Regulation of 6S RNA biogenesis by switching utilization of both sigma factors and endoribonucleases

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ABSTRACT

In Escherichia coli, 6S RNA functions as a modulator of RNA polymerase $\sigma^{70}$-holoenzyme activity, but its biosynthetic pathway remains uncharacterized. In this study, to further understand the regulatory circuit of 6S RNA biosynthesis for the modulation of $\varepsilon^{70}$ activity, we have characterized the biogenesis of 6S RNA. We reveal that there are two different precursors, a long and a short molecule, which are transcribed from the distal P2 and proximal P1 promoter, respectively. Transcription from the P2 promoter is both $\sigma^{70}$- and $\sigma^S$-dependent, whereas, in contrast, P1 transcription is $\sigma^{70}$- but not $\sigma^S$-dependent. Both precursors are processed to generate the 5' end of 6S RNA, and while the long precursor is processed exclusively by RNase E, the short precursor is processed by both RNase G and RNase E. Our data indicate that the switching of the utilization of both sigma factors and endoribonucleases in the biogenesis of 6S RNA would play an essential role in modulating its levels in E.coli.

INTRODUCTION

Small non-coding RNAs (sRNAs) ranging from 40 to 500 nt in size are involved in a number of cellular processes in bacteria, such as transcription, RNA processing, messenger RNA (mRNA) turnover, protein degradation, and secretion (1–4). sRNA molecules are generally transcribed as longer precursor products of RNA polymerase (RNAP) which then undergo a series of processing reactions to remove extra residues at either the 5' or 3' ends by various ribonucleases to generate the mature, functional forms (5). Although the biogenesis of rRNA and transfer RNA (tRNA) has been extensively studied (6,7), relatively little is yet known about the synthesis of other sRNA types. Nonetheless, the biogenesis of some sRNAs is now well characterized, such as M1 RNA, the catalytic subunit of the RNase P (8,9). M1 RNA is transcribed from the rnpB gene as a precursor molecule (10–12) and eventually formed through a processing event involving RNase E and exoribonucleases (13–16). Another well-characterized example is tmRNA, which is involved in removing products translated from truncated mRNAs (17). tmRNA is transcribed from the ssrA gene and the primary transcript is processed to generate mature tmRNA. This processing reaction requires endoribonucleolytic cleavages by RNase P, RNase III and RNase E, followed by exoribonucleolytic trimming (14–20).

6S RNA, an sRNA molecule, was initially identified by polyacrylamide gel analysis of in vivo labeled total RNA (21). Its abundance in the Escherichia coli genome was estimated to be 1000–1500 molecules, which is ~25% of the total number of ribosomes (22). 6S RNA does not associate with ribosomes, but appears to be complexed with a number of proteins and migrate at about 11S (22). Functional studies of 6S RNA had, however, originally been hampered by the lack of a detectable phenotype for either 6S RNA null mutants (23) or 6S RNA overexpressing cells (24). It was eventually discovered that 6S RNA specifically interacts to the majority of RNAP $\sigma^{70}$-holoenzyme ($E\sigma^{70}$) and reduces its activity, making it possible to alter the utilization of $E\sigma^{70}$ to $E\sigma^S$ in cells following their transition into the stationary phase of growth (25). Furthermore, a recent study on growth phenotypes of 6S RNA-deficient cells suggests that 6S RNA is required for long-term cell survival (26). The fact that 6S RNA accumulates throughout cell growth (25) also suggests that it may mediate growth-dependent changes in the physical properties of RNAP.

A precursor 6S RNA, with six to eight additional bases at the 5' terminus, was described previously (27), indicating that 6S RNA is derived from a larger primary transcript. In addition, 6S RNA is transcribed from the ssrS gene as part of a dicistronic message containing an open reading frame (ORF) (ygfA) at the 3' end (24). However, the ssrS gene promoter has not been well characterized. Furthermore, the maturation mechanism of 6S RNA from its precursor has not been elucidated, except that a plausible 3' trimming mechanism has been hypothesized (14). Given that 6S RNA functions as a regulator of the modulation of $E\sigma^{70}$ activity, the biogenesis of 6S RNA should be regulated according to the requirements for $E\sigma^{70}$ activity. However, the mechanisms underlying this regulation remain obscure, mainly due to the lack of knowledge of 6S RNA biosynthesis.

Our present study was therefore designed to determine the mechanism of 6S RNA synthesis in E.coli and the association

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of this biosynthetic pathway with the regulatory circuits required for the modulation of transcription, in response to a change in growth conditions. We show that two 6S RNA precursors are generated and that they are transcribed from two tandem promoters. A longer precursor is transcribed from a distal $\sigma^{70}$-dependent promoter, P2, and is then processed exclusively by RNase E, whereas the shorter precursor is expressed via a proximal $\sigma^{70}$-dependent promoter, P1, and is processed by both RNase E and RNase G. The findings of this study indicate that the modulation of the cellular levels of 6S RNA during cell growth could be accomplished through the coordination of both transcriptional and post-transcriptional regulation by switching the utilization of sigma factors and endoribonucleases.

Materials and Methods

Bacterial strains

*E. coli* K-12 strain JM109 was used for the construction of plasmids. Strains GW11 (W3110 but cfaA::cat), GW20 (W3110 but amsl), and GW21 (the same as GW20 but cfaA::cat) were kindly provided by Dr. Wachi, and SK7622 (MG1693 but $\Delta$nc-38 $\text{Km}^R$) was provided by Dr. Kushner (28,29). Strain W3110 or MG1693 was used as wild-type controls. A rpoS$^S$ strain, KS1000 (rpoS$^S$::$\text{Km}$), was constructed in a W3110 background by inserting the kanamycin resistance gene ($\text{Km}^R$) into codon 53 of rpoS as described previously (30,31). Briefly, a kanamycin cassette was amplified by PCR from $\text{Tn}3$ using primers $\text{kml}$ (5$'$ TAT GGA CAG CAA GGC AAC CG) and $\text{km}2$ (5$'$ TCA GAA GAA CTC GTC AAG AAG). Then, a recombinant cassette (km-rpoS) was constructed by PCR using the kanamycin cassette as a template with primers km-rpo1 (5$'$ GAT AAC GAT TTG GCC GAA GAG GAA CTT TTA TCG CAG GGA GCA GCC ACA CAG CGA TGG AGA ACA GAC GAA CCC) and km-rpo2 (5$'$ CTT CTT CTT CAG TAC TTT TTA TTA CTA CTT GTC TTA CCT TGG TGA TGG TTA TTT ACT). The resulting PCR product was directly introduced into strain DY330 (30) by electroporation. Recombinants were selected as km$^R$ clones and were confirmed by PCR with rpoS-specific primers (5$'$ GTA GAA CAG GAC CCC AGT GA and 5$'$ TAC GGG TTT GGT TCA TAA TC). The defective prophage in the recombinant was removed as described in the method by Yu et al. (30) to generate strain KS330. Bacteriophage P1-mediated transduction was used to construct KS1000 (rpoS::$\text{Km}$) by employing W3110 (wild type) as the recipient strain, and KS330 as the donor strain. KS1000 was confirmed by sequencing the PCR product amplified with the rpoS-specific primers.

Preparation of total cellular RNA

*E. coli* cultures were grown overnight in Luria–Bertani (LB) broth and diluted 1:100 in the same medium and grown further to an OD$_{600}$ of 0.5 at 30$^\circ$C. In the case of rnc$^{E}$ cells, the cells were grown at 30$^\circ$C to an OD$_{600}$ of 0.5, and then shifted to 44$^\circ$C for 1 h before RNA preparation. For RNA preparation from cells at a specific stage of growth, *E. coli* cells grown overnight were diluted 1:100 in LB broth and continued to be grown at 37$^\circ$C. Aliquots of the cell culture were taken at intervals. Total cellular RNA was isolated by hot phenol extraction as described previously (13). To remove contaminating DNA, DNA-free$^\text{TM}$ (Ambion) was added to the RNA samples according to the manufacturer’s instructions. RNA isolates were further cleaned up using an RNeasy$^\text{TM}$ Mini Kit (Qiagen).

Northern blot analysis

Total cellular RNA extracts of 30 $\mu$g were fractionated on a 5% polyacrylamide gel containing 7 M urea and electrotransferred onto a Hybond-N+ membrane (Amersham Pharmacia). To construct plasmid pKS200, a template DNA generating an antisense 6S RNA probe, an $\text{ssrS}$ DNA fragment between $-3$ and +197, was amplified by PCR and cloned into the HindIII/BamHI site of pGEM3. For the preparation of the antisense 6S RNA probe, the HindIII-digested pKS200 was used as a template for *in vitro* transcription with T7 RNAP. *In vitro* transcripts were labeled internally with $[^{32}\text{P}]$CTP. Oligonucleotides anti-5S (5$'$ CGG CAT GGG GTC AGG TGG) and anti-rnaI (5$'$ GTG GTT GTT GTG TCG GAT) were labeled at the 5$'$ end with $[^{32}\text{P}]$ATP and polynucleotide kinase, and they were used as probes for 5S RNA and RNA I, respectively. Hybridization was performed as described previously (13). Quantitative analysis of hybridization signals was performed using an Image Analyzer BAS1500 (Fuji).

RACE assays

5$'$ RACE (rapid amplification of cDNA ends) analysis was carried out as described previously (32), with minor modifications. Briefly, 1 $\mu$g of total cellular RNA was treated with 1 U of tobacco acid pyrophosphatase (TAP) in a 50 $\mu$l reaction. The TAP-treated RNA was then ligated with 500 pmol of A3 (32), as a 5$'$ adaptor RNA, at 15$^\circ$C for 16 h with 50 U of T4 RNA ligase (New England Biolabs) in a 50 $\mu$l reaction. Adaptor-ligated RNA was then reverse transcribed and PCR amplified using a Titan One-Tube RT–PCR kit (Roche Applied Science) according to the manufacturer’s instructions. PCR products were separated on a 3% agarose gel and purified by gel elution. They were analyzed by DNA sequencing after cloning into a pGEM-T-easy vector (Promega). 3$'$ RACE analysis was carried out identically to 5$'$ RACE analysis except that total cellular RNA (1 $\mu$g) was first dephosphorylated with 0.01 U of calf intestine alkaline phosphatase (Promega) in a 50 $\mu$l reaction, ligated with E1 3$'$ adaptor RNA (32).

Primer extension analysis

To identify the 5$'$ ends of 6S RNA transcripts, primer extension analysis was performed using primer $a$ (5$'$ TTC TAT TGG TTA TTA TGG TGA ATA TCG G) and primer $b$ (5$'$ ACT TGC CCG GTA GTC ACG AGT). Primer cat (5$'$ ACG GTG GTA TAT CCA GTG AT) was used to analyze $\text{ssrS}$-CAT fusion transcripts. These primers were labeled with $[^{32}\text{P}]$ATP at the 5$'$ end with T4 polynucleotide kinase, and total cellular RNA (30 $\mu$g) was annealed to 2 pmol of each labeled primer in a 25 $\mu$l reaction with four subsequent incubation steps: at 70$^\circ$C for 5 min, 42$^\circ$C for 20 min, 25$^\circ$C for 1 h and on ice for 10 min. The primer extension reactions were performed at 42$^\circ$C for 90 min according to the manufacturer’s instructions with 20 U of AMV-RT (Promega) and a dNTP mixture (0.5 mM each).
The resulting products were then analyzed on an 8% denaturing polyacrylamide gel. Quantitative analysis was performed using an Image Analyzer BAS1500 (Fuji).

**S1 nuclease mapping**

S1 nuclease mapping was performed essentially as described previously (33). Briefly, for the preparation of the DNA probe, plasmid pKS840, which was constructed by cloning a PCR-amplified ssrS DNA fragment between −420 and +420 into the HindIII/BamHI site of pGEM3, was digested with SphI and labeled at the 3′ end with [α-32P]dATP (Amersham Pharmacia) by terminal transferase (Roche Applied Science) according to the manufacturer’s instructions. The labeled DNA was then digested with EcoRI, and a 330 bp SphI–EcoRI DNA fragment (corresponding to +88 to +418 of ssrS) was gel purified and this 3′ end-labeled DNA probe (0.2 pmol) and total cellular RNA (50 μg) were hybridized in 30 μl of S1 hybridization buffer (40 mM PIPES, pH 7.5, 1 mM EDTA, 0.4 M NaCl and 80% formamide). The hybridized RNA was subsequently treated with 300 U of S1 nuclease (Promega) and analyzed on an 8% polyacrylamide sequencing gel.

**In vivo promoter assay**

Promoter-containing DNA fragments were obtained by PCR from genomic DNA with the primer pairs SSR1A/SSR1B and SSR2A/SSR2B: SSR1A, 5′ AGA CTC GTG ACT ACG CGG CAA; SSR1B, 5′ CCC CAC AAT TCA ATC GCC TCA GGG GAC; SSR2A, 5′ CGC GGA TAC GCC GTT CAC TGC GTG TGA ACT; SSR2B, 5′ CCC CAG CTT TGT GCT TCT GTC GCT AAC. The resulting PCR products were then digested with BamHI and HindIII and ligated into pKK232-8 (Amersham Pharmacia) to generate the ssrS-CAT fusion plasmids pSSR1and pSSR2, respectively. The overnight culture of JM109 cells containing the ssrS-CAT fusion plasmids was diluted to 1:300 in LB supplemented with ampicillin (50 μg/ml) followed by the addition of different concentrations of chloramphenicol. Following a 3 h incubation at 37°C, the OD600 of the cultures was measured to assess cell growth inhibition and used to determine the concentration of chloramphenicol required for the 50% inhibition of the growth (IC50) of cells as described previously (34). Alternatively, total cellular RNA was isolated from the exponentially growing JM109 cells and subjected to primer extension analysis as described above.

**In vitro transcription by E.coli polymerase**

The DNA templates were constructed by replacing the rnpB promoter, containing a HindIII–EcoRI fragment, in pLMd23-wt (35) with the ssrS promoter-containing DNA fragments. The promoter-containing DNA products were obtained by PCR with primer pairs SSRP1f/SSRP1r for P1, and SSRP2f/SSRP2r for P2: SSRP1f, 5′ CGC GGA TAA ACT AAG CAA AAT TTG GAA TG; SSRP1r, 5′ CGC GGA TAA ACT AAG CAA AAT TTG GAA TG; SSRP2f, 5′ CGC GGA TAA ACT AAG CAA AAT TTG GAA TG; SSRP2r, 5′ CGC GGA TAA ACT AAG CAA AAT TTG GAA TG. The PCR products were then digested with HindIII and EcoRI, and cloned into the HindIII–EcoRI site of pLMd23-wt to generate the pKSP series of constructs. To knock-out the promoter activity, the −10 region of P1 and P2 was changed from ‘TAGAGT’ to ‘CTCGAG’ by a site-directed mutagenesis kit (Stratagene) using primer pairs P1mu/P1md for P1 and P2mu/P2md for P2: P1mu, 5′ GGT TTA CTT GGG CGC GAG AAC CTT GGA GAC; P1md, 5′ GTC TTC AGC GTT CTT GCC CCA CAG TAA ACC; P2mu, 5′ CGC AAA GAA CGC ACC TCG AGC ACA AAT ACT GAA C; P2md, 5′ GTT CAG TAT TTG TCG TAC ACG TGT CTT CTA G. In vitro transcription, using E.coli RNAP, was carried out basically as described previously (36). Briefly, the RNAP σ32-holoenzyme (Eσ32) and the core enzyme were purchased from Ambion, and σ32 was a gift from Dr Gutierrez. Eσ32 was reconstructed by combining the core enzyme and σ32 in a ratio of 1:5. RNAP (4 nM) was incubated at 37°C for 30 min in reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 5 mM DTT, 50 mM KCl, 50 μg/ml BSA) with 3 nM of template DNA. RNAI and DNA were preincubated for 15 min, and the reaction was started by adding rNTP mixtures (200 μM of ATP, GTP, and UTP, and 20 μM of CTP including 5 μCi of [α-32P]CTP). After 30 min, reactions were terminated by the addition of a 0.2 vol of 0.2 M EDTA in 40% glycerol with dyes. The products were analyzed on a 5% polyacrylamide sequencing gel and quantitated by BAS1500 (Fuji).

**Preparation of RNA substrates**

For the preparation of RNA substrates to be used in in vitro processing assays, the ssrS regions of −221 to +285 and −9 to +285 were PCR amplified. The PCR fragments were then cloned into pGEM3 to generate the constructs pSP6SP2 and pSP6SP1. The pSP6SP2 and pSP6SP1 constructs were mutagenized with the QuikChange kit so that in vitro transcription would be initiated at positions −221 and −9, respectively, and generate run-off transcripts having a 3′ end at position +191 when the mutagenized plasmids were cleaved with SmaI. The resulting plasmids pKS221 and pKS9 were digested by SmaI. The DNA template for RNA I was obtained by PCR from pKK232-8 with a pair of primers 5′SP-RNAI and 3′RNAI: 5′-SP-RNAI, 5′-GCA TCC TAA TAC GAC TCA CTA TAG GGA CAG TAT TTG GT; 3′RNAI, 5′-AAC AAA AAA CCA CGC TAC CAG C from pKK232-8. In vitro transcription was carried out using SP6 RNAp. When required, in vitro transcripts were internally labeled with [α-32P]CTP, 5′-labeled with [γ-32P]ATP and polynucleotide kinase, or 3′-labeled with [32P]pCP and T4 RNA ligase, as described previously (37).

**In vitro processing reactions**

N-terminal catalytic half of RNase E (NTH-RNase E) and full-length RNase G were purified as his-tagged fusion proteins to homogeneity from E.coli containing pNRE or pRNG as described previously (16). RNA substrates were incubated with either NTH-RNase E or RNase G in 50 μl of reaction buffer [20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 0.1% (v/v) Triton X-100, 1 mM DTT and 20 U RNasin (Promega)] at 37°C. These reactions were quenched with phenol/chloroform, and the products were analyzed on polyacrylamide sequencing gels and quantitated by BAS1500 (Fuji). Alternatively, the reaction products were analyzed by primer extension with primer a as described above.
RESULTS

Involvement of RNase E in the processing of 6S RNA transcripts

Since the processing of primary transcripts for sRNAs is generally initiated by endoribonucleases (13,15,18,38), we examined whether 6S RNA biosynthesis would be disrupted in RNase E-, RNase III- and RNase G-deficient E.coli strains. We analyzed 6S RNA transcript levels by northern blotting (Figure 1) and observed two major bands (b and c) and one minor band (a) in each strain including wild type. Band a was found to predominantly accumulate in RNase E-deficient cells (rne/ts cells at 44°C), suggesting the involvement of RNase E in the processing of 6S RNA. In addition, we detected two minor bands between bands a and b in RNase-deficient cells.

Identification of different 6S RNA transcripts

To further identify the different 6S RNA species that are expressed in E.coli, we first identified their 5’ and 3’ ends using RACE assays. For 5’ RACE, total RNA isolates prepared from wild-type and RNase-deficient cells were treated with pyrophosphatase to convert any 5’-triphosphoryl groups to 5’-monophosphoryl groups. The treated RNA samples were then ligated with a 5’ adaptor, amplified by RT–PCR and the resulting PCR products were then analyzed on an agarose gel (Figure 2). Two bands, e and f, were detectable at comparable levels in each of the strains, whereas band d was observed only in rne/ts cells at 44°C (Figure 2). Each band was then eluted from the gel and subjected to DNA sequencing analysis (Table 1). The sequencing data indicated that bands d, e and f were derived from 6S RNA species with 5’ ends beginning at base positions −224, −9 and −1/+1/+2, respectively. These results suggest that the mature 6S RNAs have heterogeneous 5’ ends beginning at base positions ranging from −1 to +2 and that 6S RNA precursors have 5’ ends that are transcribed from either position −224 or −9.

Figure 1. Biosynthesis of 6S RNA in cells lacking either RNase E, RNase III or RNase G. Total cellular RNA isolates were prepared from strains MG1693 (wild type), W3110 (wild type), GW20 (rne/ts), SK7622 (rne−) and GW11 (rng−) grown to an OD600 of ~0.4. Each RNA sample (30 μg) was fractionated on a 5% polyacrylamide gel containing 7 M urea. In vivo occurrence.

Figure 2. RACE products (5’ and 3’) were analyzed on a 3% agarose gel. M, 100 nt size markers; ytRNA, yeast tRNA control; wt, total cellular RNA from strain W3110 grown at 44°C; rne+, total cellular RNA from GW20 grown at 44°C; rne−, total cellular RNA from SK7622 grown at 30°C; and rng−, total cellular RNA from GW11 grown at 30°C.

Table 1. Sequencing analysis of the RACE products

<table>
<thead>
<tr>
<th>RACE products</th>
<th>5’ or 3’ ends of RNA(^{b,c})</th>
<th>rne(^{ts})</th>
<th>rne(^{−})</th>
<th>rng(^{−})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ RACE</td>
<td>d</td>
<td>ND(^{d})</td>
<td>−224 (5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>−9 (3)</td>
<td>−9 (4)</td>
<td>−9 (4)</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>−1 (1), +1 (3), +2 (3)</td>
<td>−1 (1), +1 (3), +2 (3)</td>
<td>−1 (1), +1 (2), +2 (3)</td>
</tr>
<tr>
<td>3’ RACE</td>
<td>g</td>
<td>+184 (4), +185 (3), +186 (3)</td>
<td>+184 (5), +185 (2), +186 (4)</td>
<td>+184 (5), +185 (2), +186 (3)</td>
</tr>
</tbody>
</table>

\(^{a}\)The RACE products shown in Figure 2 were cloned and analyzed by DNA sequencing.

\(^{b}\)The 5’ or 3’ ends of 6S RNA transcripts were determined by analyzing the sequencing data of the RACE products. The numbers in parentheses indicate frequency of occurrence.

\(^{c}\)RNA extracts used in the RACE assays are as follows: wt, total cellular RNA from strain W3110 grown at 44°C; rne\(^{+}\), total cellular RNA from GW20 grown at 44°C; rne\(^{−}\), total cellular RNA from SK7622 grown at 30°C; and rng\(^{−}\), total cellular RNA from GW11 grown at 30°C.

\(^{d}\)ND, not determined.
For 3' RACE analysis, total RNA isolates were ligated to a 3' adaptor, followed by RT–PCR amplification. Only one PCR product (band g) was observed, however, in each of the strains under study and subsequent sequencing analysis of this fragment suggested that all the 6S RNA variants have heterogeneous 3' ends that terminate at base positions ranging from +184 to +186 (Table 1). Hence, our RACE analyses suggest that two 6S RNA precursors exist with different 5' ends that begin at either base positions −224 or −9, corresponding to bands a (long precursor) and b (short precursor) in Figure 1, respectively, and a mature 6S RNA species corresponding to band c, also in Figure 1. However, we could not detect RT–PCR products corresponding to two minor bands between bands a and b shown in Figure 1, which we suspected to be due to their low concentration. We predict that these RNA species are processing intermediates derived from the long 6S RNA precursor, as there are no promoter homologies near their predicted 5' ends (see below).

To further confirm the position of the 5' and 3' ends of the 6S RNA species, primer extension and S1 mapping analyses were performed using total cellular RNAs that were prepared from both wild-type and RNase-deficient cells. For 5' end mapping of the short and long precursor, two separate primer extension reactions were carried out with primers a and b, respectively (Figure 3A). The products extended from base positions −9 and +1/2 to −224 were observed as major bands in all the strains examined, whereas the product that was extended from the −224 position was only observed as a major fragment in the rne<sup>α</sup> cells at 44°C. These results are consistent with our 5' RACE data. In the primer extension experiments using primer a, products that were extended to positions −216/−215 and −210 were also detected. RNA species corresponding to these products were not identified in both the northern blot analysis and the RACE experiment. Therefore, they might contain the upstream sequences only, which would be produced as processing remnants from the long precursor.

The 3' ends of the 6S RNA transcripts were further analyzed by S1 mapping using a DNA probe labeled at position +88 of the 6S RNA sequence. The DNA probes protected from subsequent digestion were found to range in size from 97 to 104 nt in length, which corresponds to 3' ends that terminate at positions ranging from +184 to near +191 (Figure 3B). This result is consistent with our findings in the 3' RACE experiments except that longer ends of +187 to +191 were detected. We presumed that the longer ends might be derived from incomplete digestion of S1 nuclease. It has been previously reported that 6S RNA is produced from a dual-functional transcript, containing 6S RNA and an adjoining ygfA ORF (24), indicating that the 3' ends of 6S RNA should be generated by a 3' processing reaction. It remains to be demonstrated that how the 3' processing occurs.

### Characterization of transcription of the precursor 6S RNA molecules

A homology search upstream of the 5' ends of both the short and long 6S RNA precursors for the consensus *E. coli* promoter sequence revealed two putative promoter regions, from which transcription could initiate at the 5' end of the corresponding precursor (Figure 4). These were designated as P1 and P2, in increasing distance from the mature 6S RNA sequence. The proximal P1 region corresponds to the promoter sequence that had been previously predicted for the *ssrS* gene (24). To test whether the P1 and P2 promoters were functional in vivo and correctly started from the predicted sites, we subcloned DNA fragments containing these sequences upstream of the promoterless *cat* gene of plasmid pKK232-8. We then determined the concentration of chloramphenicol required for the 50% inhibition of growth (IC<sub>50</sub>) of bacterial cells expressing these...
constrasts and performed primer extension analysis to detect ssrS-CAT fusion transcripts (Figure 5). Both P1 and P2 DNA fragments showed promoter activities, indicating that they are functional in vivo. Analysis of the ssrS-CAT transcripts from cells in the exponential phase grown to an OD_{600} of 0.5 showed that the activity of P1 was ~5-fold higher than the activity of P2 that was comparable to the rnpB promoter. Analysis of primer extension products also revealed that P1 and P2 start transcription at 224 in vivo, respectively.

To analyze the promoter activities of P1 and P2 in vitro, we replaced the rnpB promoter-containing DNA fragment of pLMd23-wt with the 6S RNA promoter fragments to generate P1- and P2-rnpB terminator transcription units. The resulting plasmid DNAs were used as templates for subsequent in vitro transcription assays (Figure 6). pLMd23-wt, which was used as a control, generates a 143 nt truncated rnpB transcript (35). If transcription initiates at −9G or −224A for P1 and P2, respectively, the sizes of the expected transcripts are 189 nt for P1 and 195 nt for P2. The P1- and P2-rnpB terminator units produced transcripts of the expected sizes, P1 showed an ~3-fold higher activity than P2. When the −10 region of each promoter was mutagenized from ‘TAGATG’ to ‘CTCGAG’, both promoters no longer generated the transcripts (Figure 6).

The in vitro transcription data, together with the in vivo data, indicate that the short and long 6S precursors are derived from transcription from P1 and P2, respectively. One may argue that the short precursor could be generated by processing from the long precursor. However, we conclude that the 5′ end of the short precursor is originated from the P1 transcription rather than by processing from the long precursor, because P1 is more active than P2. This conclusion is consistent with a

Figure 4. E.coli ssrS gene. (A) Schematic map of the ssrS transcription unit. (B) Nucleotide sequence of the ssrS gene. Promoter regions (−35 and −10) of the ssrS gene encoding 6S RNA are underlined, and their transcription start sites are indicated by arrows. Thick lines indicate the regions corresponding to the gene encoding 6S RNA I are indicated below lanes pKSP1 and pKSP2. M, size markers; asterisk, nonspecific transcripts.

Figure 5. In vivo promoter activity. (A) Primer extension analysis of ssrS-CAT fusion transcripts. The regions −160 to +50 (containing P1) and −334 to −160 (containing P2) of the ssrS gene were cloned into pKK232-8 to generate the constructs pSSR1 and pSSR2, respectively. Plasmid pKM1, an rnpB-CAT fusion plasmid, was used as a control. The 32P-labeled primer (0.2 pmol) and total cellular RNA (13 μg) isolated from cells containing CAT fusion plasmids were used for each reaction. Primer extension reactions were performed in duplicate (lanes 1 and 2) for each plasmid. The relative amounts of RNA I in the total cellular RNA preparations were determined by northern blot analysis and used for assessing relative promoter activities. (B) Comparison of in vivo promoter activities. In vivo promoter activities were assessed by determining the concentration of chloramphenicol required for the 50% inhibition of growth of E.coli cells (IC_{50}) and calculating relative amounts of primer extension products normalized to RNA I. Plasmid pKM1, an rnpB-CAT fusion plasmid, was used as a control. The value represents the average of four independent experiments. N.D., not determined.

Figure 6. In vitro transcription analysis. In vitro transcripts generated from the ssrS promoter-rnpB terminator fusion plasmids by E.coli RNAP. In vitro transcription with either E_{70} or E_{α} was performed using plasmid DNAs as templates. The resulting RNA products were analyzed in a polyacrylamide sequencing gel. pKSP1, pKSP2 and pLMd23 contain the following transcription units: P1 (−109 to +40 of ssrS-rnpB), P2 (−319 to −120 of ssrS-rnpB), truncated rnpB (13), respectively. pKSP1m and pKSP2m are derivatives of pKSP1 and pKSP2, where the −10 region was mutagenized from ‘TAGATG’ to ‘CTCGAG’. The data are representative of three independent experiments. Relative amounts of transcripts normalized to RNA I are indicated below lanes pKSP1 and pKSP2. M, size markers; asterisk, nonspecific transcripts.
levels of the short transcript (referred as the P1 transcript) were decreased at the stationary phase in both wild-type and rpoS− cells, indicating that P1 driven transcription is exclusively carried out by Eσ70. We performed additional in vitro transcription experiments with Eσ (Figure 6B, right), which was found to generate transcripts from the P2 promoter, but not P1, further confirming our finding that the P2 region is recognized by Eσ.

5′ processing of 6S RNA precursors by endoribonucleases

The fact that the long 6S RNA precursor was found to accumulate in rneΔ cells suggested the involvement of RNase E in the processing of 6S RNA. We therefore examined whether the long precursor was in fact cleaved by this endoribonuclease. To determine this, a substrate of 412 nt (spanning −221 to +191 of the srsS gene) was synthesized in vitro and labeled at the 5′ ends for use in cleavage reactions with the NTH-RNase E (43–44). NTH-RNase E generated a 5′ upstream fragment by cleavage near position +1 (Figure 8A, left) leading to the generation of 6S RNA with a 5′ end at about the +1 position, which was then confirmed by an NTH-RNase E reaction with a 3′-labeled substrate (Figure 8A, right). NTH-RNase E reaction with a 3′-labeled substrate also generated 6S RNA containing extra 5 nt as a minor product. The long precursor can be digested near positions −53, −58, −62 and −66 (Figure 8A, left). As the amount of the enzyme in the reaction was increased, the initially cleaved product levels decreased with the increase of the digested products at these positions. This suggests that the digestions near positions −53, −58, −62 and −66 occurred after the initial digestion at position +1 (Figure 8A). These sites are located in A/U-rich regions, which is consistent with the previously published preferential RNase E recognition sequence (45). On the other hand, 6S RNA with a mature 5′ end was not further digested by NTH-RNase E, as shown in the reaction with the 3′-labeled substrate (Figure 8A, right).

Since RNase G is homologous to NTH-RNase E (29, 46–49), we also tested whether it would also cleave the long precursor transcript (Figure 8B). RNase G did not, however, generate mature 6S RNA, even at the highest enzyme concentrations, suggesting that it is not involved in the 5′ processing of the long precursor.

Next, the short precursor was investigated for cleavage by NTH-RNase E and RNase G, although no prominent accumulations of this precursor were observed in RNase E- and RNase G-deficient cells (Figure 1). A substrate of 200 nt (spanning −9 to +191 of srsS), synthesized in vitro, was labeled at the 5′ end and used in the reactions (Figure 8B). In contrast to our findings with the long precursor, both enzymes were found to cleave the short precursor at comparable efficiencies. However, the cleavage site preference was slightly different between the two enzymes. RNase G cleaved preferentially at a position that was 1 nt downstream of the major cleavage site of RNase E.

The size markers (alkaline hydrolysis or G-specific fragments) used for the analysis of the reaction products had a 3′- phosphorol group instead of a 3′-hydroxyl group generated by RNase E or RNase G. Since RNA molecules with a 3′-hydroxyl group migrate more slowly than RNA species with a
3'-phosphoryl group (50), it is difficult to determine the precise 5' end of products with this size marker ladder. To circumvent this problem, we carried out primer extension analysis with the cleavage products (Figure 8C). The resulting data indicated that RNA with a 3'-hydroxyl group migrated at a rate that was ~1 nt slower than RNA with a 3'-phosphoryl group. Hence, we conclude that RNase E cleaves predominantly at position +1, regardless of whether the molecule is the long or short precursor, whereas RNase G cleaves the short precursor at positions 1+ and 2 with a slight preference for the +2 site.

We also compared cleavage efficiency of the long and short precursors by either NTH-RNase E or RNase G in vitro (Table 2). In this experiment, we used internally labeled substrates with a triphosphoryl group at their 5' end because natural 6S RNA precursors have a 5'-triphosphoryl group as primary transcripts. We confirmed that the two RNase preparations had comparable functional purities, using RNA I as a control substrate. The cleavage rate of RNA I by NTH-RNase E

Table 2. Comparison of cleavage rates of the long and short precursor 6S RNA transcripts by NTH-RNase E and/or RNase G

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrates</th>
<th>Rate (10⁻³ × pmol/min ng of protein)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTH-RNase E</td>
<td>Long precursor</td>
<td>19.18 ± 2.26</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>Short precursor</td>
<td>1.41 ± 0.64</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>p23 RNA</td>
<td>5.28 ± 0.94</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>RNA I</td>
<td>6.14 ± 0.84</td>
<td>4.4</td>
</tr>
<tr>
<td>RNase G</td>
<td>Long precursor</td>
<td>&lt;0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Short precursor</td>
<td>1.19 ± 0.26</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>p23 RNA</td>
<td>&lt;0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RNA I</td>
<td>1.28 ± 0.42</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Substrates internally labeled with [α-32P]CTP were used and therefore contained a 5'-triphosphoryl group.

Rates are represented as picomole of substrates in 1 min by 1 ng of enzyme under our cleavage reaction conditions.

Figure 8. In vitro processing reactions. (A) The long 6S RNA precursor, transcribed in vitro, was 32P-labeled at the 5' end (left) or 3' end (right). It is noteworthy that the 5'-labeled substrate had a 5'-monophosphoryl group, while the 3'-labeled substrate had a 5'-triphosphoryl group. The labeled substrate was incubated with either NTH-RNase E or RNase G in a 50 µl reaction at 37°C for 10 min. The products were then analyzed on 5% polyacrylamide sequencing gels. OH, partial alkaline hydrolysis ladders; G, G-specific (RNase T1) cleavage products; --, no enzyme; asterisk, the position of label. The quantities of NTH-RNase E or RNase G used are indicated each lane. (B) Cleavage of the 5'-labeled short 6S RNA precursor by either NTH-RNase E or RNase G was analyzed on a 15% polyacrylamide gel, as described in (A). OH, partial alkaline hydrolysis ladders; U2, A-specific (RNase U2) cleavage products; --, no enzyme; asterisk, the position of label. The alkaline hydrolysis ladders and A-specific (RNase U2) cleavage products used as size markers have a 3'-phosphoryl group, while NTH-RNase E or RNase G cleavage products have a 3'-hydroxyl group. Since they migrate differently on the gel, the precise cleavage sites differ from the corresponding sites of the size markers up to several bases. (C) Assignment of the precise cleavage sites. The unlabeled substrates were cleaved with NTH-RNase E or RNase G, and cleavage sites were determined by primer extension analysis with primer α as in Figure 3A. All data are representative of three independent experiments.

Figure 9. Effect of the rneG/rngG double mutation on the processing of 6S RNA. (A) Total cellular RNA (30 µg) was fractionated on a 5% polyacrylamide gel containing 8 M urea, and 6S RNA transcripts were analyzed by northern blot as described in Figure 1. The different strains and growth temperatures are indicated above each lane. wt, W3110; rngG, GW11; rneG, GW20; rneG-rngG, GW21. (B) The relative amount of transcripts represents the northern signal of each transcript relative to that of 5S RNA. The values were calculated from three independent experiments.
was 4.8-fold higher than that by RNase G. This difference in the cleavage rate between two enzymes is comparable to other investigators’ data (51). NTH-RNase E cleaved the long precursor ~14-fold more efficiently than the short precursor, and the efficiency of short precursor processing by RNase G was similar to that by NTH-RNase E. Hence, the long precursor may contribute to the overall 6S RNA level more significantly than expected from the lower promoter activity of P2.

Our in vitro cleavage data (Figure 8 and Table 2) clearly showed that the long precursor is processed exclusively by RNase E, whereas the short precursor is processed by both RNase G and RNase E. To test whether this differential involvement of endoribonucleases in the 5' processing of 6S RNA occurs in vivo, we examined the cellular levels of the long and short precursors in rne<sup>ts</sup>, rng<sup>-</sup> and in rne<sup>ts</sup>-rng<sup>-</sup> double mutant strains (Figure 9). The long precursor transcript was found to accumulate in rne<sup>ts</sup> cells, but not in rng<sup>-</sup> cells. On the other hand, the short precursor transcript accumulated primarily in rne<sup>ts</sup>-rng<sup>-</sup> double mutant cells. These data suggest that the differential recognition of these two precursors by RNase E and RNase G occurs in the cell as well as in vitro.

**DISCUSSION**

We found that *E.coli* 6S RNA is transcribed from two tandem promoters, designated P1 and P2, which are proximal and distal to the mature 6S RNA sequence, respectively. P1 is a canonical σ<sup>70</sup>-dependent promoter, while P2 is both a σ<sup>70</sup>- and a σ<sup>S</sup>-dependent promoter. Hence, transcription of 6S RNA can be regulated by switching σ factors for the formation of specific RNAP holoenzymes, in response to environmental signals. We also found that the transcripts that are generated by these two promoters are differentially processed at their 5' ends by RNase E and RNase G. The P2 transcript is processed exclusively by RNase E, whereas the P1 transcript is processed by both RNase E and RNase G. From these findings, we propose a working hypothesis for the biosynthetic pathways of 6S RNA (Figure 10). In this model, 6S RNA is supplied from two sources. One is the long primary precursor transcript, the expression of which is driven by the P2 promoter. The other is a short primary precursor transcript generated from the P1 promoter. Both transcripts are generated by the RNAP σ<sup>70</sup>-holoenzyme (Eσ<sup>70</sup>) during the exponential phase of bacterial growth. In the stationary phase, however, transcription from P1 declines, presumably due to the inactivation of Eσ<sup>70</sup>, whereas expression from the P2 promoter region remains active via the switching from Eσ<sup>70</sup> to Eσ<sup>S</sup> in the RNAP holoenzyme. These primary transcripts are subjected to both 5' and 3' processing to generate mature 6S RNA, but the enzymes involved in 3' processing have not been yet identified, except that final trimming requiring exoribonucleases has been reported (14). 5' processing of 6S RNA is accomplished by both RNase E and RNase G. The short 6S RNA precursor molecule, transcribed from P1, is processed by RNase E and RNase G at the 5' end, but the long precursor, expressed from the P2 promoter, is processed exclusively by RNase E. 5' processing of the short precursor is also far less rapid when compared with the long precursor. Hence, these two precursor molecules differentially contribute to the synthesis of *E.coli* 6S RNA via different processing enzymes and with differing efficiencies. As a result of this biosynthetic pathway, the generation of 6S RNA can be regulated via the coupled action of sigma factors and endoribonucleases.

6S RNA levels accumulate throughout the bacterial growth cycle and, subsequently, the levels of mature 6S RNA strongly increase in the stationary phase. 6S RNA inhibits

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**Figure 10.** A model for the biogenesis of 6S RNA. Eσ<sup>70</sup> RNAP acts during the exponential growth phase of *E.coli* for the transcription of both the long and short precursor molecules. When the cell cultures enter into the stationary phase, Eσ<sup>S</sup> does not act as the major machinery to transcribe 6S RNA as Eσ<sup>s</sup> now takes on this role, but only in the transcription of the long precursor. The 5' ends of 6S RNA are formed from the short precursor by processing with either RNase E or RNase G, and from the long precursor by processing exclusively with RNase E. The processing remnant derived from the long precursor is also further cleaved by RNase E. The enzymes involved in the 3' end formation of 6S RNA have not yet been identified, although it has now been shown that exoribonucleases are involved in final trimming (14).
the utilization of $\sigma^{70}$-dependent promoters in the stationary phase by interacting with $\sigma^{70}$ (25). An important question, therefore, is how the transcription of 6S RNA occurs throughout the growth cycle and our findings provide a possible answer. $\sigma^{70}$ transcribes 6S RNA from both the P1 and P2 promoters in the exponential growth phase, but it stops transcription when cells are entering the stationary phase. $\sigma$ then takes over the control of 6S RNA transcription by recognizing the P2 promoter in the stationary phase. 6S RNA inhibits transcription from some, but not all, $\sigma$-dependent promoters, which have mostly the extended $-10$ sequence element as a common feature (26). The P1 promoter has the extended $-10$ element with the additional conserved sequence of TGG, whereas P2 does not. Therefore, P1 transcription by $\sigma^{70}$ may be inhibited by the accumulation of 6S RNA above a specific threshold upon transition into stationary phase. If this is the case, the regulation of $\sigma^{70}$ would be a striking example of feedback inhibition of gene expression.

Another important finding in our current study is that the two 6S RNA precursor transcripts are differentially processed. The long precursor is processed exclusively by RNase E, whereas the short precursor is processed by both RNase E and RNase G. RNase G shares a high degree of homology with the N-terminal catalytic domain of RNase E (48–49). These two enzymes also share a number of common properties, such as a propensity to cleave RNA located in single-stranded regions rich in U and A nucleotides and a preference for 5'-monophosphorylated over 5'-triphosphorylated RNA substrates (51–53). However, these two enzymes may function in a distinct manner in cells because the respective deletion mutants show different cell growth effects: RNase G is dispensable for cell growth but RNase E is essential (29,47,54–56). This may be explained by a specificity difference between these two enzymes as we have found that RNase G can cleave only the short precursor despite the fact both precursors of 6S RNA contain the same consensus region for this enzyme. A possible explanation for such a specificity difference may also be that RNase G preferentially recognizes cleavage regions that are immediately proximal to the 5'-end of the precursor. The preference of RNase G for cleavage regions close to the 5'-end was previously observed for substrates, such as RNA I, ompA mRNA and 95S RNA (53). The limited recognition of the consensus cleavage site by RNase G may also be due to high order structures within the RNA substrates that prevent the enzyme from gaining access to this region. However, the explanation as to why RNase G, but not RNase E, is affected by these structures is still uncertain. In any case, it is likely that RNase G is not a versatile enzyme, at least with respect to substrate specificity, compared with RNase E.

The reaction rates of NTH-RNase E for the processing of E. coli 6S RNA long and short precursors are quite different. NTH-RNase E cleaves the long precursor much more efficiently than the short precursor, and although RNase G cleaves the short precursor, its efficiency is also much lower (Table 2). Hence, the rapid generation of 6S RNA from the long precursor molecule may be essential for the synthesis of large quantities of mature 6S RNA during the stationary growth phase where this precursor molecule is the only source of unprocessed transcripts. In addition, the difference in the processing efficiency of these two precursors can affect the supply of mature 6S RNA in other growth phases. The cellular levels of 6S RNA could therefore be post-transcriptionally controlled by the coordinate action of both RNase E and RNase G.

We showed from our current data that the 5'-end of 6S RNA is heterogeneous and that this seems to result from the cleavage of precursor 6S RNAs at multiple sites by RNase E and RNase G. Our in vitro experiments indicate that RNase E generates 6S RNA with 5'-ends at position +1 as the major product, –1 and +2 as minor products, and that RNase G generates 6S RNA of +1 and +2 5'-ends with a slight preference for the +2 position. However, primer extension analysis of in vivo RNA transcripts showed that 6S RNA molecules with 5'-ends at positions +1 and +2 are present at almost equal levels, even in RNase G-deficient cells. The discrepancy between our in vivo and in vitro data suggests that a portion of the 6S RNA molecules with a 5'-end at +1, generated by RNase E, may be further somehow digested by 1 nt in vitro. It is noteworthy, however, that the in vivo cleavage sites of RNase E, or possibly RNase G, could be changed by a cellular factor. For example, NTH-RNase E cleaves pM1 RNA with a preference for position +379 in vitro, but its cleavage site preference changes to position +378 in the presence of a cellular factor (16). Hence, the cleavage site preference of RNase E and RNase G may differ under in vitro and in vivo conditions. It remains to be demonstrated whether 6S RNA, with different 5'-ends, have different cellular functions or different fates during RNA metabolism.

Since 6S RNA participates in the modification of general transcription upon changes to environmental growth conditions, its cellular levels should be regulated in response to environmental signals. To gain insight into this regulation circuitry, we characterized the biosynthetic pathway of 6S RNA at both the transcriptional and post-transcriptional levels and provided a molecular basis for the regulation of 6S RNA biosynthesis. Recently, it has become apparent that sRNAs are far more abundant and have more important roles in regulatory networks, modulating various cellular processes, than previously envisaged (2–4,57,58). In many cases, sRNAs are involved in these control networks by adding another layer of regulation to the existing molecular pathways. In this respect, the finding that the biogenesis of 6S RNA is regulated by switching the utilization of both sigma factors and endoribonucleases can improve our understanding of these sRNA regulation mechanisms.

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REFERENCES


function in the mechanism of chromosome segregation and cell division. 


