TWO PRECURSOR 5 S RNA SPECIES IN *BACILLUS LICHENIFORMIS*: CHARACTERIZATION AND PARTIAL ANALYSIS OF PRIMARY STRUCTURE

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1. Introduction

Both in pro- and in eukaryotic cells the genes for the two large ribosomal RNA species constitute a single transcriptional unit [1–6]. The primary transcript of this unit is processed to the final products in a fairly complex sequence of events [1–6], which in bacteria starts while transcription is still in progress [7–11]. Apart from posttranscriptional modification of nucleotides, maturation involves removal of a large part of the primary transcript: about 25% in prokaryotes [7–10] and up to 50% in eukaryotes [1–5].

Although the general outlines of the maturation process of pro- and eukaryotic rRNA are fairly well understood, study of the fine mechanism is hampered by the large size of these RNA's. Therefore, the formation of the smaller bacterial 5 S rRNA has attracted much attention as a model system for study of maturation processes. This attention has been focused mainly on *B. subtilis*, since, in contrast to Gram-negative organisms where only one short precursor of 5 S RNA is present [12,13], *B. subtilis* contains two, independently maturing, precursor 5 S RNA species with 37 and 63 extra nucleotides respectively [14]. It has been reported that the extra sequences are located at both the 3′- and the 5′-termini [6]. The existence of precursor 5S RNA's of similar length in *B. megatherium* has been described [14].

Here we report the existence of two precursor 5 S RNA species of even larger size in *B. licheniformis*. These precursors are about twice as long as their mature counterpart and are processed independently of each other. The extra nucleotides are shown to be located at both the 3′- and the 5′-termini.

2. Materials and methods

2.1. *Growth and labeling conditions*

B. licheniformis (laboratory strain S244) was grown in flasks on a synthetic medium as described previously [15]. In case the cells were to be labeled with $^{32}P_i$ the phosphate concentration of the medium was reduced to 0.2 mM.

Cells were labeled uniformly with either [2-^{14}C] uracil (New England Nuclear Corp., Boston, Mass., U.S.A.; 0.1 μCi/ml) or $^{32}P_i$ (Philips Duphar, Petten, The Netherlands; 25 μCi/ml) by incubating an exponentially growing culture with the isotope for about three doubling times.

Labeling in the presence of chloramphenicol was carried out by adding 200 μg/ml of the inhibitor to an exponentially growing culture at a density of 2×10^8 cells/ml, followed three min later by [5-^{3}H]uridine (New England Nuclear Corp.; 1.0 μCi/ml) or $^{32}P_i$ (125 μCi/ml). Cells were harvested 20 min later by rapid cooling and centrifugation.

B. subtilis (strain 168) was grown and labeled in the same way.

2.2. *Isolation of low molecular weight RNA*

Labeled cells were converted to spheroplasts by treatment with lysozyme (Sigma Chem. Co., St. Louis, Mo., U.S.A.), lysed by addition of Brij-58 and the lysate was incubated with desoxyribonuclease (Sigma; 5.0 μg/ml) for 10 min at 0°C [15]). The lysate was then centrifuged for 5 min at 10 000 *g*, and RNA was extracted from the pellet at 0°C with phenol-SDS as described by Retèl and Planta [16]. Low molecular weight RNA was isolated by sucrose gradient

centrifugation [17], precipitated with ethanol and dissolved in the buffer used for polyacrylamide gel electrophoresis containing 5% sucrose or 10% glycerol.

2.3. *Polyacrylamide gel electrophoresis*

Analytical as well as preparative separation of low molecular weight RNA species was carried out by disc gel electrophoresis on 10% polyacrylamide gels [17] using the discontinuous buffer system described by Richards et al. [18]. In some experiments 7% glycerol was added to the gels to facilitate slicing. RNA labeled with $^{32}P_i$ for fingerprinting was eluted from the slices with 1.0 M NaCl, dialysed and lyophilized [17].

2.4. *Digestion of RNA and analysis of oligonucleotides*

^{32}P-labeled RNA was mixed with carrier RNA and digested with either T_1-ribonuclease (Sankyo Co., Ltd., Tokyo, Japan) or pancreatic ribonuclease (Sigma) as described previously [17,19]. The digestion products were separated by two-dimensional electrophoresis according to Sanger et al. [19]. Oligonucleotides were further analysed by digestion with either KOH or snake venom diesterase (Sigma) as described by Jeppesen [20].

3. Results and discussion

When a culture of *B. licheniformis* is labeled with [^{3}H]uridine in the presence of a high concentration of chloramphenicol no labeled mature 5 S RNA can be detected, while two low molecular weight RNA species with a much lower electrophoretic mobility than 5S RNA appear [fig.1A]. Although the positions of these particular RNA species on the polyacrylamide gel indicate a length appreciably greater than reported for the two precursor 5 S RNA species from *B. subtilis* [14], it seemed likely that they represented precursors of *B. licheniformis* 5 S RNA; the more so because (i) like 5 S RNA they were found to be unmethylated (data not shown) and (ii) they were found to be present in the electrophoretic pattern of RNA obtained from pulse labeled cells (data now shown), but not in the pattern of RNA from uniformly labeled cells (Fig.1). The two RNA species were therefore tentatively designated as p_1 5 S and p_2 5 S.

The existence of a precursor-product relationship between p_1 5 S and p_2 5 S on the one hand, and mature 5 S RNA on the other is supported by the experiment shown in fig. 1. Both p5 S RNA species, accumulated

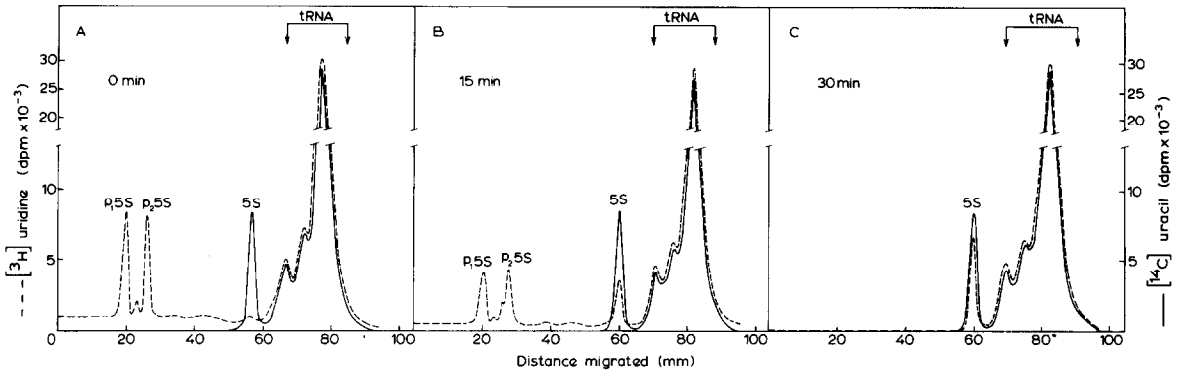

Fig.1 A. Polyacrylamide gel electrophoresis of low molecular weight RNA isolated from *B. licheniformis* labeled in the presence of chloramphenicol. Exponentially growing cells of *B. licheniformis* were labeled uniformly with [2-^{14}C]uracil (0.1 μCi/ml). Chloramphenicol (200 μg/ml) was added when the culture reached a density of 2 × 10^8 cells/ml followed 3 min later by [5-^{3}H] uridine (1.0 μCi/ml). After an additional 15 min incubation, rifampicin (Lepetit, 150 μg/ml) was added and 2 min later the culture was rapidly cooled and the cells harvested by centrifugation. Low molecular weight RNA was isolated from a portion of the cells and analysed on 10% polyacrylamide gels. Figs.1B and C.Metabolic fate of the two large low molecular weight RNA species present in chloramphenicol-treated cells. The rest of the labeled cells was resuspended in fresh prewarmed medium containing 150 μg/ml of rifampicin and incubated at 37°C. At different times after resuspension aliquots were taken, cooled rapidly and low molecular weight RNA was isolated and analysed on 10% polyacrylamide gels. Panels B and C: labeling pattern 15 and 30 min after removal of chloramphenicol respectively.

in the presence of chloramphenicol (fig. 1A), disappear when the inhibitor is removed, concomitant with the appearance of mature 5 S RNA, even when de novo RNA synthesis is blocked by addition of rifampicin (figs. 1B and 1C). Moreover, one can calculate that, at any moment after removal of chloramphenicol, the increase in the amount of label present in 5S RNA is equal to half the decrease in the amount of label originally present in the sum of p_1 5S and p_2 5S. This is in agreement with the electrophoretic mobility of the two precursor species which indicates a length approximately twice that of mature 5S RNA.

Fig.2 shows the two-dimensional separation of the oligonucleotides obtained after ribonuclease T_1 digestion of the three ^{32}P-labeled RNA species. The results leave no doubt that both p_1 5S and p_2 5S are structurally related to mature 5S RNA; all spots present in the fingerprint of 5S RNA are also present in the fingerprints of both p_1 5S and p_2 5S, except for spot 18 which represents the 5'-terminal fragment of 5S RNA (see below). Moreover, all spots present in the fingerprint of p_2 5S RNA, the shortest of the two precursors, can also be detected in the fingerprint of p_1 5S RNA. Although this suggests that maturation

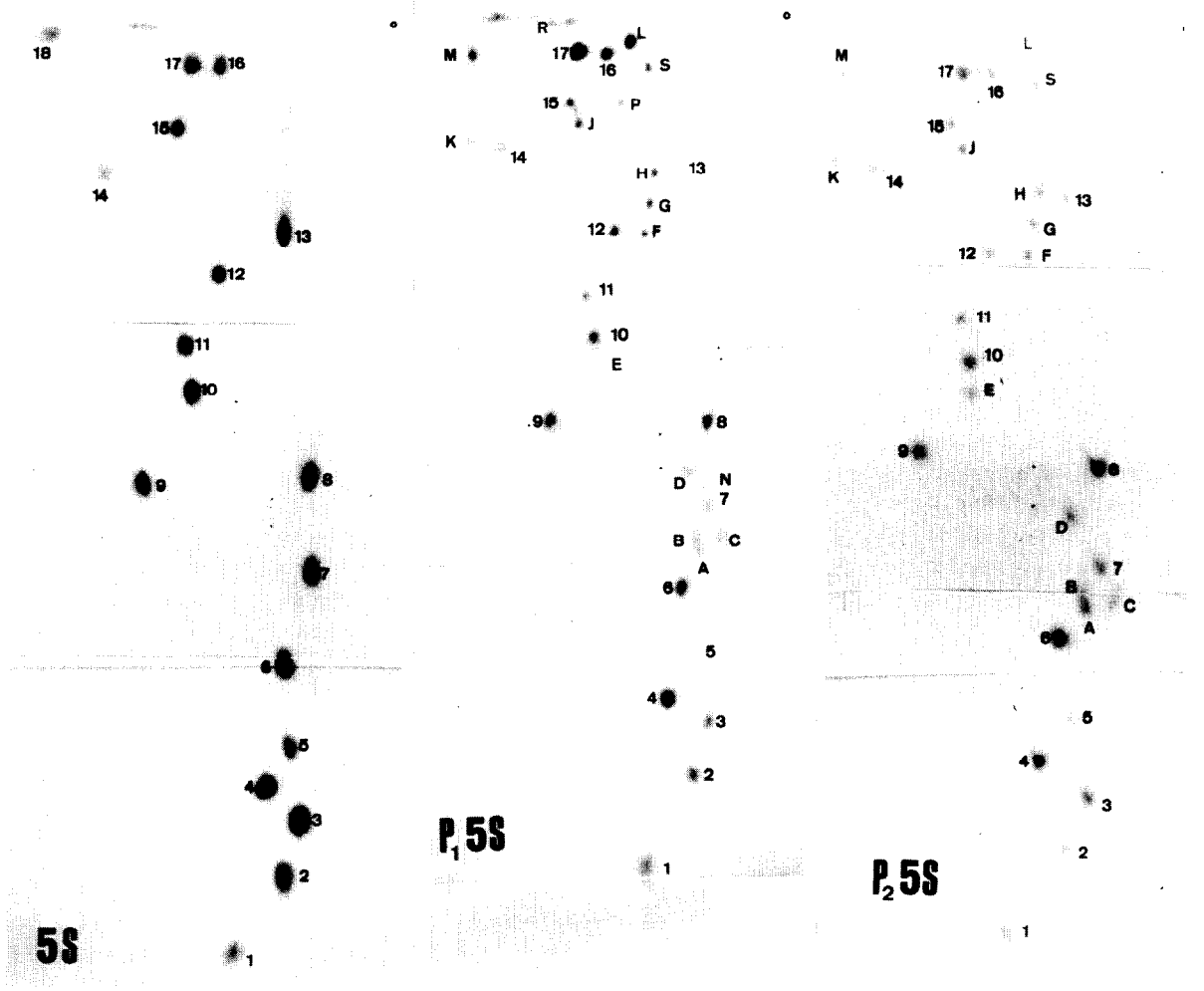

Fig.2. Two-dimensional separation of the oligonucleotides obtained after digestion of ^{32}P-labeled p_1 5S, p_2 5S and 5S RNA by ribonuclease T_1. For experimental details see Materials and methods.

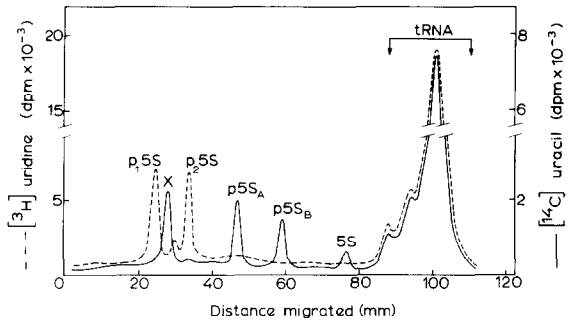

Fig. 3. Comparison of the sizes of precursor 5S RNA species from *B. subtilis* and *B. licheniformis*. Cultures of *B. subtilis* 168 and *B. licheniformis* S244 were labeled for 20 min in the presence of 200 μg/ml chloramphenicol with [^{14}C] uracil (0.75 μCi/ml) and [^{3}H]uridine (1.5 μCi/ml) respectively. Low molecular weight RNA was isolated and analysed on 10% polyacrylamide gels. Nomenclature for the *B. subtilis* precursors is according to Pace et al. [14]. Peak X represents an RNA species not structurally related to 5S RNA [14].

might proceed in a sequential fashion from p_1 5S via p_2 5S to mature 5S RNA, kinetic analysis of the data from the experiment shown in fig.1 indicates that, as in *B. subtilis* [14], maturation of the two precursor species from *B. licheniformis* proceeds independently.

The much greater length of the *B. licheniformis* precursors as compared to those from *B. subtilis*, also indicated by the large number of extra spots in the fingerprints of p_1 5S and p_2 5S, is confirmed by the data shown in fig.3. In this experiment low molecular weight RNA was isolated from a mixture of *B. subtilis* and *B. licheniformis* labeled in the presence of chloramphenicol with [^{14}C]uracil and [^{3}H]uridine respectively. Assuming a length of 77 nucleotides for the bulk of tRNA [21], 115 for mature 5S RNA [14] and 178 and 152 nucleotides, respectively, for $p5S_A$ and $p5S_B$ from *B. subtilis* [14], one arrives at a length of about 240 nucleotides for p_1 5S RNA and about 220 nucleotides for p_2 5S RNA. Similar values were obtained when the total recovery of radioactive oligonucleotides from the fingerprints was divided by the average value of cpm per phosphate residue in known unimolar oligonucleotides.

To determine the location of the extra sequences present in p_1 5S and p_2 5S RNA, their 3'- and 5'-termini were compared to those of mature 5S RNA. Spot 18 in the fingerprint of 5S RNA (fig.2) was found to have the sequence pUpUpUpGp-, as determined from an alkaline digest of this fragment, and thus represents the 5'-terminal fragment. The same 5'-terminal sequence is present in *B. subtilis* 5S RNA [14] while *B. stearothermophilus* 5S RNA possesses a radically different 5'-terminus [22]. The 5'-termini of both p_1 5S and p_2 5S are represented by spot K (fig.2). Alkaline digestion of this fragment showed the sequence to be pUpGp- also identical to the 5'-termini of the *B. subtilis* precursors [14]. Since both p_1 5S and p_2 5S contain the sequence -UpUpUpGp- (spot M in fig.2) at least some of the extra nucleotides are located at the 5'-end.

The 3'-terminal sequence of mature 5S RNA from *B. licheniformis* was identified by digestion of the RNA with pancreatic ribonuclease and subsequent separation of the digestion products by two-dimensional electrophoresis [19]. The separate oligonucleotide fragments were further digested with KOH and snake venom phosphodiesterase. The 3'-terminal fragment, which can be identified by the absence of either Up or Cp from its alkaline digest, was found to have the sequence-Ap-ApGpC_{OH}. This sequence is similar, though not identical, to the 3'-terminal sequence of 5S RNA from *B. stearothermophilus* [22].

Both p_1 5S and p_2 5S RNA have 3'-terminal sequences different from that of mature 5S RNA. In the fingerprints of p_1 5S and p_2 5S RNA, obtained after digestion with pancreatic ribonuclease, no radioactivity could be detected at the spot where the -ApGpC_{OH} fragment should be located. The extra nucleotides present in the two precursors of 5S RNA, thus, are located at both the 3'- and the 5'- end. It proved to be impossible to identify unequivocally the 3'-terminal fragments of p_1 5S and p_2 5S RNA in the same way as described above for mature 5S RNA, due to the complexity of the fingerprints in these cases. However, preliminary analysis of the two precursor RNA species, using the two-dimensional diagonal electrophoresis technique developed by Dahlberg [23], suggests the existence of slight differences between the 3'-termini of p_1 5S and p_2 5S RNA. As shown in fig.2, the 5'-termini of both precursor 5S RNA's are identical. If we assume that the 3'-terminal sequence of precursor 5S RNA is the final sequence to be transcribed from the transcriptiona unit for the 3 ribosomal RNA species, our result indicates the existence of two types of cistrons for 5S

RNA in *Bacillus licheniformis,* one about 20 nucleotides longer than the other at the 3′ end. If, however, the transcriptional unit extends beyond the sequence for precursor 5S RNA, $p_1$5S and $p_2$5S RNA could be different cleavage products from the same primary transcript.

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