METHODS**

**Strains and Media** — *B. subtilis* strains DE1 (prototrophic revertant of 168 *rpC2* (1)) and KK1 were used for RNA isolation. In strain KK1 the *pur* operon promoter, leader region and part of the first structural gene from nucleotides ~193 to +303 (+1 is the start of transcription, as shown in Fig. 1) have been replaced by the chromosone by the chloramphenicol acetyltransferase gene from pC194 (3). Cells were grown in minimal medium (1) supplemented with purines or purine nucleosides as noted. Strain KK1 required 10 μg/ml adenine for growth due to deletion of the *pur* operon promoter and a portion of the *purE* gene.

**Isolation of RNA** — Cells were grown in 50 ml of medium supplemented with purine nucleosides at 200 μg/ml for repressing conditions. At a Klett reading of 70, using a 66 filter, the cells were poured onto ice chilled to ~20 °C and collected by centrifugation for 4 min at 3000 × g at 4 °C. The cell pellet was then resuspended in ice-cold TE (10 mM Tris, pH 7.6, 0.1 mM EDTA), and RNA was isolated by hot phenol extraction as described (4). The final RNA pellet was dissolved in H2O and aliquots frozen at ~70 °C. The yield was approximately 200 μg of RNA. A second method employing rapid cell harvest was used to confirm steady state RNA levels. Cells were grown as described above, and 10-ml samples were harvested quickly by filtration onto 2.4-cm glass fiber filters. Filters containing harvested cells were immediately placed into phenol solution (1.0 ml of phenol, 1.3 ml of phenol-chloroform, 2 ml of 0.25 M NaCl, 5 mM EDTA, 63 mM Tris, pH 7.5, 0.1% sodium dodecyl sulfate) at 75 °C. Cell contents were washed from the filters by gentle shaking for 15 min at 75 °C. The solution was then shaken vigorously for 5 min at 75 °C, and RNA was isolated as described above. A yield of approximately 40 μg of RNA was obtained from 10 ml of cell culture. The A260/A230 was 2.0-2.2.

**Nuclease S1 Mapping** — Nuclease S1 mapping was carried out as described (5). Single-stranded DNA probes complementary to the RNA were synthesized by primer extension of oligonucleotides 1 and 7 (Table I) using 1 μg of M13 template DNA (1). The [α-32P]dCTP-labeled probes were isolated from 5% polyacrylamide gels by electroelution and then ethanol precipitated. The probe (10-15 ng) was dissolved in water and mixed with 25 μg of *B. subtilis* RNA. This mixture was precipitated with ethanol, dried briefly, and then dis-
solved in hybridization buffer. Each 350-μl reaction contained 500 units of nuclelease S1 and was incubated at 22°C for 1 h. Digestion products were precipitated with 1.0 ml of ethanol, resuspended in 0.3 M NaOAc, pH 8.0, 0.1 mM EDTA and precipitated with ethanol a second time. After washing with 75% ethanol, the pellet was resuspended in 10 μl of H2O and 10 μl of gel-loading buffer (80% formamide, 1 mM EDTA, 0.1% dyes) was added. The samples were heated at 90°C for 3 min, and 5 μl was loaded on an 8% polyacrylamide–sequencing gel. Dideoxy nucleotide sequencing (6) ladders were used as size standards.

**Primer Extension Mapping**—Primer extension was carried out using Moloney Murine Leukemia Virus reverse transcriptase. A 25-μM nucleotide probe was annealed to 0.1 pmol of template DNA, and the mixture was heated at 90°C for 5 min, cooled at 65°C for 5 min, and then placed at room temperature for the following additions: 10 μl of 5X buffer, (5 × buffer = 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 50 mM dithiothreitol, 15 mM MgCl2, dNTPs to 0.5 mM, bovine serum albumin to 100 μg/ml, 20 units of RNasin (Promega Biotec) and 800-1600 units of reverse transcriptase. The 50-μl mixture was incubated for 1 h at 37°C. Alternatively, 800 units of reverse transcriptase were added, and after 30 min another addition of 800 units was made. Primer-extended products were ethanol precipitated, rinsed with 75% ethanol, and resuspended in 10 μl of H2O and 10 μl of gel-loading buffer. Samples were heated for 3 min at 90°C, and 5 μl was loaded on an 8% polyacrylamide–sequencing gel. Dideoxy nucleotide–sequencing ladders were used as size standards.

**Northern Blot Analysis**—Total cellular RNA (25 μg) was fractionated by electrophoresis on an 8% polyacrylamide gel (38:2, acrylamide/bis) containing 8 M urea. After electrophoresis the gel was soaked in transfer buffer (12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA, pH 7.5), for 1 h and then electroblotted to a nylon membrane (Hybond-N, Amersham Corp.) for 4 h at 20 V. Prehybridization was at 42°C in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.6% NaPPi, 1× Denhardt's reagent (0.02% Ficoll, 0.02% polyvinylpyrrodilene, 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate, and 0.1 mg/ml sonicated calf thymus DNA for at least 6 h. Hybridization was in 6× SSC, 0.05% NaPPi, 1× Denhardt's reagent, 20 μg/ml Escherichia coli RNA, and 100 ng of 32P 5'-end-labeled 30-mer oligonucleotide probe for at least 12 h. Membranes were hybridized at 42°C unless otherwise indicated. Membranes were washed in 6× SSC, 0.05% NaPPi at room temperature for 1 h, and then at 42°C for 1 h. Alternatively, 3 M tetramethylammonium chloride was used for low and high stringency washing conditions (7). Membranes were washed twice in 6× SSC, 0.05% NaPPi in TMA buffer (5 M tetramethylammonium chloride, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0), once at room temperature for 30 min in TMA buffer containing 0.2% sodium dodecyl sulfate and finally for 7 min in TMA buffer containing 0.2% sodium dodecyl sulfate at 45°C for low stringency, or 65°C for high stringency. Membranes were then rinsed in room temperature for 10 min in TMA buffer. Autoradiographs were exposed at −70°C with an intensifier screen. Exposure times ranged from 6 to 24 h. Densitometry of X-ray films was used to estimate the relative levels of RNA species.

In some cases membranes were stripped and rehybridized. The first hybridization and autoradiography of a membrane with Probe 1 is shown in Fig. 3, lane 2. The probe was removed by treatment with TMA buffer plus 0.2% sodium dodecyl sulfate at 85°C for 20 min. Autoradiography of the washed membrane for 30 h verified that no probe remained. Hybridization was performed successively with Probe 3 (not shown), and with Probe 6, shown in Fig. 3, lane 11. Similar results were obtained for at least five cycles of hybridization and stripping.

32P-labeled, single-stranded DNA preparations synthesized in vitro were used for size standards for Northern blot analysis. DNA was obtained by extending universal primer on an M13 template. After extension of template M13mp19-H-1.4, of known sequence (1), products were cut with restriction enzymes to generate the following fragments with length given in nucleotides: BamHI, 39; HindIII, 53; HindIII, 69; PvuII, 94 and 165. With template M13mp19-HC, containing a HindII to Clal site, (−31 to +97) pur operon fragment in the HindII site of M13mp19, digestion with PvuII and SphI generated sizes of 195, 185, and 94 nucleotides.

**Measurement of Attenuated Transcript Half-life**—For RNA half-life measurements, 200-ml cultures of strain DE1 were grown in log phase, Klett 70, 190 μg/ml rifampicin was added to inhibit transcription initiation (8). Immediately, a 10-ml sample of cells was collected by filtration onto a 2.4-cm glass fiber filter and RNA isolated as described under Isolation of RNA. Times for cell harvest were measured from the time of rifampicin addition to the time of submersion of the glass fiber filter in the hot phenol solution. The time points were 0.6, 1.4, 2.6, 5.0, 10, and 20 min.

RNA from each time point (17 μg) was loaded on each of two 8% polyacrylamide 8 M urea gels. After electrophoresis the RNA was electroblotted to nylon membranes as described above. One membrane was hybridized with Probe 1, the other with Probe 6 (Table I). Autoradiographs were used as templates to excise membrane slices corresponding to hybridized RNA bands. Radioactivity was quantitated by liquid scintillation counting. Background radioactivity was obtained from slices of equal dimensions excised from various parts of the membrane.

**Calculation of Corrected Half-life**—For a series of first order reactions A→B→C, where λA and λB are the rate constants, the half-life, TH, is proportional to the rate constant λ = ln2 / TH. If a semi-log plot of concentration versus time is used to measure the half-life of B or C, the half-life will be overestimated. This is due to an increased concentration of B resulting from the decay of A. The conversion of A to B makes the rate of disappearance of B slower than it would be in the absence of A. The Bateman equation for radioactive decay (9) can be used to correct for this effect and determine the true half-life. The equation is:

\[
N_B = C_A e^{-\lambda_A t} + C_B e^{-\lambda_B t} + N_{B0} e^{-\theta t},
\]

where

\[
C_A = \frac{\lambda_A}{\lambda_B - \lambda_A} N_B
\]

and

\[
C_B = \frac{\lambda_B}{\lambda_B - \lambda_A} N_B
\]

and

\[
N_B = \text{amount of } A \text{ at } t = 0
\]

\[
N_B = \text{amount of } B \text{ at } t = 0
\]

\[
N_B = \text{amount of } B \text{ at time } t.
\]

Solving the equation for λA gives the corrected half-life for B. The half-life of each degradation intermediate was corrected for the decay of the next larger precursor.

**RESULTS**

**Detection of the Attenuated Transcript and Its Accumulated Degradation Intermediates**—The nucleotide sequence of the *B. subtilis pur* operon promoter–leader region is shown in Fig. 1. Transcription that initiates mRNA synthesis at adenine residue +1 can either continue into the structural genes or may prematurely terminate between base pairs 195–205. We have used Northern blot analysis to detect the *in vitro*, apparently full length, attenuated mRNA transcript and several degradation intermediates. The results of the Northern blot analysis are summarized in Fig. 2. RNA was obtained from repressed cells grown in medium containing 200 μg/ml of guanosine. RNA from cells grown in the absence of purine nucleosides or in media containing adenosine show much lower levels of these truncated species, presumably due to the lower amount of the attenuated mRNA transcript produced under these conditions. The hybridization probes shown schematically in Fig. 2 are described in Table I. Fig. 2 provides a schematic representation of the transcripts detected by each of the probes, as well as an estimate of the abundance of each degradation intermediate relative to the initial attenuated mRNA. Each of the hybridization probes detected the ~200-nucleotide–attenuated transcript as well as discrete degradation intermediates. Evidence supporting the assignments...
Probes 3 detected the ~200-nucleotide-attenuated mRNA and the 97-nucleotide degradation intermediate when hybridization was conducted at room temperature and was followed by washing at either high or low stringency (Fig. 3, lane 4). However, with the standard hybridization at 42°C, an additional RNA species of ~86 nucleotides was detected (Fig. 3, lane 3). Hybridization of Probe 3 to the ~86-nucleotide RNA was independent of washing stringency. Probe 3 did not detect the ~58-nucleotide RNA even with low stringency washing.

Since the ~86-nucleotide RNA hybridized to Probe 3 only at elevated temperature and was not detected by any other probe at either temperature, it was necessary to verify that this species was derived from the attenuated pur operon mRNA. The RNA blots in Fig. 3, lanes 5–8, show that accumulation of the ~86-nucleotide RNA was dependent upon guanine nucleotide-mediated attenuation (compare lanes 5 and 7). Furthermore, accumulation was repressed by adenine nucleotides (Fig. 3, lane 6). Finally, this RNA species, as well as the ~200- and ~97-nucleotide molecules, was not produced in a pur operon promoter deletion strain (Fig. 3, lane 8).

Because of the unusual hybridization properties of the ~86-nucleotide RNA, and difficulties in assigning likely 5'- and 3'-ends (see below), the termini are shown by dashed lines in Fig. 2.

Probes 4–6 each hybridized to the ~200-nucleotide transcript and to an ~93-nucleotide degradation product (Fig. 3, lanes 9–11). RNA blots for Probes 5 and 6 were identical and only that for Probe 6 is shown. Probes 5 and 6 also hybridized to an ~88-nucleotide RNA degradation intermediate as shown for Probe 6 in Fig. 3, lane 11.

The hybridization of Probe 4 to the ~93-nucleotide RNA was dependent upon the washing temperature. The stringency of washing was examined using 3.0 M tetramethylammonium chloride which eliminates the differential melting of A·T and G·C base pairs and allows the melting of DNA-DNA hybrids to be controlled solely as a function of hybridized probe length (10). Although we cannot directly relate the melting temperature curves for RNA-DNA hybrids with DNA-DNA hybrids, we have assumed that the melting temperature for RNA-DNA hybrids is independent of base composition and is likewise solely a function of the hybridized probe length. Hybridization of Probe 4 to the ~93-nucleotide RNA was maintained after washing at 45°C (Fig. 3, lane 9), but the probe was dissociated by washing at 65°C (Fig. 3, lane 10). In contrast hybridization of Probe 4 to the ~200-nucleotide-attenuated transcript was retained at 65°C (Fig. 3, lane 10). Thus, the ~93-nucleotide RNA-Probe 4 duplex is less than 30 base pairs. We have estimated this duplex to be between 10 and 20 base pairs, based on the melting curve for DNA-DNA hybrids in 3.0 M tetramethylammonium chloride (7) and the fact that RNA-DNA hybrids are more stable than DNA-DNA hybrids (10). The 3'-end assignment of the ~93-nucleotide RNA at nucleotide 93 (considered below) would provide a 13-base pair duplex with Probe 4 (Fig. 2).

Probes 7 and 8, listed in Table I, are not shown in Fig. 2. Probe 7 anneals within the coding region of purE, at a position downstream of the 3'-end of the attenuated transcript. There was no hybridization of Probe 7 with small RNA species. Attenuation reduces the level of purE mRNA, and this RNA is too large to enter the gel that was used. Probe 8 matches a segment upstream of the start of transcription and did not detect any RNA species.

### Steady State RNA Levels

**Steady State RNA Levels** — Steady state levels of the degradation intermediates were estimated by densitometry from the Northern blots shown in Fig. 3. Levels of these RNA species relative to the attenuated transcript are tabulated in...
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Table I
Oligonucleotide probes

| No. | Nucleotide position* | Sequence 5'→3' |
|-----|----------------------|----------------|
| 1   | 195-166              | CCGGCTCTGCGATTGCAGTTATGAGGAAGCA |
| 2   | 174-147              | GAGGAAGCAATACAAACCAGGGCATGTTACT |
| 3   | 150-121              | GGTACATGTATCGTCTCGGTCTTCG     |
| 4   | 110-81               | ATGTCAGTTTATCGATCTTCTGCCAT    |
| 5   | 80-51                | AGTCCGGCAATTACAGTTGGCAGTGAA   |
| 6   | 44-15                | GGGCCATATTCCCAAGATATTAGCTCG    |
| 7   | 275-246              | GCTTCCCATGATTTCTACCAGGCGTCG   |
| 8   | -22 to -51           | GGATAATGCACGCATATTATCGAAGATA  |

*Numbering used is that of Fig. 1.

Fig. 2 and Table III. All of the degradation intermediates accumulated to levels exceeding that of the ~200-nucleotide-attenuated transcript, with maximal accumulation of the ~93- and ~88-nucleotide species.

These steady state levels were confirmed using an alternative method. Cells were harvested by rapid filtration and counted for radioactivity. The average relative abundance of species 200:97:88 estimated by the three different methods was 1:4:12:19:43, respectively.

Determination of 5' Ends by Primer Extension Mapping—Northern blot analysis provides information on the approximate size and origin of the intermediates in the degradation of the pur operon-attenuated RNA. Primer extension mapping of B. subtilis RNA was used to determine the 5' termini of the RNA species detected by Northern blot analysis. From this information we deduced the position of the likely 3'-end for each fragment (Table II).

The 5'-end of the attenuated transcript was mapped by primer extension using Probes 1, 2, and 5. The largest cDNA using Probe 1 was 192–195 nucleotides in length (Fig. 4, lane 3), corresponding to RNA 5'-ends at positions +1 to 3. With Probe 2, a cDNA of 172 nucleotides was obtained (Fig. 4, lane 4). This cDNA corresponds to an RNA 5'-end at nucleotide 3. Primer extension of the ~88- and ~93-nucleotide intermediates with Probe 5 yielded a single cDNA of 77 nucleotides, corresponding to RNA transcripts with 5'-ends at nucleotide 4 (Fig. 4, lane 10). Collectively, primer extension mapping with Probes 1, 2, and 5 therefore provides evidence that places the 5'-end of the attenuated transcript as well as the ~93- and ~88-nucleotide degradation intermediates between nucleotides +1 and 4 (Table II). These experiments thus confirm the previous nuclease S1 mapping of the termi-
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Fig. 4. Primer extension mapping of RNA 5'-ends. Sizes indicated were derived from DNA-sequencing ladders. Probe 1: lane 1, T-track from dideoxy-sequencing ladder; lane 2, products of 192–195, 89 and 52 nucleotides; lane 3, longer exposure of lane 2. Probe 2: lane 4, products of 171–173, 98, 96, and 68 nucleotides; Probe 3: lane 5, major bands of 52 and 42 nucleotides; Probe 5: a single strong cDNA of 77 nucleotides is marked in lane 10; lanes 6–9 are GACT sequence tracks. Sequencing ladders were generated using pur operon DNA (−193 to 308) cloned into M13mp18 and the same oligonucleotide primers as used for primer extension mapping.

The 5'-end of the ~97-nucleotide degradation intermediate was mapped using Probes 1–3. With Probe 1, the 89-nucleotide cDNA (Fig. 4, lane 2) provides evidence for an RNA 5'-end at nucleotide 107. The 88-nucleotide cDNA obtained with Probe 2 (Fig. 4, lane 4) also supports an RNA 5'-end at nucleotide 107. Finally, primer extension of Probe 3 gave a cDNA of 42 nucleotides (Fig. 4, lane 5) corresponding to an RNA 5'-end at position 109. Results with Probes 1–3 thus place the 5'-end of the ~97-nucleotide degradation intermediate at nucleotides 107–109 (Table II).

The 5'-end of the ~58-nucleotide degradation intermediate was assigned by the 52-nucleotide cDNA obtained with Probe 1 (Fig. 4, lanes 2 and 3). This cDNA places the 5'-end of the ~58-nucleotide degradation intermediate at position 144 (Table II).

The 52-nucleotide cDNA and the minor background cDNA transcripts obtained with Probe 3 (Fig. 4, lane 5) are unrelated to the pur operon. These products were also detected using template RNA from the pur operon promoter deletion strain KK1 as well as with RNA from repressed cells grown with adenine (not shown). We did not detect a cDNA corresponding to the anomalous ~66-nucleotide RNA. Either its 5'-end overlaps with the ~97-nucleotide RNA or its 5'-end is within a few nucleotides of the 3'-end of Probe 3 and was obscured by the labeled primer.

With Probe 2, a 96–98-nucleotide doublet was obtained and is marked in lane 4 of Fig. 4. This doublet appears to correspond to a reverse transcriptase pause site at a potential secondary structure rich in G-C base pairs. The level of doublet was reduced by addition of increased amount of reverse transcriptase (not shown). This doublet was visible in a longer exposure of lane 3 (not shown).

We deduced positions for the 3' termini of the attenuated transcript and the degradation intermediates from the RNA sites obtained by Northern blot analysis and the 5' RNA ends determined by primer extension mapping. These deduced 3'-ends are tabulated in Table II. The deduced 3'-ends for the terminated transcript and the ~97- and ~58-nucleotide degradation intermediates are all within the region of nucleotides 200–205. This region is within the stretch of uracil residues, nucleotides 197–208, of the transcription termination site (Fig. 1).

Mapping the 3' Termini of Degradation Intermediates—We have attempted to directly identify the 3' termini of the ~97- and ~58-nucleotide degradation intermediates by nuclease S1 mapping. A minimal amount of nuclease S1 was used in order to avoid overdigestion of A-U base pairs at the RNA 3'-end. Two uniformly labeled hybridization probes, designated Probes 7A and 1A, were synthesized from an M13 template using 30-mers 7 and 1, respectively. A schematic diagram of the probes and the mapping experiment is given in Fig. 5 (top). The longer probe 7A was designed to anneal to and protect the intact in vivo RNA fragments. The shorter probe

Fig. 5. Nuclease S1 mapping of mRNA 3' ends. Top, schematic representation of mapping experiment. The top line shows B. subtilis DNA from the ClaI site at position 99 to the early part of purE. The black bar represents 22 nucleotides of M13 polylinker sequence. The converging arrows show the position of the terminator structure. Hybridization probes 7A and 1A were extended from primers 7 and 1, respectively (shown as open bars), and were cut at the polylinker EcoRI site. Protected fragments containing uridylate residues at the 3' end of the attenuated transcript will be longer using Probe 7A than with Probe 1A since the 3'-uridylate residues extend past position 195 and will be protected by Probe 7A (dotted 3' ends). Bottom, protection of the ~97- and ~58-nucleotide RNA species. Lane 1, protected fragments of 95–98 and 57–61 nucleotides from Probe 7A. Lane 2, protected fragments of 90–92 and 54–55 nucleotides from Probe 1A; Lanes GACT, sequencing ladder size standard.
1A was designed to align with and protect fragments having a 3′-end at nucleotide 195. Using the short probe, nuclease S1 should remove any of the uracil and 2 intervening adenine residues that may remain at the 3′ terminus of the RNA degradation intermediates. A difference in protected fragment size using the two probes must be due to a 3′ stretch of uracil residues that may remain at the 3′ terminus of the RNA. The results of nuclease S1 mapping are shown in Fig. 5 (bottom).

Protected fragments of 95–98 nucleotides and 90–92 nucleotides were obtained for nuclease S1 mapping of the ~97-nucleotide degradation intermediate using the two probes (Fig. 5, bottom, lanes 1 and 2). Therefore, the ~97-nucleotide RNA extends 5–6 nucleotides downstream of position 195 and has a 3′ terminus at nucleotides 200–201 (Table II).

The nuclease S1 mapping of the 3′-end of the ~58-nucleotide RNA was less satisfactory due to a smear of closely spaced bands using the large probe (Fig. 5, bottom, lane 1). Nevertheless, we estimated the 3′ terminus of the fragment by choosing the darkest bands corresponding to the largest fragments. Protected fragments of 57–61 and 54–55 nucleotides were estimated for nuclease S1 mapping of the ~58-nucleotide degradation intermediate using the two probes (Fig. 5, bottom, lanes 1 and 2). By difference, the ~58-nucleotide RNA has a 3′-terminal stretch of 3–6 residues past nucleotide 195. The deduced 3′ terminus is thus at nucleotides 198–201 (Table II). The 3′ termini of the ~97- and ~58-nucleotide degradation intermediates estimated by two methods are in reasonable agreement (Table II).

Estimation of Half-lives for the Attenuated Transcript and Degradation Intermediates—We have estimated the half-life of the attenuated transcript and each of the major degradation intermediates after inhibition of transcription with rifampicin. Northern blots were hybridized with Probes 1 and 6, and the resulting autoradiographs were used as templates to excise RNA bands from the membrane for measurement of radioactivity. Semi-log plots of the hybridized radioactivity versus the time of RNA sampling after rifampicin addition are shown in Fig. 6. A straight line was drawn through each set of data points, consistent with pseudo-first order decay. Levels of the ~200-nucleotide-attenuated transcript were similar using Probes 1 and 6, thus these values from the two blots were combined. The initial high values for the ~93- and ~88-nucleotide intermediates deviate from first order decay, perhaps as a result of exceeding the linear range for hybridization of probe to RNA. In a preliminary experiment using less RNA, initial decay of these species was linear (not shown).

Extrapolation of the decay rates to zero time yields an estimate of the relative steady state level of each RNA species at the time of rifampicin addition. These values are summarized in Table III. The relative levels for species 200:97:58:93:88 of 1:5:10:26:44 are in reasonable agreement with steady state values estimated from analyses of Northern blots.

Apparent half-times for decay of the attenuated transcript and the degradation intermediates were calculated from the data in Fig. 6 and are given in Table III. Apparent half-times ranged from 0.70 min for the attenuated transcript to 5.0 min for the ~88-nucleotide intermediate. However, the half-time values of the degradation intermediates are overestimated, since the amounts of the smaller products were supplemented by the decay of the larger precursor species. For example the decay of the ~93-nucleotide RNA adds to the amount of the ~88-nucleotide intermediate, resulting in an apparent increase in the half-time for decay of the ~88-nucleotide RNA. For first order processes, this effect can be corrected using the Bateman equations for radioactive decay (9). Thus, the decay rate of each degradation intermediate was corrected for the contribution of the next larger species. The decay rate of the ~97-nucleotide RNA was corrected for the amount of attenuated transcript, and the ~58-nucleotide RNA was corrected for the amount of the ~97-nucleotide RNA. These corrections decrease the half-times for first order decay (Table III). The calculated half-times of 0.70–0.83 min are similar for the attenuated transcript and the ~97- and ~58-nucleotide intermediates. The values of 3.6 and 3.1 min for the ~93- and ~88-nucleotide intermediates, respectively, are also similar and are 4–5-fold larger than those for the ~200-, ~97-, and ~58-nucleotide species.

FIG. 6. Semi-log plot for decay of the attenuated transcript and degradation intermediates. Times are minutes after addition of rifampicin. Open symbols, Probe 1; solid symbols, Probe 6.

Table III

| RNA     | Half-life | Relative steady state RNA level |
|---------|-----------|---------------------------------|
|         | Observed  | Correcteda | Extrapolated from Fig. 6 | From Northern Blots |
|         | min       |           |                          |                      |
| 200     | 0.70      | 0.70      | 1.0                       | 1.0b                 |
| 97      | 0.85      | 0.74      | 5.0                       | 6.0                  |
| 58      | 1.2       | 0.83      | 10                        | 17                   |
| 93      | 3.8       | 3.6       | 26                        | 20                   |
| 88      | 5.0       | 3.1       | 44                        | 40                   |

a Corrected half-lives were determined using the Bateman equations.
b Values in this column were obtained by densitometry of Northern blots. RNA was isolated from cells harvested by centrifugation.

c Values in this column were obtained by hybridization with 32P-labeled probes and measurement of radioactivity by liquid scintillation counting. RNA was prepared from cells harvested rapidly by filtration. Values are averages from two Northern blots using 15 and 30 µg of RNA.
FIG. 7. Deduced secondary structures of the attenuated transcript and degradation intermediates. Arrows indicate proposed sites of endonucleolytic cleavage. A, the -200-nucleotide-terminated transcript, 200, is drawn to maximize secondary structure. The 5'-single-stranded end and the terminator region are outlined. B, it is proposed that intermediate 93L is produced by cleavage of 200, between nucleotides 106-107 and trimming the 3'-end up to guanosine 93. Cleavage in the A-rich bulge of 93L, followed by 3'-trimming yields 88L. C, the 97R intermediate is produced by endonucleolytic cleavage of 200 between nucleotides 106-107. Endonucleolytic cleavage of 97R between nucleotides 143-144 and removal of 3 nonbase-paired uracil residues from the 3'-end generates an intermediate which folds into 58R. It is proposed that 3 nonbase-paired uracil residues at the 3'-end are removed during the conversion of 97R to 58R. D, schematic representation of the degradation pathway showing the relative steady state amounts of intermediates in parentheses, normalized to 1.0 for the 200 RNA.
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DISCUSSION

A combination of Northern blot analysis, primer extension mapping, and nuclease S1 mapping was used to characterize the attenuated transcript of the B. subtilis pur operon as well as stable intermediates which accumulate during its degradation. The attenuated transcript is ~200-nucleotides in length with a 5'-end between position +1 to 4 and 3'-ends between nucleotides 200–203 (Table II). This truncated mRNA thus very likely results from transcription termination at the cluster of uracil residues at nucleotides 196–208, which is part of the ρ-independent terminator. Four major intermediates accumulated from the degradation of the attenuated mRNA. Two of these intermediates were derived from the 5'-half of the attenuated transcript and two from the 3'-half. Based on size and mapping data, it is possible to align the degradation intermediates with the nucleotide sequence and to assign probable transcript secondary structures. These structures are drawn in Fig. 7. The terminator secondary structure and a single-stranded 5' segment are boxed. The RNA molecules are named according to their approximate sizes: the attenuated transcript shown in Fig. 7A is designated 200. The 200-attenuated transcript has been drawn to maximize its secondary structure. The exact positions of the 5'- and 3'-ends are arbitrarily shown as nucleotides +1 and 201. The degradation intermediates that are derived from the 5'-half of the attenuated transcript are named 93L and 88L (Fig. 7B), those from the 3'-half, 97R and 58R (Fig. 7C). It is apparent that the attenuated transcript and each of the degradation intermediates can be drawn as extensively base-paired structures. We propose that secondary structure formation stabilizes the degradation intermediates and accounts for their steady state accumulation.

A scheme shown in Fig. 7D summarizes the deduced steps of the degradation pathway. Additional intermediate steps are possible but have not been investigated. The anomalous 86-nucleotide species detected by Probe 3 is not included in this scheme because its termini could not be mapped. Based on the accumulation of the two largest intermediates, it is proposed that endonucleolytic cleavage in the single-stranded region between nucleotides 94–106 (Fig. 7A) initiates the degradation of the attenuated transcript. The initial endonucleolytic cleavage of 200 is drawn on the 5' side of adenosine 107. Cleavage at this position is compatible with the postulated roles of RNase III and 3' exonuclease in mRNA degradation (11). RNase III is the only well-characterized endonuclease from B. subtilis. It cleaves phage SP82 early mRNA at the 5' side of specific adenosine residues located in the loop of a stem-loop structure (12). Thus, RNase III is a candidate for catalyzing an initial cleavage of the attenuated transcript at position adenosine 107. A cut at this position would generate species 97R having a base paired 5'-end plus an intermediate with an exposed single-stranded 3' tail. 3' Exonuclease digestion of the single-stranded 3' tail would yield species 93L. In E. coli two 3' exonucleases, RNase II and polynucleotide phosphorylase have been implicated in mRNA degradation (13). There is no evidence for a 5' to 3' exonuclease in E. coli (14), and it is not known whether such an activity is present in B. subtilis. Persistence of the 5'-end of species 200, 93L, and 88L is consistent with the absence of a 5' to 3' exonuclease in B. subtilis.

An endonucleolytic cleavage on the 5' side of adenosine 89 is arbitrarily suggested to initiate conversion of intermediate 93L to 88L. This cleavage could be followed by 3' exonuclease removal of adenosine 88. The 3' base-paired stem in species 88L could provide a barrier to further rapid 3' exonuclease decay and might account for the enhanced half-life of 88L.

An endonucleolytic cleavage on the 5' side of adenosine 144 in a 4 nucleotide bulge could initiate conversion of 97R to 58R. This might be followed by removal of 3' non-base-paired uracil residues at the 3'-end of the resulting intermediate to yield 58R. This proposed scheme for decay of the pur operon-attenuated transcript thus involves a series of endonucleolytic cleavages, perhaps catalyzed by RNase III, followed by progressive 3' exonuclease trimming up to a base-paired secondary structure. The decay of several mRNAs in E. coli follows this same general scheme (11).

The scheme shown in Fig. 7D lists the approximate steady state levels of degradation intermediates relative to the attenuated mRNA. The steady state RNA levels shown in Fig. 7D are mean values obtained by three different methods. These steady state RNA levels imply that the endonucleolytic cleavage of the attenuated transcript is rapid compared to its further degradation. In addition the steady state and half-time measurements indicate that the degradation of the 3'-half of the attenuated transcript proceeds faster than that of the 5'-half. At least two factors are expected to contribute to the half-life of each of the observed species. These are (i) accessibility of sites for endonucleolytic cleavages to initiate decay and (ii) stability of secondary structures that provide a barrier to the action of 3' exonucleases (11).

Although half-life measurements for the degradation intermediates are consistent with slower decay of the 5'-half of the attenuated transcript compared to the 3'-half, the experimentally determined RNA half-lives do not correlate well with the observed steady state RNA levels. For a reaction, A → B → C, first order kinetics predict that the ratio of the steady state concentrations of A, B, and C should be the same as the ratio of their half-lives. Thus, degradation of the attenuated transcript apparently cannot be fully explained by simple first order kinetics. This raises the question of how the degradation intermediates can accumulate in such large amounts under steady state conditions. One possibility is that effective half-lives may differ during synthesis and decay prior to rifampicin treatment.

Does the decay of full length pur operon mRNA generate the degradation intermediates shown in Fig. 7D? Northern blot analysis indicates that the ~200-nucleotide-attenuated transcript is not generated from decay of full length pur operon mRNA (Fig. 3, lanes 1, 5). Previous nuclease S1 mapping indicates synthesis of comparable amounts of pur operon mRNA from derepressed cells and attenuated mRNA from gramine nucleotide-repressed cells (1). Since maximal accumulation of the degradation intermediates requires attenuation (Fig. 3, compare lanes 1 and 2, and 5 and 7) intermediates 97R and 58R, as well as 93L and 88L, were not produced stoichiometrically from the decay of pur operon mRNA. It thus appears unlikely that the leader portion of pur operon mRNA and the attenuated transcript decay by similar pathways. The 3' terminator secondary structure is a major distinguishing feature of the attenuated transcript that is not found in pur operon leader mRNA. However, it has not been established that the 3' terminator secondary structure actually determines whether the 93L, 88L, 97R, and 58R degradation intermediates are produced stoichiometrically from the attenuated transcript.

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