Small cytoplasmic RNA of Bacillus brevis: transcriptional and phylogenetic analysis

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Using a DNA fragment of Bacillus subtilis scRNA as a probe, a Bacillus brevis gene encoding the small cytoplasmic RNA was cloned and characterized. B. brevis scRNA consists of 273 nucleotides; the sequence has comparatively low homology (approximately 70%) with other Bacillus sequences. Phylogenetic analysis indicated that B. brevis forms a line of descent distinct from other Bacillus species. However, despite the low overall homology, both functional nucleotide sequence and secondary structural features defined among signal recognition particle (SRP) RNA family members were well conserved.

Keywords: small cytoplasmic RNA, signal recognition particle, Bacillus brevis, phylogenetic analysis

INTRODUCTION

Signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein which targets the ribosomes translating nascent secretory proteins to the endoplasmic reticulum membrane (Walter & Lingappa, 1986). The extensively studied canine particle is composed of one RNA molecule (here referred to as SRP RNA) and six polypeptides (Walter & Blobel, 1980, 1982). SRP RNA-like RNAs have been identified in a wide range of eukaryotes, including humans, plants and yeast, and in a wide variety of archaeobacteria (Larsen & Zwieb, 1991).

The small cytoplasmic RNA (scRNA; 271 nucleotides) of Bacillus subtilis is considered to be a member of the SRP RNA family. Although dissimilar in size and overall primary structure to mammalian SRP RNA, it contains domains of which the secondary structures correspond to domains I, II and IV of the SRP RNA-like RNAs of archaeobacteria and eukaryotes. The nucleotide sequence in domain IV is highly conserved (Poritz et al., 1988; Kaine, 1990). Functional analyses have shown that this RNA is essential for the cell viability of B. subtilis (Nakamura et al., 1992a). To identify the functional domains of scRNA, knowledge of its structure is required, and phylogenetic comparisons between homologous RNAs have proved useful in elucidating a higher-order RNA structure (Romero & Blackburn, 1991; Haas et al., 1991). It is therefore useful to compare scRNAs from more closely related species. The scRNA cataloguing studies have so far been restricted to two Bacillus species, B. subtilis and B. steaothermophilus (Struck & Erdmann, 1990). Consequently, it has not been possible to draw reliable conclusions about the phylogenetic structure of the genus. To identify the conserved nucleotides and structural elements of scRNA, we determined the nucleotide sequence of scRNA from various bacilli (Nakamura et al., 1992b). DNA fragments corresponding to the mature scRNA of ten Bacillus species were obtained by polynucleotide chain reaction (PCR), using primers based upon comparisons of the nucleotide sequence of scRNAs between B. subtilis and B. steaothermophilus. A positively amplified DNA fragment was not obtained in B. brevis.

In this report, we describe the cloning and nucleotide sequencing of the entire structural portion of the B. brevis scRNA gene. We also determined the 5' and 3' ends of mature scRNA. A pairwise comparison of scRNA sequences from 13 Bacillus species indicates that B. brevis forms a distinct line of descent in the genus Bacillus.

METHODS

Bacterial strains. Escherichia coli JM109 (Yanisch-Perron et al., 1985) and NM514 [hidR314 (r5mC) argH5 galE galX strA thyB (His')'] were used as cloning hosts. B. brevis ATCC 5246 was obtained from the Bacillus Genetic Stock Center (Ohio State University). The cells were cultured in L broth at 37 °C.

DNA and RNA preparation. Chromosomal DNA of B. brevis ATCC 5246 was prepared by the method of Saito & Miura

Abbreviations: scRNA, small cytoplasmic RNA; SRP, signal recognition particle.

The GenBank accession number for the nucleotide sequence reported in this paper is D14472.
K. NAKAMURA and OTHERS

from diethyl pyrocarbonate (DEPC). DNA Southern hybridization. 1992a) and stored at -80 °C in distilled water treated with diethyl pyrocarbonate (DEPC).

**DNA Southern hybridization.** DNA preparations digested with restriction enzymes were separated by agarose gel electrophoresis and transferred onto Gene Screen Plus nylon membranes (NEN Research Products). Hybridization under low stringency proceeded as described previously (Nakamura et al., 1990). The 271 bp DNA fragment, corresponding to mature scRNA, was amplified from the chromosomal DNA of B. subtilis 168 using PCR with the synthetic oligonucleotide primers TTTGCCGTGCTAAGCGGG 5' and CGGCACATGAGAGGTAATTCCCAT-3'. The amplified DNA fragment was inserted at the Smal site of M13mp10. To prepare the specific probe which cannot hybridize to the 4S rRNA gene of E. coli but can hybridize to Bacillus scRNA, 42 nucleotides in domain IV, corresponding to +144 to +185, were deleted by oligonucleotide-directed mutagenesis, using the mutagenic oligonucleotide 5' CGGCACATGAGAGGTAATTCCCAT- TGCCGA 3'. After KpnI-BamHI digestion of the double-stranded M13 phage DNA, the 234-nucleotide fragment of Bacillus subtilis scRNA gene without domain IV was isolated by polyacrylamide gel electrophoresis. This fragment was labelled with [α-32P]dCTP using a random primer DNA labelling kit (Takara Shuzo Co.) and used as the probe.

**RESULTS AND DISCUSSION**

**Cloning and sequencing of the scRNA gene region**

To verify the existence of the **scr** gene in chromosomal DNA of B. brevis ATCC 5246, Southern hybridization was performed using the B. subtilis scRNA gene without domain IV as a probe. A 3.8 kb PvuII-HincII fragment specifically hybridized with the probe. PvuII–HincII-digested chromosomal DNA from B. brevis ATCC 5246 was resolved by agarose gel electrophoresis. Fragments of 3–5 kb were extracted from the gel and ligated with the EcoRI–NotI adaptor (5' dAAATTCGCGGCCGCT 3', 5' dAGCGGCGCCC CGG 3') to create EcoRI sites at both ends. The resultant DNA fragments were inserted into λgt10, packed *in vitro*, then transfected into E. coli NM514. Among 5000 plaques, 25 carried the 3.8 kb insert. Plasmid pTUE825 was constructed by subcloning the 3.8 kb NotI fragment from the phage DNA into the NotI site of pBluescript II KS(+). Physical mapping followed by Southern analysis of pTUE825 revealed that a NotI–DraI fragment of about 1.1 kb contains the scRNA coding region (Fig. 1) and the entire region of this fragment was sequenced (Fig. 2a). The sequences were aligned with the mature form of B. subtilis scRNA (Fig. 2b). The scRNA coding region of B. brevis deduced from this alignment, underlined in Fig. 2(a), is about 70% identical to that of B. subtilis scRNA. About 40 bp upstream from the putative scRNA coding region, a possible promoter region was identified (−35 box, TTGCTA; −10 box, TATTCT), which was highly homologous with the consensus sequence recognized by the B. subtilis σ^A RNA.
Characterization of *B. brevis* scRNA

Fig. 2. (a) Nucleotide sequence of the chromosomal segment containing the *B. brevis* scRNA gene. The nucleotide sequence of the *Dral–Not1* fragment in pTUE825 was determined and numbered arbitrarily. The sequence of mature scRNA of *B. subtilis* deduced by alignment with that of *B. brevis* is underlined. The presumed promoter elements (–10 and –35) are boxed. The positions of the 5′ and 3′ ends of the mature scRNA determined in Fig. 3 are indicated by single and double asterisks, respectively. Palindromic sequences which may serve as transcriptional terminator are indicated by converging arrows. A nucleotide sequence complementary to the synthetic oligonucleotide primer used in primer extension is overlined.

Fig. 3. Determination of the 5′ and 3′ ends of *B. brevis* scRNA. (a) Mapping the 5′ terminus by primer extension using primer NK1 (Fig. 2a). RNA samples were prepared from *B. brevis* cultured for 6 (lane 1), 9 (lane 2) and 12 h (lane 3) after inoculation. Sequencing reactions (G, A, T and C) served as size markers. (b) Mapping the 3′ terminus by means of an RNase protection assay of the scRNA gene transcript. RNA samples were prepared from *B. brevis* cultured for 3 (lane 1), 6 (lane 2), 9 (lane 3) and 12 h (lane 4). Sequencing reactions (G, A, T and C) served as size markers. The lengths of some fragments are indicated. Protected major and minor fragments are indicated by arrows. (c) Suggested secondary structure of the 3′ portion of the *scr* gene, highlighting the secondary structure that may serve as the p-independent terminator. Arrows are according to (b).

Mapping the 5′ and 3′ ends of scRNA

To examine the *in vivo* functionality of these transcriptional regulatory elements, both the 5′ and 3′ ends of scRNA were mapped. In addition, we investigated whether processing events occurred in *B. brevis* scRNA, since RNA processing is involved in the maturation of the *B. subtilis* scRNA. The 5′ end was identified by primer extension. Using the NK1 primer, only a 75 nucleotide band appeared (Fig. 3a), indicating that transcription starts from the T residue at nucleotide position 473 (Fig. 2a). This position matched well with the 5′ end predicted by polymerase (–35 box, TTGACA; –10 box, TATAAT). About 30 bp downstream from the scRNA coding region there is an almost perfect palindromic sequence comprising 26 bp (ΔG = –25.7 kcal mol⁻¹; –107.5 kJ mol⁻¹) as calculated by the method of Tinoco et al. (1973), followed by a run of thymidine residues (Fig. 3c). This region resembles a characteristic *E. coli* p-independent transcription terminator (Adhya & Gottesman, 1978).
Table 1. Similarity and nucleotide distance data among scRNA gene sequences of Bacillus spp.

The upper right half of the table gives similarity values, \( H \), for all pairwise comparisons of the scRNA sequences from 13 Bacillus species. \( H \) is defined as in Sogin et al. (1986), where \( H = m/(m + u + g/2) \); \( m \) is the number of sequence positions with matching nucleotides in the two sequences, \( u \) is the number of sequence with nonmatching nucleotides, and \( g \) is the number of sequence positions that have a gap in one sequence opposite a nucleotide in the other. The absolute number of base changes and gapped positions is shown in the lower half of the table. The species names are abbreviated as follows: B. subtilis 168 (B. sub), B. amylobacteriaceus H (B. amy), B. polymyxa ATCC 842 (B. pol), B. sphaericus 1593 (B. sph), B. pumilus BP1 (B. pum), B. macerans BKM B-51 (B. mac), B. megaterium 899 (B. meg), B. thuringiensis B4039 (B. thu), B. cereus T (B. cer), B. circulans ATCC 4513 (B. cir), B. stearothermophilus 799 (B. ste), alkaliphilc Bacillus C-125 (B. C125) and B. brevis ATCC 5246 (B. bre).

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Alignment with the B. subtilis mature scRNA sequence (Fig. 2b). No other minor pre-scRNA was detected by primer extension.

For 3' end mapping, a 0.6 kbp BamHI-EcoRI fragment encompassing the 3' portion and the 3' flanking region of the B. brevis scRNA gene (Fig. 1) was cloned into the riboprobe plasmid pBluescript II SK(−) and used to generate a 32P-labelled antisense RNA probe. RNA was prepared 3, 6, 9, and 12 h after inoculation. Fig. 3(b) shows three major and several minor protected RNA species. The three major species comprised 210, 206, and 204 nucleotides, among which the longest (210 nucleotides) may represent the 3' end of mature scRNA. The 3' end heterogeneity is probably an artifact caused during RNA preparation or the RNase protection assay, since the smaller protected RNA fragments varied in length with the experiment and RNA preparation. This suggests that the transcription of the scRNA gene terminates at the A residue at nucleotide position 745. The 5' and 3' mapping study shows that the mature form of B. brevis scRNA consists of 273 nucleotides.

Phylogeny of Bacillus scRNA

Including the nucleotide sequence of B. brevis scRNA reported here, sequence data of scRNAs from 13 Bacillus species are now available. Gram-positive, rod-shaped, aerobic or facultatively anaerobic spore-forming bacteria have traditionally been assigned to the genus Bacillus. This genus as constituted in Bergey's Manual of Systematic Bacteriology (Claus & Berkeley, 1986) is phenotypically heterogeneous, demonstrating an extremely wide range of nutritional requirements, growth conditions, metabolic diversity and DNA composition. A phylogenetic matrix study of the scRNA sequence also shows that the genus Bacillus is phylogenetically very heterogeneous (Table 1). B. brevis scRNA exhibited remarkably low sequence homologies with the other Bacillus groups. Based upon a comparison of the sequences of small-subunit-ribosomal RNA, Ash et al. (1991) have reported that the genus Bacillus can be separated into phylogenetically distinct groups. Among these, a group consisting of B. brevis and B. laterosporus forms a distinct line of descent exhibiting low levels of relatedness to all other Bacillus groups. This phylogenetic relationship agreed well with that deduced from scRNA. In contrast, a pairwise comparison of small-subunit-ribosomal RNA reveals the very wide percentage sequence homology variation (range approximately 88–99%). Therefore, the overall rate of divergence for scRNA is greater than that for 16S rRNA.

Despite the relatively low sequence homology of the scRNA sequence between B. brevis and other Bacillus species, almost all nucleotide substitutions in scRNA are covariant and do not affect the secondary structure. That is, mutations that individually affect base pairing are often compensated by base changes on the other strand. Consequently, a strikingly similar secondary structure can be drawn for B. brevis scRNA based on that of B. subtilis (Struck & Erdmann, 1990). The presence of a terminal loop, corresponding to domains I and II of human SRP RNA, is restricted to spore-forming Bacillus species among eubacteria. In domain I of B. subtilis scRNA,
nucleotides 24–28 (5’ AGCGG 3’) can pair with nucleotides 46–50 (5’ CCGCU 3’). A potential tertiary interaction has been detected within this region (Fig. 4a). This interaction can be maintained in \( B. \) \textit{brevis} scRNA by complementary base changes at nucleotide positions 24 and 50 (Fig. 4a). Therefore, this interaction is considered to also be important for the function of scRNA. It is notable that the same pairing can form in human SRP RNA and archaeobacterial 7S RNA (Fig. 4b, c), but not in yeast homologues.

On the other hand, the 22-nucleotide sequence in domain IV is completely identical among all \textit{Bacillus} scRNAs. In eukaryotic SRP RNA and \textit{E. coli} 4.5S RNA, this chemical evidence for a secondary structure model of a small cytoplasmic RNA and archaeobacterial 7S RNA (Fig. 4b, c), but not in yeast homologues.

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**REFERENCES**


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