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Early RNAs in SP82- and SP01-Infected *Bacillus subtilis* May Be Processed

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Transcription of SP82 and SP01 DNAs in vitro by *Bacillus subtilis* RNA polymerase yielded mostly large RNA species, with many in excess of 1,500 bases in length, whereas most of the RNAs synthesized in vivo early in infection were much smaller. Addition of an extract from uninfected *B. subtilis* to reaction mixtures containing RNAs synthesized in vitro generated additional discrete RNAs whose mobilities on polyacrylamide gels matched the mobilities of some of the smaller RNAs synthesized in vivo.

The development of the closely related phages SP82 and SP01 in *Bacillus subtilis* is characterized by a complex pattern of transcription (3, 8, 17). As in many phage infections, the host RNA polymerase transcribes the injected phage DNA to produce "early" RNA; during infection by SP82 and SP01, the subsequent synthesis of "middle" and "late" RNAs requires sequential modification of the host polymerase (10, 18-20). Most of the RNA synthesized immediately after infection comes from the terminally redundant regions of the genome (9, 11, 12); phage protein synthesis is not required for the production of this class of early RNA (12, 19; A. Panganiban, unpublished data), and the same regions of DNA are transcribed in vitro by purified *B. subtilis* RNA polymerase (9, 11, 12). Recent investigations have centered on the role of polymerase modification in the regulation of transcription and on the in vitro specificities of the different forms of polymerase. However, studies of the arrays of transcripts produced by these enzymes provide an opportunity to identify other factors affecting the synthesis of specific RNA molecules in *B. subtilis*. The results reported here suggest the presence of one such factor—an activity which processes large RNA molecules synthesized in vitro to smaller-sized RNAs which have the same electrophoretic mobilities as RNAs observed in lysates of SP82- and SP01-infected cells.

Early SP82 and SP01 RNAs synthesized in vivo are shown in lanes A and D of Fig. 1. ^{32}P-labeled phage RNAs were identified by autoradiography of sodium dodecyl sulfate-polyacrylamide slab gels. To label the early RNAs, *B. subtilis* cultures were grown in a low phosphate medium (1a) at 37°C to an optical density at 600 nm of 0.25, the cultures were irradiated with UV light for 6 min and incubated for 15 min at 37°C, and chloramphenicol was added to 200 μg/ml. At 5 min after the addition of chloramphenicol, the bacteria were infected with phage at a multiplicity of 10, and the infected cells were labeled from 10 to 14 min after infection with 40 to 80 μCi of $^{32}PO_4$ (carrier-free; Amersham Corp.) per ml. It has been shown that only early phage RNAs are synthesized in the presence of chloramphenicol (7). Labeling was terminated by the addition of one-half volume of a solution containing 0.05 M potassium phosphate and 30 μg of rifampin per ml. After 30 s, the infected cells were converted to protoplasts on ice (6), harvested by centrifugation, lysed with sodium dodecyl sulfate sample buffer (4), and applied to polyacrylamide slab gels; after electrophoresis, the RNAs were visualized by autoradiography. The ^{32}P-labeled material in these lysates was identified as RNA on the basis of its sensitivity to RNase and the inhibition of labeling by rifampin.

It was readily apparent from the data shown in Fig. 1 (lanes B and E) that most of the early RNAs were smaller than 1,600 bases in length, that RNAs as small as 235 bases could be detected, that the arrays of RNA molecules produced by infection with SP82 and SP01 were similar but not identical, and that these RNAs were not seen in uninfected, UV-irradiated *B. subtilis*. Within the limits of resolution, the same RNA species were observed immediately after infection of unirradiated cells (data not shown). Chloramphenicol had no effect on the synthesis of these early RNAs since the RNAs seen in lane E of Fig. 1 were also produced in the absence of chloramphenicol by cells infected with a gene 28 mutant of SP01 (this mutant only produces early RNA [2]; data not shown). Lastly, the early RNAs seen in Fig. 1 appeared to be similar to those observed by Reeve et al. (13), but direct comparisons are difficult due to differences in the experimental systems.

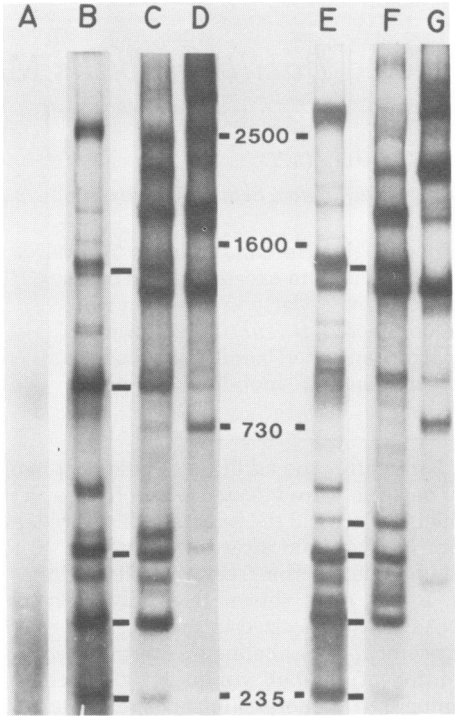

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel analysis of ^{32}P-labeled SP82 and SP01 RNAs. RNAs were labeled as described in the text. For RNA processing, 10 μl of a B. subtilis S-30 fraction (ca. 40 μg of total protein) was added to 10 μl of the in vitro RNA synthesis reaction mixture and incubated for 10 min at 37°C; incubation was terminated by the addition of 20 μl of sodium dodecyl sulfate sample buffer. The S-30 extract was prepared as described by Hagen and Young (5), except that, before disruption, the cells were washed twice with a solution containing 10 mM Tris (pH 7.5)–10 mM $MgCl_2$–5 mM EDTA–10 mM β-mercaptoethanol–10% glycerol–0.5 mM phenylmethylsulfonyl fluoride and 1 M KCl and once with the same solution containing 50 mM KCl. The conditions of electrophoresis and the composition of 3% polyacrylamide gels (acrylamide-bisacrylamide ratio, 15: 1) were essentially as described by Gegenheimer et al. (4). Lanes: A, lysate of uninfected UV-irradiated B. subtilis; B, lysate of SP82-infected B. subtilis; C, SP82 processed RNA; D, SP82 in vitro RNA; E, lysate of SP01-infected B. subtilis; F, SP01 processed RNA; G, SP01 in vitro RNA. Examples of in vivo RNAs and processed RNAs with matching mobilities are indicated with bars between lanes A and B and lanes D and E. The approximate molecular sizes of selected RNAs, estimated from the sizes of known markers (11), are shown between lanes C and D and are given in bases. Equal amounts of ^{32}P-labeled RNA were applied to lanes B, C, E, and F on the same gel, and autoradiography of these lanes was done identically.*

RNAs were synthesized in vitro in the presence of [α-^{32}P]ATP as described by Panganiban and Whiteley (11); lanes D and G of Fig. 1 show the RNA species synthesized by *B. subtilis* RNA polymerase from SP82 and SP01 DNAs, respectively. The SP82 RNAs have been mapped, and most arise by transcription of genes located in the redundant ends of the genome (11). Similarly, the SP01 RNA synthesized under these conditions is derived from corresponding regions of SP01 DNA (Downard, unpublished data). Most of the SP82 and SP01 RNAs observed in these experiments were large, with many of the major species in excess of 1,500 bases in length. Despite the fact that the in vivo and in vitro SP82 and SP01 early RNAs are generally transcribed from the same regions of DNA, comparisons of lanes B and D and lanes F and G of Fig. 1 reveal little similarity.

A number of explanations may be proposed to account for the dissimilarity between the in vivo and in vitro RNAs. One is that large primary transcripts, such as those synthesized in vitro, are processed in vivo, thereby generating the smaller RNAs observed in lanes B and E of Fig. 1. To test this possibility, 10-μl portions of the RNA samples shown in lanes D and G of Fig. 1 were supplemented with a 50-fold excess of unlabeled nucleotide triphosphates and heated to inactivate the polymerase. The preparations were then incubated for 10 min at 37°C with 10 μl of a dilution of an S-30 fraction prepared from an extract of uninfected *B. subtilis* cells and analyzed by gel electrophoresis. It should be noted that the amount of trichloroacetic acid-precipitable RNA remained constant throughout the second incubation, indicating that there was no net synthesis or degradation of labeled RNA.

As shown in lanes C and F of Fig. 1, reaction mixtures which had been incubated with the S-30 fraction contained less high-molecular-weight RNA and a number of new smaller RNAs; several of these new RNAs (examples indicated with bars in Fig. 1) had mobilities which matched those of RNAs synthesized in vivo. Since most of the RNAs generated by exposure to the S-30 extract were relatively small, samples were electrophoresed on a 6% polyacrylamide gel to enhance resolution of the smaller species. Figure 2 shows at least five RNAs generated by the S-30 extract which match early SP82 RNAs (lanes B and C of Fig. 2) and five prominent "processed" RNAs which match early SP01 RNAs (lanes E and F). Several of the smaller processed RNAs and in vivo RNAs of matching mobilities (for example, the two smallest matching RNAs shown in lanes D and E of Fig. 2) were eluted from excised portions of the gel and hybridized to restriction fragments of SP82 and SP01 DNAs which had been separated by gel electrophoresis and transferred to nitrocellulose (16). No differences were found in the restriction

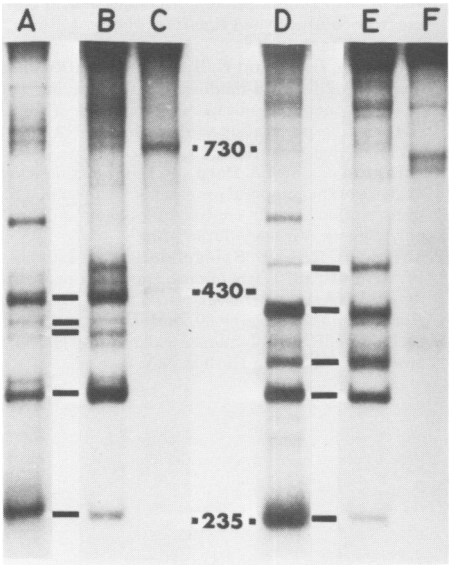

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel analysis of ^{32}P-labeled SP82 and SP01 RNAs. The same RNA samples used in Fig. 1 were analyzed on a 6% polyacrylamide gel. The composition of the gel was as in Fig. 1, except that the acrylamide-bisacrylamide ratio was 30:8. Lanes: A, lysate of SP82-infected B. subtilis; B, SP82 processed RNA; C, SP82 in vitro RNA; D, lysate of SP01-infected B. subtilis; E, SP01 processed RNA; F, SP01 in vitro RNA. Examples of in vivo RNAs and processed RNAs with matching mobilities are indicated with bars between lanes A and B and lanes D and E. The approximate molecular sizes of selected RNAs are shown between lanes C and D and given in bases.*

fragments hybridized by in vivo RNAs and their matched processed RNAs (data not shown). Preliminary experiments were performed attempting to process in vitro RNA with an extract of *Escherichia coli* or with purified RNase III from *E. coli*, but no RNAs were generated which exhibited matching mobilities with in vivo RNAs.

The processing of *E. coli* tRNA and rRNA, phage T4 tRNA, and phage T7 mRNA have been well characterized, and several processing enzymes have been identified and purified (1). Only one example of processing in *B. subtilis* has been carefully documented, that of the maturation of 5S rRNA by RNase M5 (14, 15). The results presented in Figs. 1 and 2 and the preliminary experiments on hybridization suggest strongly that RNAs produced in vitro by transcription of SP82 and SP01 DNAs are cleaved to generate smaller molecules which have mobilities which match those of RNAs produced in vivo; direct proof that matching RNAs are indeed identical will require two-dimensional fingerprinting of RNase T_1-generated oligonucleotides or sequencing. The present observations also suggest that some of the RNAs produced in vivo may arise by processing; additional mechanisms (e.g., transcriptional termination factors) may be involved in the production of other RNAs in vivo. Conclusive demonstration of RNA processing in vivo in this system awaits the elucidation of precursor-product relationships among the observed in vivo and in vitro RNAs and the isolation of mutants defective in the processing enzymes(s). The results of this study provide an assay for the processing activity and, when combined with the detailed transcription map of early in vitro RNA, should allow mapping of the sites for specific RNA cleavage.

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