Rho-dependent Termination of ssrS (6S RNA) Transcription in Escherichia coli

IMPLICATION FOR 3'-PROCESSING OF 6S RNA AND EXPRESSION OF DOWNSTREAM ygfA (PUTATIVE 5-FORMYL-TETRAHYDROFOLATE CYCLO-LIGASE)

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It is well known that 6S RNA, a global regulatory noncoding RNA that modulates gene expression in response to the cellular stresses in Escherichia coli, is generated by processing from primary srrS (6S RNA) transcripts derived from two different promoters. The 5’-processing of 6S RNA from primary transcripts has been well studied; however, it remains unclear how the 3’-end of this RNA is generated although previous studies have suggested that exoribonuclease trimming is necessary for 3’-processing. Here, we describe several Rho-dependent termination sites located ~90 bases downstream of the mature 3’-end of 6S RNA. Our data suggest that the 3’-end of 6S RNA is generated via exoribonuclease trimming, rather than endoribonuclease cleavage, following the transcription termination events. The termination sites identified in this study are within the open reading frame of the downstream ygfA (putative 5-formyl-tetrahydrofolate cyclo-ligase) gene, a part of the highly conserved bacterial operon srrS-ygfA, which is up-regulated during the biofilm formation. Our findings reveal that ygfA expression, which also aids the formation of multidrug-tolerant persistor cells, could be regulated by Rho-dependent termination activity in the cell.

Small noncoding RNAs (sRNAs)3 mediate a number of cellular processes in bacteria, such as protein tagging for degradation, modulation of RNA polymerase activity, regulation of mRNA stability and translation, and secretion (1–4). Many sRNAs are transcribed as primary transcripts by RNA polymerase (RNAP), and the transcripts are then subjected to processing by removing extra residues at the 5’- and/or 3’-ends to form functional mature forms (5).

The 6S RNA was first identified as an abundant sRNA in Escherichia coli, migrating as an 11S particle without ribosome association (6). A recent study revealed that 6S RNA binds to RNAP α70-holoenzyme (Eα70) and represses transcription from α70-dependent promoters and activates α5-dependent promoters (7–9). The 6S RNA is highly induced during stationary growth, during which time it plays an important role in modifying the utilization of Eα70 to Eα5 (7, 8, 10). The characteristic secondary structure of 6S RNA consists of a largely double-stranded helix and a central bulge (11, 12). This highly conserved structure resembles DNA templates in terms of its open promoter complex and might therefore mimic DNA promoters to encourage the binding of Eα70 (7, 8). Interestingly, 6S RNA can act as a template for the transcription of pRNA, a very short RNA molecule (10).

6S RNA is cotranscribed with ygfA, a gene expressed as a second gene from the srrS promoters, that encodes a putative 5-formyl-tetrahydrofolate cyclo-ligase (7, 13). This dual-functional operon structure is highly conserved in α- and γ-proteobacteria, as well as in certain members of the class β-proteobacteria (14, 15). Recent reports have suggested that ygfA expression aids the formation of persistor cells (13) and is up-regulated during the biofilm formation (16). The expression of ygfA is of particular interest, considering that the antibiotic recalcitrance of biofilm infections is largely caused by persistor cells. However, the mechanisms of ygfA regulation remain unclear.

The transcription of primary 6S RNA transcripts and processing of their 5’-ends has been well characterized in E. coli (17). Two alternative promoters (i.e. a proximal canonical α70-dependent srrS P1 and a distal α70- and α5-dependent P2) are involved in the transcription of 6S RNA. These promoters produce two transcripts: a short P1 transcript that begins at nucleotide position −9 and a long P2 transcript that begins at position −224 relative to the mature 5’-end of 6S RNA (+1). Interestingly, transcription from these promoters changes in response to cellular stress: P1 transcription is the predominant type of transcription during exponential phase, while P2 transcription increases upon entry into stationary phase (17). Furthermore, the 5’-ends of the transcripts are removed by two functionally similar enzymes with different specificities: RNase E acts on the long transcript, while RNase E and RNase G act on the short transcript (17). On the other hand, the mechanism responsible for the formation of the 3’-end of 6S RNA is not clear, although it has been shown that exoribonucleases are involved in a plausible 3’ trimming...
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TABLE 1
Oligonucleotides used in this study

| Primer             | Sequence (5' to 3') | Use                                      |
|--------------------|--------------------|------------------------------------------|
| 6SKO_up            | CCA CAA GAA TGT GCC GCT CCG CGG TGG AGT CAT GCA | Construction of MG1655 ssrS::kan         |
| 6SKO_dn            | GCA GTT TTA AGG CTT CTC GGA GAG ACC GAG CAT GTA | DNA templates for in vitro transcription (Figs. 1, 2, and 4) or construction of pCAT (ssrS-CAT fusion) plasmids (Fig. 2) |
| ssr5-92_F          | CGG GGA TTC TTA CTT CTA GAA CAA GAT CCG A | DNA templates for in vitro transcription (Fig. 1) or construction of pCAT (ssrS-CAT fusion) plasmids (Fig. 2) |
| ssr5+185_R         | CCC AAG CTT GGG AAT CTC CGA GAT GCC | Anti-6S RNA probe or DNA templates for in vitro transcription (Figs. 3, 4, and 6) |
| ssr5+238_R         | CCC AAG CTT ATA ATG TCA GGG GAA GAT CTC | Anti-tRNA<sup>Arg</sup> probe (Fig. 3) |
| ssr5+260_O         | CCC AAG CTT ATG TCA GGG GAA GAT CTC | Construction of ssrS-tRNA<sup>Arg</sup>-CAT fusion plasmids (Fig. 3) |
| ssr5+330_O         | CCC AAG CTT GGG TGG AAT CTC CGA | tyrT template for in vitro transcription (Fig. 4) |
| ssr5+488_O         | CCC AAG CTT GGG TGG AAT CTC CGA | DNA templates for in vitro transcription (Fig. 4) |
| ssr5+488_R         | CCC AAG CTT GGG TGG AAT CTC CGA | Anti-6S RNA probe or DNA templates for in vitro transcription (Figs. 3, 4, and 6) |
| ssr5+511_1         | CCC AAG CTT ATA CCA ACA AAC GAG AAG AGG G | Anti-tRNA<sup>Arg</sup> probe (Fig. 3) |
| ssr5+1044_R        | CCC AAG CTT GGG TGG AAT CTC CGA | Construction of anti-rho expression plasmid (Fig. 5) |
| ssr5+781_F         | GCC GGA TCC ACA GAT TCG AAA GTC TGG GA | DNA templates for in vitro transcription (Fig. 4) |
| cat_R              | ATC CGG GGT AGC CAT CGA | Anti-tRNA<sup>Arg</sup> probe (Fig. 3) |
| a6S+185            | GGS AAT CAT CTC GAT GCC | Construction of anti-rho expression plasmid (Fig. 5) |
| atRNA<sup>arg</sup> | ACC TGT ACT TTA CTA TCC AAC | Reverse transcription of rho mRNA (Fig. 5) |
| tRNA<sup>F</sup>   | TAC AAG CTT GCC CTC GTA GTC CAG | Reverse transcription of ssrS::ygfA dicistronic RNA (Fig. 5) |
| tRNA<sup>R</sup>   | TAT AAG CTT TGG TGC GGC CGG CCG | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| TyrT<sup>F</sup>   | GGC TCC TAT TGG TTC TTA AGT GTA TAC T | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| TyrT<sup>R</sup>   | GAT TCG TTT GAG AAT TCC GGG G | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| ERIT765_S          | CGG AAT TCT TAA TAC GAC TCA CTA TAG GAT TTC TCT | DNA templates for in vitro transcription (Fig. 4) |
| ArRh<sup>ol</sup>_F| GGC GAC GTT ATC ATG GGG GGT TCT TAA ACT TGG AGT AGC TAA AAA CCC | DNA templates for in vitro transcription (Fig. 4) |
| pmNTXb<sup>al</sup>_R| GCT GTA GAA AAG AAA CCC GCC GAA GCC GGT TTT | Reverse transcription of rho mRNA (Fig. 5) |
| Rho216_R           | GTA GGA GCT TGC TGC GAC CAT GCA CTG TCA CCG ACT CTC | Reverse transcription of ssrS::ygfA dicistronic RNA (Fig. 5) |
| 65+733_R           | CCA CAA ACT GAC CAT GAC ATC CC | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| Rho20_F            | AGA ATG CGG CGG TCT TCT CAG | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| Rho196_R           | GGA GGA AAC ACA ATC CAT CCC | RT- or qRT-PCR of rho mRNA (Fig. 5) |
| 65+104_F           | AGG CCT TTA AAC TCC GAC GAG GAA GTG TTT | DNA templates for in vitro transcription (Fig. 4) |
| 65+316_R           | GCT GAC CCA TTT CCT GTT CTT | DNA templates for in vitro transcription (Fig. 4) |
| 5S_F               | TGG CCT GGG GCC GGA GTA CGG | DNA templates for in vitro transcription (Fig. 4) |
| 5S_R               | ATG CCT GCC AGT TCT CCA CTC CTC | DNA templates for in vitro transcription (Fig. 4) |

mechanism that forms heterogeneous 3’-ends from +184 to +191 (17, 18).

Considering that 6S RNA is transcribed as part of a dicistronic ssrS::ygfA message, the 3’- processing of 6S RNA likely occurs via endoribonuclease cleavage followed by exoribonucleolytic trimming. However, this model is not consistent with past observations that formation of the 3’-end of 6S RNA is not altered in the endoribonuclease RNase E-, RNase G-, or RNase III-deficient cells (17).

In the present study, we found that in vitro transcription from the ssrS promoter continues beyond ygfA and can extend beyond sibc<sub>R</sub>, a gene that encodes an anti-toxin small RNA (19, 20). However, in vivo studies suggested that transcription from the ssrS promoters results in very low levels of ygfA expression. This low expression could reflect either internal cleavage of the polycistronic RNA or termination of transcription somewhere beyond the 3’-end of 6S RNA in the cell. We identified several Rho-dependent termination sites located ~90 bases downstream of the 3’-end of 6S RNA. No accumulation of Rho-terminated ssrS transcripts in cells lacking the four endoribonucleases, RNase E, RNase G, RNase III, and RNase P suggest that the 3’-end of 6S RNA is generated via exoribonucleolytic trimming after termination. The location of Rho-dependent termination sites within the N-terminal coding region of ygfA implies that ygfA expression depends on ssrS transcription continuing through the termination sites.

EXPERIMENTAL PROCEDURES

Bacterial Strains—E. coli—K-12 strain JM109 was used for the construction of plasmids and for in vivo analysis of ssrS transcription. Plasmid-borne transcripts were analyzed using 6S RNA knock-out strains. MG1655 ssrS::kan was constructed based on the previously described report (21) with a corresponding primer pair (Table 1). The 6S RNA knock-out derivatives of the following endoribonuclease-deficient strains were constructed by employing bacteriophage P1-mediated transduction with MG1655 ssrS::kan as the donor strain (21): GW10 (rne<sup>−</sup> rmg<sup>−</sup>), GW11 (rne<sup>−</sup> rmg<sup>−</sup> cat), GW20 (GW10 me<sup>I</sup>), GW21 (rne<sup>−</sup> rmg<sup>−</sup> cat) for RNase E or/and RNase G (22); SDF204 (rne<sup>−</sup>) and SDF205 (rne<sup>−</sup> cat) for RNase III (23); NYH312 (rne<sup>−</sup>) and NYH322 (rneA49) for RNase P (24).

Preparation of Total Cellular RNA—E. coli—Overnight cultures grown at 37 °C were diluted 1:100 in LB broth containing ampicillin (50 μg/ml) or tetracycline (10 μg/ml) and grown to an A<sub>600</sub> of 0.5 at the same temperature. If necessary, IPTG was added to the cell culture at 1 mM of final concentration and the culture was incubated further for 30 min. In the case of rne<sup>−</sup> or rnpA49 cells, the cells were grown at 30 °C.
and then shifted to 44 °C for 1 h. rng::cat cells also treated as above to compare with rne-1 effect even though the strain itself is not temperature-sensitive. Total cellular RNA was isolated by hot phenol extraction as described previously (25).

Northern Blot Analysis—Total cellular RNA (15 μg) was fractionated on a 5% polyacrylamide gel/7 M urea and electro-transferred onto a Hybond-XL membrane (GE Healthcare). 5’-End-radiolabeled oligonucleotides a6S + 185 and atRNA Arg (Table 1) used as probes for 6S RNA and Brevibacterium albidum tRNAArg respectively. Hybridization was performed according to the manufacturer’s instructions. The Northern blots were visualized and quantified using Image Analyzer FLA7000 (Fujifilm).

In Vitro Transcription by E. coli RNA Polymerase—The linear DNA templates used for in vitro transcription reactions were obtained with chromosomal or plasmid DNA via PCR with corresponding primer pairs (Table 1). Some primers had extra linker sequences at the 5’-end. In vitro transcription reaction was conducted using E. coli Eo70 (Epcenter) according to the manufacturers’ instructions with minor modifications. Briefly, the DNA templates (6 nm) were incubated at 37 °C for 5 min in reaction buffer (40 mM Tris-Cl, pH 7.5, 150 mM KCl, 10 mM MgCl2, 0.05% Triton X-100, 10 mM DTT) containing Eo70 (1 unit) and 2 mM ATP. The reaction was then initiated by adding mixtures containing rNTPs (0.5 mM rGTP, 0.5 mM rUTP, and 0.025 mM rCTP including 10 μCi of [α-32P]CTP) and rNasasin (4 units, Promega). After 25 min, the reactions were terminated by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended with gel-loading buffer II (Ambion) and loaded on a 5% polyacrylamide/8 M urea sequencing gel. The gel was visualized and analyzed by FLA7000 (Fujifilm).

Analysis of Transcription from ssrS-P1 Promoter in Vivo—ssrS-P1 promoter-containing DNA fragments (i.e. ranging from nucleotide position −92 to various downstream bases) were obtained via PCR amplification of genomic DNA with corresponding primer pairs (Table 1). The resulting PCR products were digested with BamHI/HindIII and ligated into pKK232–8 (GE Healthcare) to generate pCAT plasmids. Cells containing fusion plasmids were exponentially grown in LB broth (1:300 dilution) of different chloramphenicol concentrations. The IC50 was measured as previously described (26).

Alternatively, a B. albidum tRNAArg sequence (27) was amplified by PCR with a corresponding primer pair (Table 1) and inserted immediately upstream of the CAT gene in the pCAT plasmids to measure the exogenous amount of TRNAArg (i.e. as an indicator of transcription activity). Total RNAs isolated from cells containing the exogenous RNA gene were subjected to Northern blot analysis as described above.

Analysis of Rho-dependent Termination—His-tagged Rho and IcdA (isocitrate dehydrogenase) proteins were purified from cells containing ASKA-rho and ASKA-icdA plasmids, respectively, as previously reported (28). The linear DNA templates were prepared and subjected to in vitro transcription analysis as described above except that Rho, or IcdA protein as a control, was added at 40 nm.

Gel Mobility Shift Assay—DNA templates for in vitro transcription of 6S RNA and precursor 6S RNA carrying the downstream sequence to +330 were obtained via PCR using pCAT330 DNA with primer pairs of ERIT76S/a6S + 185 and ERIT76S/cat R (Table 1), respectively. In vitro transcription was carried out using T7 RNA polymerase (Promega). Gel-purified 6S RNA and precursor 6S RNA were 5’-end labeled with [γ-32P]ATP and re-purified by gel elution. Labeled RNAs (2 nm) were incubated with the purified Rho protein in 10 μl of binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 1 mM DTT, 100 mM NaCl, 0.06% Triton X-100, 0.25% glycerol, 10 μg of yeast RNA) for 15 min at 25 °C. The reaction mixtures were then analyzed on 5% polyacrylamide gels, as described previously (20).

3’-RACE Assay—3’ RACE analysis was performed on the in vitro transcribed RNAs in the presence of Rho protein with the ssrS-containing DNA ranging from −92 to +448 as previously described (29), with following modifications. The Adaptor-ligated RNA was reverse transcribed and PCR amplified using a One-Step RT-PCR Premix kit (Intron) according to the manufacturer’s instructions. The PCR products were separated on a 2% agarose gel, purified, and analyzed by DNA sequencing after cloning into a pGEM-T-easy vector (Promega).

Analysis of Rho-knockdown Effects on ygfA Expression—Plasmid pAKA (20), a derivative of pACYC184, was used to construct an anti-rho RNA expression plasmid. The anti-rho RNA sequence linked to the rnpB terminator was amplified with a primer pair of ARrho1_F/pnMTXbal_R by PCR and cloned into the AttII/EcoRI sites of pAKA so that anti-rho RNA could be induced by IPTG. Total RNAs were isolated from cells containing the anti-rho RNA expression plasmid pARRho after the IPTG induction for 30 min as described above, and treated with Turbo DNase (Ambion) to remove contaminating DNA. DNase was heat-inactivated and RNA samples were subjected to reverse transcription with MMLV RT (Enzymomics) with primers Rho216_R for rho mRNA, 6S + 733_R for ssrS-ygfA dicistronic RNA, and 55_R for 5S rRNA. The resulting cDNAs were amplified using Taq polymerase premix (Solgent) with primer pairs Rho200_F/Rho196_R for rho mRNA, 6S + 104_F/6S + 316_R for ssrS-ygfA dicistronic transcripts, and 55_F/55_R for 5S rRNA. The primers used were listed in Table 1. The PCR products were electrophoresed on 2% agarose gels, stained by SYBR Safe (Invitrogen), and photographed by GelDoc 1000 (Bio-Rad). Quantitative real-time RT-PCR was done by ABI 7500 Real-time PCR (Applied Biosystems) with QuantiTect SYBR Green PCR premix kit (Qiagen) in triplicate experiments. The abundance of each RNA was normalized to the amount of 5S RNA and represented as a fold change. Data were analyzed using ABI 7500 SDS software (Applied Biosystems, ver. 1.3). The cycle threshold (Ct) values obtained were an average of the triplicates.

RESULTS

In Vitro Transcription of ssrS Extends to sibC—Although it has been reported that ssrS and ygfA are co-transcribed, it remains unknown where this transcription is terminated. To examine the presence of intrinsic termination sites in vitro first, we prepared DNA templates that extended downstream...
of the SibC sRNA gene and used these templates for in vitro transcription to initiate transcription at the ssrS P1 promoter. Because all the DNA constructs had a 9-bp linker sequence at the ends, run-off transcripts were expected to have extra 9 nucleotides. The Constructs 1 and 2 produced transcripts (marked with a and b), with the same sizes as the P1 run-off transcripts of expected 256 and 866 nucleotides, respectively. Construct 4 contained the sibC transcription unit where transcription is initiated at position +869 and terminates at +1009 by an intrinsic terminator (T_{sibC}) (19, 20). This construct generated a transcript (marked with c) with the estimated size of 141 nucleotides. We did not observe apparent run-off transcripts of expected 181 nucleotides in this construct, suggesting that the sibC terminator is very effective. Construct 5 generated two transcripts (marked with d and c) of estimated 1018 and 141 nucleotides, which would initiate from the ssrS P1 and sibC promoter, respectively, but both of which would be terminated at the T_{sibC}. In Construct 6, which lacks the T_{sibC}, we observed a run-off transcript (marked with e) of estimated 949 nucleotides. We did not observe apparent run-off transcripts of expected 181 nucleotides in this construct, suggesting that the sibC terminator is very effective. Construct 5 generated two transcripts (marked with d and e) of estimated 1018 and 141 nucleotides, which would initiate from the ssrS P1 and sibC promoter, respectively, but both of which would be terminated at the T_{sibC}. In Construct 6, which lacks the T_{sibC}, we observed a run-off transcript (marked with e) of estimated 949 nucleotides.
nucleotides that would begin at the ssrS P1 promoter. These in vitro transcriptional data suggest that transcription from the ssrS P1 promoter continues through to sibC and terminates at the T_{sibC} in vitro.

**In Vivo Transcription from ssrS P1 Promoter**—Our in vitro transcription data revealed that transcription of ssrS extends to sibC (Fig. 1). To determine if this transcription extension occurs in vivo, we constructed reporter fusion plasmids by inserting ssrS P1 promoter-containing DNA fragments with unique downstream sequences into plasmid pKK232–8, which contained a promoterless CAT gene. We measured IC_{50} in cells containing the ssrS-CAT fusion plasmids (Fig. 2). Interestingly, the IC_{50} decreased significantly when the ssrS-CAT fusions contained the downstream sequences of 6S RNA beyond position +239 up to +260. The further decrease of IC_{50} was observed when the downstream sequences were extended to or past position +330. These data can be explained in two ways. First, it is possible that the in vivo transcription termination sites in the intergenic sequence between +239 and +330 resulted in ssrS transcription termination. Alternatively, ssrS transcription might extend into the downstream CAT gene, but the intergenic region would be susceptible to endoribonucleolytic cleavage and the cleaved 3' RNA fragment would be rapidly degraded. To discriminate between these two possibilities, we inserted a B. albidum tRNA{Arg} sequence between the ssrS sequences and the CAT coding sequence in the ssrS-CAT fusion constructs, and examined exogenous tRNA expression as well as 6S RNA in a 6S RNA knock-out strain (Fig. 3). We used heterologously expressed B. albidum tRNA{Arg}, which was previously shown to be metabolically stable in E. coli (30) and could be detected by Northern blot analysis without cross-hybridization with E. coli tRNAs. If our second hypothesis, of the susceptibility of the intergenic region to endoribonucleolytic cleavage and the rapid degradation of the cleaved 3' RNA fragment, were correct, we would expect to observe tRNA{Arg} expression in all the constructs even if the endoribonucleolytic cleavage could lead to RNA degradation. However, the amount of tRNA decreased in the presence of the sequences downstream of +239 to +260, and the decrease was prominent with the downstream sequences extended to or past +330 (Fig. 3B), consistent with the IC_{50} data obtained from the ssrS-CAT fusion constructs (Fig. 2). These findings suggested that the intergenic sequence between 6S RNA and CAT genes has in vivo termination sites rather than undergoing endoribonucleolytic cleavage. Therefore, it is likely that the in vivo termination sites lie within the sequence ranging from +239 to +330.
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FIGURE 4. Function of Rho protein as an \textit{ssrS} transcription termination factor in vitro. \textit{A}, in vitro transcription of the \textit{ssrS} gene in the presence of the Rho factor. In vitro transcription reactions were carried out in the presence or absence of Rho protein. IcdA protein was used as a control protein. The Rho-dependent terminated transcripts are indicated by \textit{f} (for \textit{ssrS}) or \textit{g} (for \textit{tyrT}). \textit{B}, schematic representation of DNA templates used for \textit{in vitro} transcription. The DNA templates for \textit{in vitro} transcription were prepared by PCR using the corresponding pCAT plasmids. The DNA templates contained 94 bp of the vector sequences (open rectangles). The major Rho-dependent sites, estimated from the data shown in panel \textit{A}, are indicated by \textit{F}on each substrate. The \textit{tyrT} transcription unit was used as a positive control for Rho-dependent termination and the termination site is indicated by \textit{g}. \textit{C}, binding of Rho protein with precursor 6S RNA containing 3’ downstream sequences. Mature 6S RNA (65) and precursor 6S RNA carrying the 3’ downstream sequences to +330 (Pre-6S) were labeled with \textsuperscript{32}P and subjected to gel mobility shift assay. The labeled RNA of 2 nM was incubated for 15 min in the absence (lanes 1 and 6) of protein or presence (lanes 2–5 and 7–10) of increasing amounts of Rho protein (1–50 nM). IcdA protein (50 nM) was used as a control (lane 11).

(minor sites in the region of +239 to +260 and major sites in +261 to +330).

\textbf{Rho Factor Is Involved in Termination of 6S RNA—}It is well known that many \textit{in vivo} transcription terminations are mediated by termination factors. As Rho factor is the major termination factor in \textit{E. coli} (31), we examined whether Rho factor is involved in transcription termination in the region ranging from positions +239 and +330. We performed \textit{in vitro} transcription assays in the presence of Rho factor (Fig. 4), using DNA templates spanning from −92 to +238, −92 to +330, and −92 to +448, which were marked with 238, 330, and 448 above each lane of the figure, respectively. The −92 to +330 and −92 to +448 templates generated Rho-dependent termination products (marked with \textit{f}), while the −92 to +238 template did not. This is consistent with our \textit{in vivo} results suggesting that the transcription termination is located beyond +238 (Figs. 2 and 3).

We then examined whether this Rho-dependent termination occurs through binding of Rho protein to the 3’ downstream sequences. Mature 6S RNA and precursor 6S RNA carrying the 3’ downstream sequences to +330 were synthesized \textit{in vitro} and used for a gel shift assay with Rho protein. Rho protein bound to the precursor 6S RNA, not mature 6S RNA (Fig. 4C), suggesting that Rho protein terminates the \textit{ssrS} operon transcription through its binding to RNA. To identify the precise termination sites, we analyzed the \textit{in vitro} transcripts produced in the presence of Rho protein by 3’ RACE. The RACE data revealed the 3’ heterogeneity of the \textit{in vitro} transcripts, with the 3’-end of +279 being predominant (Table 2). These data suggest that transcription is terminated by Rho at multiple sites and that the major termination occurs at +279 (Fig. 1C). We also found \textit{in vitro} transcripts with 3’-end of +253, +255, +260, which could explain the observation that the sequence of +239 to +260 provided a partial termination signal in the \textit{ssrS}-CAT fusion assay (Figs. 2 and 3).

To determine whether the Rho-dependent termination takes place \textit{in vivo}, we constructed a plasmid expressing anti-rho RNA by IPTG induction. This anti-rho RNA was designed to bind to the translation initiation region \textit{rho} mRNA (the region of −22 to +6 with the first nucleotide of the start codon as +1) of \textit{rho} mRNA so that the cellular level or translation level of \textit{rho} mRNA would be reduced. Cells containing the plasmid were treated with 1 mM of IPTG and total RNAs were subjected to RT-PCR to analyze the cellular levels of \textit{rho} mRNA and \textit{ssrS-ygfA} dicistronic RNA. The level of \textit{ssrS-ygfA} dicistronic RNA was estimated by determining the amount of transcripts containing the region of +104 to +316 in the \textit{ssrS-ygfA} transcription unit (Fig. 1C). The \textit{rho} mRNA level was reduced to about 50% by induction of anti-rho RNA, while \textit{ssrS-ygfA} dicistronic RNA as run-through transcripts at the Rho termination sites increased by more than 1.5-fold (Fig. 5), suggesting that \textit{ygfA} expression could be regulated by Rho-dependent termination activity.

\textbf{Rho-terminated ssrS Transcripts Are Not Accumulated in Endoribonuclease-deficient Cells—}Our previous study showed that 3’ processing of \textit{ssrS} transcript was not affected in RNase E-, RNase G-, or RNase III-deficient cells (17), while Deutscher and co-workers (18) reported that multiple exoribonucleases can participate in the 3’ trimming reaction of 6S RNA and that any one of five exoribonucleases, RNase II, D, BN, T, and PH, can carry out the trimming reaction although either RNase T or RNase PH appear to be the most effective

\begin{table}[h]
\centering
\caption{Sequencing analysis of the 3’ RACE products}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
3’-Ends of RNA & 253 (3), & 255 (1), & 260 (+1), & 271 (1), & 275 (3), & 277 (2) \\
\hline
& 279 (8), & 281 (1), & 282 (1), & 284 (1), & 298 (1), & 301 (1) \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} The numbers in parentheses indicate the frequency of occurrence.
exoribonucleases. They also showed that the mutant strains lacking four of the five enzymes accumulated 6S RNA with extra sequences of up to 6 nt at the 3′-end. Because mutant cells lacking all five exoribonucleases are not viable and the enzymes functionally overlap in vivo (32), we thought that it would be impossible to observe the accumulation of Rho-terminated ssrS transcripts in any of exoribonuclease-deficient cells. Therefore, we re-examined whether Rho-terminated ssrS precursors would be accumulated in endoribonuclease-deficient cells. In this experiment, we included cells lacking the endoribonuclease RNase P, which had not been included in our previous work (17). First, the mutant strains lacking RNase E, RNase G, RNase III, and RNase P were converted to the corresponding 6S RNA knock-out strains by transduction with ssrS::kan from strain MG1655 ssrS::kan. In these 6S RNA knock-out mutant cells, we analyzed 6S RNA transcripts generated from ssrS-CAT fusion plasmids (Fig. 6). The fusion plasmids generate only P1 transcripts without P2 transcripts, making it easier to analyze 3′-ends of 6S RNA transcripts. All the mutant strains did not show any accumulation of Rho-terminated transcripts of about 280 nucleotides from plasmid pCAT848 carrying the Rho-dependent termination site. On the other hand, plasmid pCAT238, which did not carry the termination site, generated a large precursor (marked with *h*) in rne-1 cells at 44°C, suggesting that this precursor needs the action of RNase E before the 3′ trimming by exoribonucleases. All together, these data suggest that the four endoribonucleases are not involved in 3′ processing of 6S RNA.

**DISCUSSION**

In this study, we found that ssrS transcription is terminated by Rho factor. This finding completes the current model of 6S RNA biogenesis in the cell (Fig. 7). Transcription of 6S RNA from the P1 and P2 promoters is typically terminated by Rho factor at position +279, generating P1 transcripts of 288 nucleotides and P2 transcripts of 503 nucleotides. The mature 6S RNA then forms from these transcripts. Processing and maturation of the 5′-end occurs via different utilization of RNase E or RNase G on P1 and P2 transcripts (17), while processing at the 3′-end seems to precede this 5′ processing because 5′ precursors with the already matured 3′-end were observed as major ssrS transcripts in vivo (17). Both transcripts are subjected to 3′ processing to remove ~90 extra nucleotides. Processing at the 3′-end appears to be carried out by exoribonucleases because premature 3′-ends of 6S RNA were observed in exoribonuclease-deficient cells (18), not in endoribonuclease-deficient cells such as RNase E−, RNase G−, RNase III−, and RNase P− strains (Ref. 17 and Fig. 6). However, we do not exclude the possibility that unidentified endoribonucleases are involved in 3′ processing of 6S RNA before the final trimming although RNase I, known as a non-specific endoribonuclease, appears to be irrelevant because 3′ processing of 6S RNA was not affected in RNase I− cells (33). We also found that mature 6S RNA was generated from two different CAT fusion transcripts containing ssrS sequences up to +185 or +238 (i.e. transcripts from pCAT185 and pCAT238), suggesting that correct 3′ processing can occur even in the absence of Rho-dependent termination. Because a large 6S RNA precursor was observed in cells carrying pCAT238 in rne-1 cells at the nonpermissive temperature, RNase E might assist 3′ processing to promote the generation of mature 6S RNA. In addition, the formation of mature 6S RNA from pCAT185 and pCAT238 implies that 3′ processing is guided by the structural sequence of 6S RNA rather than by extra 3′ sequences.

A recent genome wide study found a class of Rho-terminated loci leading to transcription termination of noncoding RNAs including tRNAs, but not 6S RNA, at the ends of their genes (34). Therefore, the Rho-terminated ssrS locus is likely to belong to this class. Rho factor prefers to bind to naked, untranslated, and C-rich RNA, reiterating C residues with about 12 ± 1 nucleotide-spacing (35–37). The Rho-dependent termination complex consists of a hexamer of Rho proteins that can contact at least six C residues; therefore, Rho-dependent termination requires a repeated C region of ~60 nucleotides in nascent RNA transcribed by the elongation complex. The 3′ downstream region of the 6S RNA sequence contains C residues form positions
Therefore, it is likely that the C-residues ranging from +193 to +265 interact with Rho factor to terminate the transcription. Rho-dependent termination sites are located within the ygfA open reading frame. The definitive gene function of ygfA is not yet known, but encodes a protein with a high degree of sequence identity to mammalian 5-formyl-tetrahydrofolate cyclo-ligase (7, 13). As ygfA does not have its own promoter, its expression should depend on transcription initiation from the ssrS promoters (14, 17), as well as antitermination at Rho-dependent termination sites. Consequently, ygfA expression can be regulated by the initiation rate from the ssrS promoters and the activity of Rho factor in the cell. When a rho-knockdown experiment was performed by expressing anti-rho RNA, we observed the increase of ssrS-ygfA dicistronic transcripts formed by read-through transcription at the Rho-dependent termination sites. Recently, Nudler’s group also reported a significant increase in ygfA mRNA when RNAs from cells treated with bicyclomycin, which inhibits Rho factor function, were subjected to microarray analysis (38). It is likely, therefore, Rho factor participates not only in transcription termination for generating 3'-ends of 6S RNA from the termination sites but also in regulating ygfA expression. It is noteworthy that ygfA expression is implicated in the formation of persister cells, which contribute to the antibiotic resistance of biofilm infections (13). Moreover, several microarray analyses showed that ygfA gene is up-regulated during the biofilm formation (16, 39, 40). Thus, antitermination of ssrS transcripts at Rho-dependent termination sites under some Rho-factor function suppressing conditions could play an essential role in persister cell and biofilm formations.

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