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The mRNA for an Inducible Chloramphenicol Acetyltransferase Gene Is Cleaved into Discrete Fragments in *Bacillus subtilis*

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***cat-86* is a promoter-deficient plasmid gene that encodes chloramphenicol acetyltransferase (CAT). Insertion of a promoter at a site 144 base pairs 5′ to the *cat-86* coding sequence activates transcription of the gene and allows *cat-86* to specify chloramphenicol-inducible CAT activity in *Bacillus subtilis*. Induction of *cat-86* by chloramphenicol has been shown to result from a regulatory event that activates translation of *cat-86* mRNA that is present in cells before the addition of inducer (E. J. Duvall and P. S. Lovett, Proc. Natl. Acad. Sci. USA 83:3939–3943, 1986). In the present study we show an unusual property of *cat-86* mRNA. Full-length *cat-86* transcripts, consisting of 920 nucleotides (nt), are cleaved in *B. subtilis* to yield two predominant fragmentation products: an 810-nt species that lacks sequences present at the 5′ end of the 920-nt species and a 720-nt species that lacks sequences present at the 3′ end of the 920-nt species. A third fragmentation product consisting of 620 nt may result from the cleavage of a single 920-nt transcript at both the 5′ and 3′ ends. The sequences which are missing from the 720- and 620-nt species suggest that these transcripts cannot be translated into functional CAT. The 810-nt species lacks sequences from the 5′ regulatory region, and it is not yet certain whether or not translation of this species can be induced by chloramphenicol. The ratio of 920-nt molecules/720-nt molecules in rifampin-treated cells is increased when the cells are grown in chloramphenicol. Therefore, induction may partially stabilize full-length *cat-86* transcripts against inactivation by a novel processing-like system.**

The stability of mRNA is one of several factors that contribute to the level of expression of individual genes. In bacteria, decay of mRNA is thought to involve both exonucleases and endonucleases which in concert bring about the dissolution of RNA molecules (5, 8, 12–15). It is established that different mRNA species exhibit very different half-lives (12), and regions within individual transcripts can decay at different rates (4, 13, 25). It is therefore evident that specific structural features of RNA and probably specific sequences in RNA determine the susceptibility to enzymatic attack.

We have undertaken an examination of the mechanism through which the antibiotic chloramphenicol induces expression of the gene *cat-86* that encodes the enzyme involved in inactivation of chloramphenicol, chloramphenicol acetyltransferase (16). Studies with *cat-86* indicate that induction is due to the activation of translation of *cat* mRNA (10). Other inducible *cat* genes may be regulated by a similar mechanism (6, 7). It has been proposed that chloramphenicol enables ribosomes to perform a specialized function that causes the destabilization of a stem-loop in *cat* mRNA that sequesters the ribosome-binding site (11). Because mRNA translation is the level at which *cat-86* expression is regulated it is important to understand the factors which contribute to the stability of this mRNA species as well as the mechanisms which cause decay of the molecule.

cat-86 expression can be induced by chloramphenicol in *B. subtilis* cells whose RNA synthesis has been inhibited by rifampin or streptolydigin (10). However, when *cat-86* mRNA was present in cells at a low level, a condition achieved by activating the gene with a weak promoter, chloramphenicol inducibility after rifampin inhibition declined more rapidly than the chemical half-life of the mRNA, which was estimated to be 8 min (10). It was suggested that a major fraction of the *cat-86* mRNA molecules were functionally inactivated at a rate which exceeds the rate of chemical decay. In theory, mRNA can be inactivated by a single endonucleolytic cleavage in the coding region. In the present study we show that *cat-86* mRNA is a substrate for highly specific cleavage in vivo and that this processing-like event appears to occur more rapidly in uninduced cells than in cells induced with chloramphenicol.

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MATERIALS AND METHODS

Bacteria and plasmids. *Bacillus subtilis* BR151 (*trpC2 metB10 lys-3*) was the plasmid host. Plasmids used were pPL703 and pPL703Δ39 (Fig. 1). The promoterless gene present on pPL703, *cat-86*, was activated by inserting the promoter-containing restriction fragment SpacI (30) between the *Eco*RI and *Pst*I sites of the linker that is present 144 base pairs (bp) 5′ to *cat-86* (2). Low-resolution S1 mapping of transcripts specified by pPL703-SpacI placed the transcription start site for *cat-86* within the SpacI promoter fragment (data not shown). For the purpose of calculating the length of *cat-86* transcripts we assumed that the site of transcription initiation in SpacI is the same as the initiation site in the *lac* promoter in *Escherichia coli* (24). The site of transcription termination 3′ to *cat-86* is known (21), and we calculated the distance between the sites of transcription initiation and termination to be 920 bp. Thus, full-length *cat-86* transcripts should consist of 920 nucleotides (nt), and this is the same value as that determined experimentally (see Results).

Plasmid pPL703Δ39 was derived from pPL703 by removing 39 bp from the 5′ end of the 144-bp regulatory region that precedes *cat-86* (1). However, pPL703Δ39 differs from pPL703 by 30 bp, not 39 bp, because of the method used to restore the linker. Deletion of 39 bp had no detectable effect on *cat-86* expression or on mRNA stability (1; N. P. Ambulos, E. J. Duvall, and P. S. Lovett, manuscript in preparation). Growth media and conditions, methods for plasmid transformation, and plasmid manipulations were as previously described (17, 18).

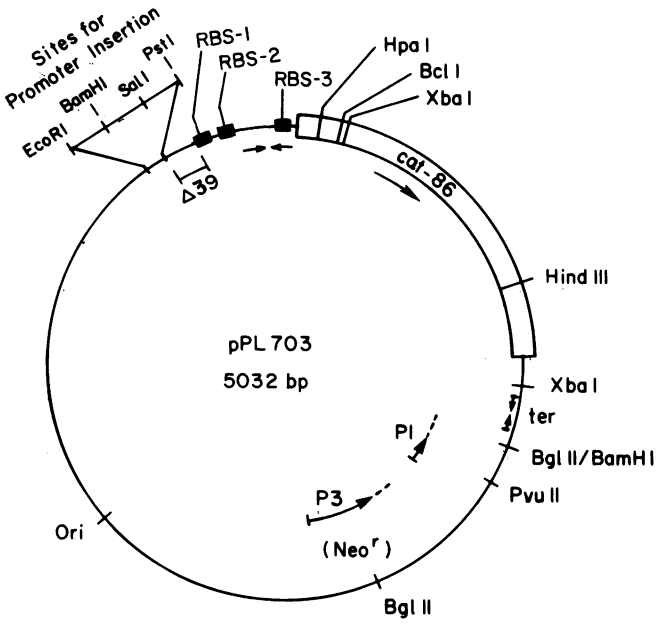

FIG. 1. Diagram of the promoter-cloning plasmid pPL703. This plasmid is composed of a 1,250-bp *Pst*I-*Bgl*II fragment of DNA from *Bacillus pumilus* NCIB 8600 containing the *cat-86* gene inserted between the *Eco*RI and *Bam*HI sites of pUB110 by use of a 21-bp *Eco*RI-*Pst*I linker that contains internal *Bam*HI- and *Sal*I-sensitive sites (20). Transcripts initiated in pUB110 are opposite to the orientation of *cat-86*, and these vector transcripts do not enter the *cat-86* coding sequence (21). The *cat-86* coding sequence is preceded by a 144-bp regulatory region that contains sequences essential to chloramphenicol inducibility of the gene. This regulatory region contains three ribosome-binding sites designated RBS-1, -2, and -3 (1). RBS-3 is the ribosome-binding site for the *cat-86* coding sequence. pPL703Δ39 was derived from pPL703 by removing 39 bp of DNA from the *Pst*I site to a site immediately 5′ to RBS-1. However, because of the method used to restore the linker, pPL703Δ39 differs from pPL703 by only 30 bp (1). pPL703 consists of 5,032 bp (2, 19).

RNA isolation, electrophoresis, and hybridization. BR151(pPL703-SpacI) cells were grown at 37°C to the mid-log phase in Penassay broth (Difco Laboratories, Detroit, Mich.) containing no antibiotic or 20 μg of chloramphenicol per ml. Rifampin was added to 100 μg/ml, and cells were harvested immediately and after 10 and 20 min of incubation in the presence of the RNA synthesis inhibitor. Denatured RNA (5 μg) purified (26) from each cell sample was electrophoresed through a 2% vertical agarose gel containing 6% formaldehyde (N. P. Ambulos, Jr., E. J. Duvall, and P. S. Lovett, Gene, in press). RNA was transferred from the gels to Gene Screen by blotting. Hybridization was performed as described elsewhere (18), using any of three probes. Probe 1 consisted of a 101-bp *Bam*HI-*Fok*I fragment from the 5′ end of the *cat-86* regulatory region inserted into M13mp19 between the *Bam*HI and *Hin*cII sites (Fig. 2). Probe 2 consisted of a 387-bp *Bcl*I-*Hin*dIII fragment inserted between the *Bam*HI and *Hin*dIII sites of M13mp9 (Fig. 2). Probe 3 consisted of a 215-bp *Hin*dIII-*Xba*I fragment inserted into M13mp19 (Fig. 2). A radioactively labeled complement to the inserted fragment in probe 3 was synthesized with the universal primer, ^{32}P-labeled nucleotides, and the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Probes 1 and 2 were made radioactive as for probe 3 except that hybridization probe primer was used to prime DNA synthesis. Standards included in each gel were prepared by digesting pPL703 with various restriction enzymes chosen to produce a range of fragments that spanned the desired molecular weight range and would hybridize with the probe being used.

When pPL703 is present in *B. subtilis* neither strand of *cat-86* is transcribed based on the results of RNA hybridization experiments with probe 2 (21). When *cat-86* is activated by inserting a promoter into the linker 5′ to *cat-86*, transcripts can be detected by probe 2, and these are complementary only to the sense strand of *cat-86* (21). To establish that hybridizations with probes 1, 2, and 3 were detecting transcripts that were dependent on the inserted promoter, we tested the ability of each probe to hybridize to RNA from BR151(pPL703). These experiments were performed in the same manner as when pPL703-SpacI was in BR151 (see above). The result was that RNA from BR151(pPL703) did not contain any species that detectably hybridized with any of the three probes (data not shown). Therefore, all RNA species detected by the three probes depend on transcription initiation in SpacI.

RESULTS

Resolution of *cat-86* transcripts into four molecular weight species during decay. The pattern of decay for *cat-86* mRNA was determined by Northern blotting and hybridization. *B.*

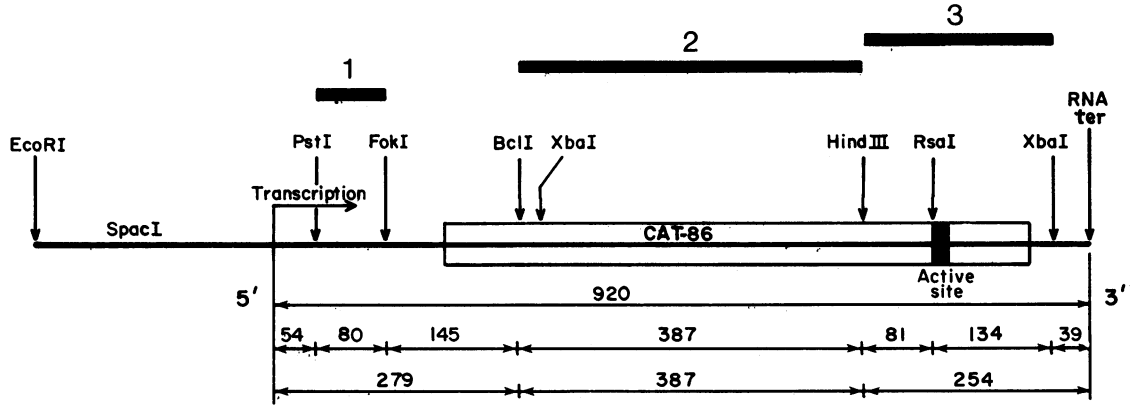

FIG. 2. Diagram of *cat-86* activated by the SpacI promoter fragment. Transcript sizes are in nucleotides. Diagram is drawn to scale. The region encoding the active site was inferred from studies on chloramphenicol acetyltransferase proteins which are homologous to the *cat-86* gene product in the vicinity of the active site (27, 28).

subtilis BR151 cells containing the plasmid pPL703-SpacI (Fig. 1), in which the *cat-86* gene is activated by the SpacI promoter fragment, were grown to the mid-log phase in drug-free Penassay broth or broth containing 20 μg of chloramphenicol per ml. Rifampin was added to 100 μg/ml, and cells were removed immediately, and again after 10 and 20 min of incubation of rifampin. RNA purified from each cell sample was subjected to electrophoresis through formaldehyde-agarose gels and blotted to Gene Screen. *cat-86* transcripts specified by pPL703-SpacI were initially analyzed by probing the blots with hybridization probe 2. This probe spans a 387-nt sequence within the *cat-86* coding region (Fig. 2). The largest species of *cat-86* mRNA detected in these RNA preparations was 920 nt in length, which is precisely the size predicted for full-length *cat-86* transcripts specified by pPL703-SpacI (Fig. 3). Hybridization to zero time RNA samples consistently detected the 920-nt species plus a smear of lower-molecular-weight species. By backlighting autoradiograms we could detect three bands in the smear which migrated at the positions expected for RNA fragments of 810, 720, and 620 nt. These bands are not visible in the photographs of the autoradiograms (Fig. 3).

RNA from cells that had been incubated in rifampin for 10 or 20 min consisted of four distinct mRNA species which hybridized with probe 2 (Fig. 3). The largest was the full-length *cat-86* transcript of 920 nt; the sizes of the smaller species were 810, 720, and 620 nt. The three species smaller than the full-length *cat-86* transcript could be decay products of the 920-nt species or could result from termination of transcription at sites upstream from the *cat-86* transcription termination signal which is 3′ to the gene (21). If the four RNA species differed only in the site where each terminated, the four species should differ in nucleotide sequence at the 3′ end but not at the 5′ end. Alternatively, if the 920-nt species was processed by RNases to generate the smaller species, it was thought that differences might be detected at the 5′ end of certain of the smaller transcript species.

The 810-nt species is due to processing of the full-length *cat-86* transcript at the 5′ end. Probe 1 hybridizes specifically to the 5′ end of *cat-86* mRNA (Fig. 2). Hybridization of this probe to Northern blots detected the 920- and 720-nt species (Fig. 4). However, probe 1 did not detect the 810-nt species or the 620-nt species, indicating that these mRNA species

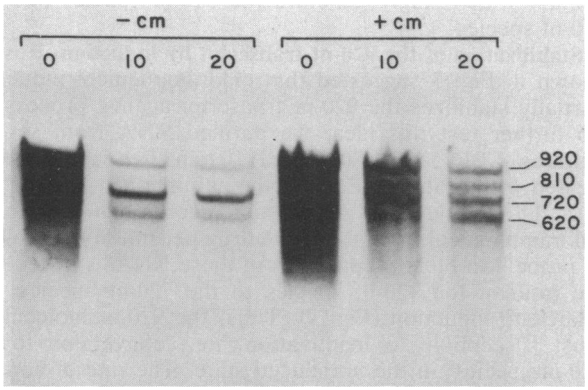

FIG. 3. Hybridization of probe 2 to electrophoretically separated RNA from BR151(pPL703-SpacI). +cm or −cm indicates the presence or absence of chloramphenicol at 20 μg/ml during cell growth. 0, 10, and 20 refer to the minutes after rifampin addition to 100 μg/ml when cell samples were removed for RNA extraction. Sizes of RNA molecules are in nucleotides.

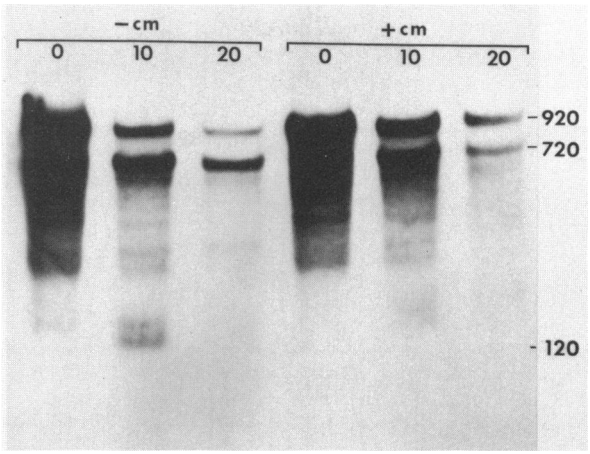

FIG. 4. Hybridization of probe 1 to electrophoretically separated RNA from BR151(pPL703-SpacI). See the legend to Fig. 3 for details.

lack sequences which are present at the 5′ end of both the 920-nt and the 720-nt species. Since all *cat-86* transcripts initiate from a site that is located upstream of probe 1 (Fig. 2), the 810-nt species is likely to have been derived from the 920-nt species by a processing type of event that removes more than 100 nt from the 5′ end of full-length *cat-86* transcripts. If this interpretation was correct, it was reasoned that small deletions into the 5′ end of the 144-bp regulatory region, which intervenes between the promoter and the *cat-86* coding sequence, should diminish the sizes of the 920- and 720-nt transcripts, but not the size of the 810-nt species. A version of pPL703 lacking the first 39 bp of this 144-bp region, pPL703Δ39, was previously generated by *Bal* 31 digestion (1). We activated *cat-86* expression in pPL703Δ39 by inserting SpacI and transformed the plasmid into BR151. RNA isolated from these cells after rifampin inhibition was hybridized with probe 2. The results showed that the Δ39 mutation reduced the size of the full-length *cat-86* transcript to 890 nt and that the "720"-nt species was reduced in size to 690 nt. However, the sizes of the 810-nt RNA species and the 620-nt species were not altered by the deletion (Fig. 5).

If the 810-nt species was the result of endonucleolytic cleavage that removed a 100-nt (approximately) fragment from the 5′ end of the 920-nt species, the small fragment should be detected by hybridization with probe 1. The autoradiogram shown in Fig. 4 reveals a band of weak hybridization at the position expected for a 120-nt RNA species. We suspect that the 120- and 810-nt species are the two products of a single endonucleolytic cleavage of the 920-nt species. The weak hybridization to the 120-nt RNA molecule may indicate that this is a relatively labile species.

Ambulos et al. (3) found that a substantial fraction of the potential *cat-86* transcripts terminated at approximately codon 2 of the *cat* gene. This terminated RNA should be 200 nt in length, and an apparently labile mRNA species can be detected at the position expected for a 200-nt molecule (Fig. 4).

The 720-nt species results from a processing event at the 3′ end of full-length *cat-86* transcripts. The 720-nt mRNA species was detected with probes 1 and 2 (Fig. 3 and 4). Moreover, this species was smaller when we probed transcripts specified by the mutant of pPL703 deleted for the first 39 bp of the *cat-86* regulatory region than when we probed

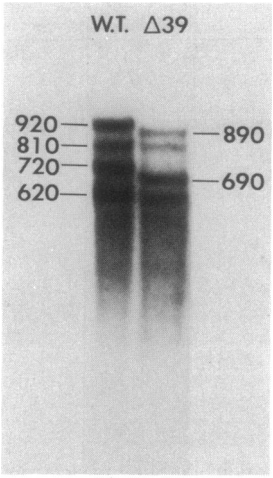

FIG. 5. Hybridization of probe 2 to electrophoretically separated RNA from BR151(pPL703-SpacI) and BR151(pPL703Δ39-SpacI). Both RNA preparations were derived from cells that had been grown in 20 μg of chloramphenicol per ml and were incubated for 10 min in rifampin. The well containing transcripts specified by pPL703-SpacI is designated W.T., and the well designated Δ39 shows transcripts specified by pPL703Δ39-SpacI. Sizes of RNA molecules are in nucleotides.

transcripts specified by the nondeleted parent plasmid (Fig. 5). These results suggested that the 720-nt species contained the same sequence at the 5′ end as did the 920-nt full-length *cat-86* transcript. We therefore anticipated that the 720-nt species would lack about 200 nt from the 3′ end relative to the 920-nt species. Accordingly, we constructed probe 3, which is designed to detect sequences at the 3′ end of full-length (920-nt) transcripts (Fig. 2). Probe 3 hybridized efficiently to the 920- and 810-nt mRNA species, but showed virtually no hybridization to the 720- and 620-nt species (Fig. 6). The very weak hybridization signal that was occasionally obtained with the 720- and 620-nt fragments probably indicates a slight overlap of the probe with both RNA species. These data suggest that the 720-nt species is a truncated version of the 920-nt species.

If the 720-nt species was derived from the 920-nt species by endonucleolytic cleavage, hybridization with probe 3 should detect an RNA species of approximately 200 nt. Inspection of Fig. 6 shows the presence of a 220-nt species, which was not detected with probe 1 or 2. We believe that the 220-nt species and the 720-nt species are the products of endonucleolytic cleavage of full-length transcripts.

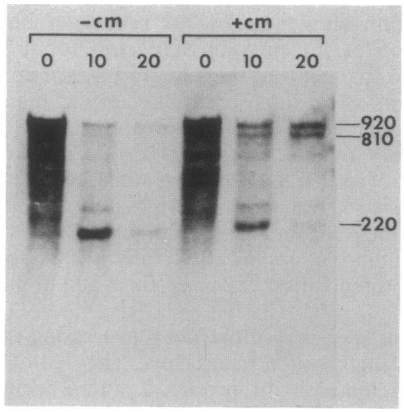

FIG. 6. Hybridization of probe 3 to electrophoretically separated RNA from BR151(pPL703-SpacI). See the legend to Fig. 3 for details.

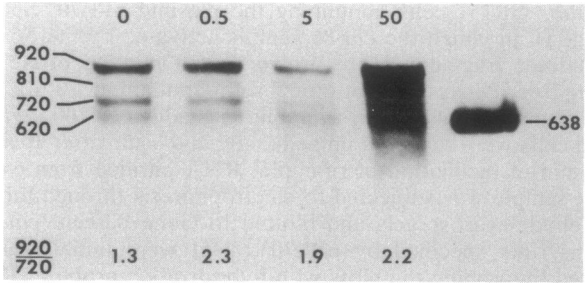

FIG. 7. Hybridization of probe 2 to electrophoretically separated RNA from BR151(pPL703-SpacI). RNA was isolated from cells grown in 0, 0.5, 5, or 50 μg of chloramphenicol per ml 10 min after the addition of rifampin to 100 μg/ml. The ratio of the 920-nt/720-nt molecules was determined by densitometer tracing and is noted at the bottom of each well. Sizes of RNA molecules are in nucleotides.

Origin of the 620-nt species. The 620-nt transcript species shares characteristics in common with both the 810- and the 720-nt species. For example, hybridization with probe 2 detected the 620-, 720-, and 810-nt species, whereas hybridization with probe 1 detected the 720-nt species but failed to detect both the 620- and 810-nt species (Fig. 3 and 4). Thus, the 620- and 810-nt species lack sequences that are normally found at the 5′ end of *cat-86* transcripts. This interpretation is also supported by our analysis of the transcripts specified by the deletion mutant pPL703Δ39-SpacI. This mutation, which deletes 39 bp from the 5′ end of the *cat-86* regulatory region, diminished the sizes of the full-length *cat-86* transcript and the "720"-nt species (Fig. 5). However, the mutation did not affect the sizes of the 810- and 620-nt species.

The similarity of the 620-nt species with the 720-nt species is suggested by hybridization analysis with probe 3 (Fig. 6). Probe 3 showed intense hybridization to the 810-nt species, but hybridization to the 620- and 720-nt species was virtually undetected. These results lead us to believe that the 620-nt species may arise from the 920-nt species by two processing events, which singly generate the 810-nt species and the 720-nt species.

Stabilization of the 920-nt transcript by induction. Results shown in Fig. 3 suggested that chloramphenicol induction partially stabilizes the 920-nt transcript against processing. To further test this idea, we purified RNA from several cultures of BR151(pPL703-SpacI) 10 min after each had been treated with rifampin. Each of the cultures had been grown in a different level of chloramphenicol, ranging from no chloramphenicol to 50 μg of the drug per ml. Hybridization of probe 2 to blots prepared from these RNAs showed that the ratio of the 920-nt species to the 720-nt species was related to induction (Fig. 7). Thus, the 920-nt molecule is most susceptible to inactivation, i.e., conversion to the 720-nt species, in the uninduced state. The rate of conversion of the 920-nt species to the 720-nt species in RNA from different batches of cells that had been grown without chloramphenicol was variable (compare Fig. 3 and 7). This ratio ranged from 0.5 up to 1.3. However, the 920/720 ratio in induced cells consistently was within a higher range of 1.9 to 2.5.

DISCUSSION

The results indicate that full-length *cat-86* transcripts of 920 nt are fragmented during decay in *B. subtilis* into four products consisting of 810, 720, 620, and approximately 220 nt and probably a fifth species of about 120 nt. We infer from the following observations that these products are due to processing of the 920-nt species rather than to premature transcription termination. The 810- and 620-nt species do not detectably hybridize with a probe designed to detect the 5′ end of full-length *cat-86* transcripts (Fig. 4). The sequences that are absent from the 810- and 620-nt species must be present at the 5′ end of primary transcripts of *cat-86* because the site for initiation of transcription of *cat-86* is within the cloned promoter-containing fragment and is therefore upstream from the missing sequences (Fig. 2). The 810-nt species, as well as the 620-nt species, could arise either from an endonucleolytic cleavage that removes a discrete fragment from the 5′ end of the 920-nt species or by limited exonuclease digestion into the 5′ end of the 920-nt transcript. If conversion of the 920-nt species to the 810-nt species was due to endonucleolytic cleavage, we would anticipate that hybridization with probe 1, which detects 5′ mRNA sequences, might detect an RNA fragment of less than 130 nt, corresponding to the 5′ end of the *cat-86* transcript. A fragment of about 120 nt was detected by hybridization with probe 1. However, this fragment may be highly labile since the intensity of hybridization was consistently weak. Thus, the 810- and 620-nt species are the result of RNase action at the 5′ end of the 920-nt species, and we believe the enzyme involved is an endoribonuclease.

The 720-nt species is thought to result from endonucleolytic cleavage into the 920-nt transcript at a site approximately 200 nt upstream from the 3′ end of the molecule. This is suggested by the inability of probe 3 to hybridize significantly to the 720-nt species (Fig. 6) and the detection of a 220-nt RNA species by hybridization with probe 3 which was not detected by hybridization with probe 1 or 2 (Fig. 3 and 4). Last, the 610-nt species exhibits hybridization properties similar to those of both the 810- and 720-nt species. Thus, the 610-nt species may result from two processing events, which individually generate the 810- and 720-nt species.

The processing event that generates the 720-nt transcript likely involves a cleavage between the *Hin*dIII site in the *cat-86* coding region and the region of the coding sequence that specifies the active site of the enzyme (Fig. 2). Therefore, the 720-nt species, and the 620-nt species, probably cannot be translated into functional chloramphenicol acetyltransferase. The sequences removed from the 5′ end of the 920-nt species in generating the 810-nt transcript appear to end in the vicinity of a regulatory region that we have previously shown to be essential to chloramphenicol induction of *cat-86* (1, 2). Although we suspect that the 810-nt molecules may be inducibly expressed (N. P. Ambulos, unpublished data), this remains to be conclusively demonstrated. Both of the processing events we identified are highly specific and are probably due to the action of one or more endonucleases. Panganiban and Whiteley (22, 23) have shown that fragmentation of early SP82 transcripts in *B. subtilis* (9), as well as processing of precursor rRNA, is due to cleavage by an RNase III-like enzyme. This enzyme, Bs-RNase III, has been shown to cut mRNA within the loop region of stable stem-loops. While this enzyme must be a candidate for the processing of *cat-86* mRNA, our estimates of the sites of cutting that potentially generate the 810- and 720-nt species do not coincide with sequences that would generate RNA secondary structure. Thus, it is possible that a processing-like enzyme(s) other than Bs-RNase III is involved.

Full-length *cat-86* transcripts of 920-nt appear more labile in uninduced cells than in cells grown with the inducer (Fig. 3 and 7). Moreover, uninduced cells seem to accumulate the 720-nt transcript, which is a form of *cat-86* mRNA that probably cannot be translated into functional chloramphenicol acetyltransferase. These observations suggest that chloramphenicol-induced translation of *cat-86* mRNA partially protects the 920-nt species from endoribonuclease attack, perhaps by changing the conformation of the RNA from a sensitive to an insensitive state or by physically blocking RNase interaction with the mRNA.

Conversion of specific mRNA molecules to discrete fragments has been previously reported in bacteria. In *E. coli*, the polycistronic *lac* and *trp* mRNA molecules are cleaved internally at sites that are at, or near, the junctions between cistrons (5, 14, 15). These cleavages are followed by apparent exonucleolytic decay of the fragments, which in the case of *lac* mRNA results in a wave of mass loss in a 5′-to-3′ direction (14). The pattern of fragmentation of *cat-86* mRNA is consistent with the presence of at least two internal cleavage sites, and fragmentation appears to precede the net loss of mRNA mass. One of the sites of cleavage in *cat-86* mRNA appears to separate the region encoding the active site for the enzyme from the 5′ portion of the message. Cleavage at this site likely causes functional inactivation of the mRNA. Cleavages in *lac* mRNA also seem to inactivate the function of the message: cleavages immediately 5′ to *lacY* and *lacA* separate the coding region from sequences essential to translation initiation (14).

The internal cleavages within the *cat-86* mRNA may represent the first step in the overall decay of this RNA species. If this is so, events which diminish the fragmentation of *cat-86* mRNA would extend the functional life of the mRNA. We therefore think it is significant that chloramphenicol, which activates translation of *cat-86* mRNA, also causes a reduction in the conversion of the 920-nt full-length transcript to the 720-nt form, which we feel is inactive. Thus, the process of translation of *cat-86* mRNA seems to increase the functional life of intact *cat-86* transcripts.

ACKNOWLEDGMENTS

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