Effect of Transforming RNA on the Synthesis of a Protein with a Secretory Signal Sequence in Vitro*

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U5 small nuclear RNA itself can act as a clastogenic and transforming agent when transfected into cells. In the previous work, the 3′ half of the U5 small nuclear RNA first stem structure (designated RNA3S) was capable of driving normal cells into tumorigenic cells when expressed with a poly(A) tail (RNA3S*). This transformation critically depended upon the polypurine sequence GGAGAGGAA in RNA3S*. In this work, we first examined the pre-β-lactamase and luciferase (model secretory and nonsecretory proteins) translation with the in vitro synthesized RNA3S in rabbit reticulocyte lysate. The capped RNA3S with a poly(A) tail suppressed the translation. In addition, the polypurine sequence played a crucial role in affecting the secretory protein synthesis, indicating a primary action of RNA3S*. Further studies revealed that the oligodeoxynucleotides, corresponding to the polypurine and its antisense sequences, directly contacted 28 S rRNA in ribosome and 7SL RNA in signal recognition particle, respectively, and differentially affected the nascent chain elongation of secretory protein synthesis. These results suggest that RNA3S* blocks a physiological regulatory function played by signal recognition particle and the ribosome in the secretory protein synthesis and support the idea that the transformation might result from a repressed cellular activity.

Carcinogenesis proceeds through a series of genetic alterations involving oncogenes and tumor suppressor genes (1–3). It is also widely believed that carcinogenic initiation is caused by genetic mutation(s) induced by carcinogens. Initiated cells continue to exhibit various unusual phenomena leading to malignant neoplasia such as morphological transformation, immortalization (4), suppression of intercellular communication (5–7), loss of extracellular matrix protein (8, 9), and autonomous growth (10). However, the mechanisms underlying such uncertain alterations associated with all stages in carcinogenesis are still unknown.

In our previous work based on the cell transformation induced by U5 small nuclear RNA (U5) (11),1 the 3′ half of the U5 first stem structure (12) (designated RNA3S) had an ability to convert normal rat fibroblastic 3Y1 cells to morphologically transformed cells at a marked frequency when expressed with a poly(A) tail as an RNA polymerase II-derived noncoding transcript (RNA3S*) (13). The morphologically transformed cells went on eventually to produce tumorigenic cells, suggesting that RNA3S* is capable of driving the normal cells into the neoplastic stage. Additionally, RNA3S* suppressed the fibronectin protein synthesis in HeLa cells, supporting the idea that it is indeed a new type of transforming agent. We thus call an RNA* having a transforming activity such as RNA3S* “transforming RNA.” Based on these results, the transforming RNA might have the ability to perturb a regulatory system to maintain the normal cellular process. This transformation was critically dependent upon the polypurine sequence GGAGAGGAA in RNA3S*. Because the cells expressing the RNA3S without a poly(A) tail (RNA3S) exhibited only a very low frequency of the morphological transformation (14), the poly(A) tail is evidently involved in the enhancement of transformation. It is known that the cap of mRNA binds to an initiation factor, eIF-4E, with several other initiation factors to be anchored to the ribosome (15, 16) and that the poly(A) tail, in cooperation with the cap, stabilizes mRNA to enhance its translation (17–20). In fact, RNA3S* in 3Y1 cells was found to be associated with polysomes (13). In this work, we examined an effect of RNA3S* on the rabbit reticulocyte lysate translation of pre-β-lactamase and luciferase (model secretory and nonsecretory proteins) mRNAs and found that RNA3S* suppressed the secretory rather than nonsecretory protein synthesis.

Proteins with a secretory signal sequence are synthesized by polysomes bound to the endoplasmic reticulum. Signal recognition particle (SRP) is made up of 7SL or SRP RNA (about 300 nucleotides) and six polypeptides (21, 22) and functions in targeting secretory and membrane proteins to the endoplasmic reticulum membranes (23). 7SL RNA has the Alu portion comprising approximately 100 nucleotides from the 5′ end and 45 nucleotides from the 3′ end of the RNA (24). In the mammalian translation system, secretory protein synthesis is paused transiently at multiple sites of ribosome-associated nascent polypeptides (25). With this fact, it has been postulated that a translational pause may be a convenient switch by which cells could adapt the secretory protein synthesis to the secretory needs of the cells (26). We also present evidence that the oligodeoxynucleotides (ODNs), corresponding to the polypurine sequence in RNA3S* and its antisense sequence, directly contacted 28 S rRNA in ribosome and 7SL RNA in SRP, respectively, and differentially affected secretory protein synthesis. Finally, we discuss a possible involvement of the transforming RNA during the process of carcinogenesis.

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‡ The abbreviations used are: U5, U5 small nuclear RNA; SRP, signal recognition particle; ODN, oligodeoxynucleotide; DTT, dithiothreitol; PIPES, piperazine-1,4-bis-2-ethanesulfonic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
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EXPERIMENTAL PROCEDURES

Plasmid Construction—As described previously, the 3′ half of the U5 first stem structure (nucleotides 50–78 of U5) was designated RNA3S, and its antisense counterpart was designated RNA3A (14). Synthetic cDNAs, corresponding to these RNAs, were inserted at the Smal site of a polylinker region in pGEM3 (Promega) to give pG35S and pG3A (13). The BamHI–SacI fragments from the pG constructs were then inserted at the Smal site of the polylinker region in pSP64(poly(A)) (Promega) to create pS35S and pS3A that express RNA3S or RNA3A with a poly(A) tail (RNA3S* or RNA3A*) by SP6 RNA polymerase. Similarly, pS35S and pS3SM were constructed to express RNA3SG* and RNA3SM*, in which the polyuridine sequence in RNA3S* was altered to GGGGG-GGGG and CCCCUCUUCU, respectively, by using pS35S and pS3SM as described previously (13). The RNA* sequences used in this study are shown in Table I. The calf thymus cDNA sequence of RNA3IS is contained in the DDBJ/EMBL/GenBankTM with accession number AB021173.

In Vitro Transcription—Plasmid DNAs were linearized at a site beyond the SP6 promoter and cDNA insert. A 20-μl reaction contained 40 mM Tris-HCl, pH 7.5, 6.5 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol (DTT), 1 unit/μl ribonuclease inhibitor (Takara), 0.5 mM each of ATP, CTP, and GTP, 12 μM of UTP, 50 μCi of [α-32P]UTP (5000 Ci/mmol, Amersham Pharmacia Biotech) to give 35S-labeled RNA3SM, and 1 unit/μl SP6 RNA polymerase (Takara) (27). Samples were incubated for 60 min at 37 °C, and the DNA templates were digested with 2 units RNase-free DNase I (Takara)/μg DNA at 37 °C for 15 min. RNA was extracted with phenol/chloroform and precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. For the poly(A) tail (RNA3S*), a reaction mixture contained 0.5 mM of magnesium DNA, and 1 unit/μl SP6 RNA polymerase (Takara) (27). Samples were incubated for 60 min at 37 °C, and the DNA templates were digested with 2 units RNase-free DNase I (Takara)/μg DNA at 37 °C for 15 min. RNA was extracted with phenol/chloroform and precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol.

mRNA was purified by electrophoresis in a denaturing polyacrylamide gel containing 7 M urea. After electrophoresis, a nitrocellulose filter was hybridized with a riboprobe as described previously (13).

Northern Blot Hybridization—RNA was electrophoresed on a 5% denaturing polyacrylamide gel containing 7 M urea. After electrophoresis, a nitrocellulose filter was hybridized with a riboprobe as described previously (13).

Determination of the Rabbit 7SL RNA Sequence—To determine the 7SL RNA sequence by using the SMART methods (CLONTECH), a GppG cap and a poly(A) were added to the gel-purified RNA at the 5′ end and the 3′ end, respectively. First, 1 μg of the gel-purified RNA was incubated with 2 units of poly(A) polymerase (Takara) in 50 μl of buffer containing 50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 2.5 mM MnCl₂, 250 mM NaCl, 1 mM DTT, 0.05% bovine serum albumin, and 0.1 mM ATP for 60 min at 37 °C and precipitated with ethanol after the phenol/chloroform extraction. The poly(A) RNA was further incubated with 2 units of guanylyltransferase (Life Technologies, Inc.) in 30 μl of buffer containing 50 mM Tris-HCl, pH 7.9, 1.25 mM MgCl₂, 6 mM KCl, 2.5 mM DTT, 0.1% bovine serum albumin, 30 units of RNase inhibitor (Takara), and has been submitted to the DDBJ/EMBL/GenBankTM with accession number AB021174.

Cloning of a Partial 7SL cDNA—Cloning of a partial cDNA of rabbit 7SL RNA was carried out by using a 5′-AmpLI FINDER RACE kit (CLONTECH). Single-stranded cDNA was synthesized by using 0.5 μg of the gel-purified RNA as described previously (13). PCR amplification of the anchor-ligated cDNA was performed in 50 μl of reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 250 mM NaCl, 1 mM DTT, 0.05% bovine serum albumin, and 0.1 mM ATP for 60 min at 37 °C and precipitated with ethanol after the phenol/chloroform extraction. The poly(A) RNA was further incubated with 2 units of guanylyltransferase (Life Technologies, Inc.) in 30 μl of buffer containing 50 mM Tris-HCl, pH 7.9, 1.25 mM MgCl₂, 6 mM KCl, 2.5 mM DTT, 0.1% bovine serum albumin, 30 units of RNase inhibitor (Takara), and has been submitted to the DDBJ/EMBL/GenBankTM with accession number AB021174.

TABLE I

| RNA sequences used for the in vitro translation assay |
|-----------------------------------------------------|
| RNA3S*   | GAAUUCGGUGGAGAGGAACAUCUCUGAGU |
| RNA3A    | ACUCAGAGGGUGUCCUCGAGGGAAAC |
| RNA3SG   | GAAUUGCCGGGGGCGGGCCAGUCUGAGU |
| RNA3SM   | GAAUUGGCGGGGGCGGGCCAGUCUGAGU |

RESULTS

RNA3S* Affects Protein Synthesis in Vitro—To examine an effect of RNA3S* on the translation of pre-β-lactamase and luciferase mRNAs in rabbit reticulocyte lysate, we synthesized RNA3S** RNA3A* (antisense RNA3S*), and RNAPL* (transcript from the pSP64(poly(A)) polylinker region) that had a cap structure at the 5′ end and a poly(A) tail at the 3′ end.
FIG. 1. Translation of pre-β-lactamase and luciferase mRNAs with in vitro synthesized RNA3S⁺, RNA3S⁻, RNA3A⁺, and RNAPL⁺ were synthesized by using SP6 RNA polymerase from pS3S, pS3A, and pSP64 linearized with EcoRI. Aliquots (3 μl) were removed at the indicated times and analyzed by SDS-PAGE and scanning densitometry as described. The gel patterns of luciferase and pre-β-lactamase synthesized and their intensities are shown in each experiment. A, dose-effect test. Mixtures of RNA3S⁺ and RNAPL⁺ were added at the indicated amount into a 20-μl translation. Set 1, 4 μg of RNA3S⁺; set 2, 2 μg of RNA3S⁺ and 2 μg of RNAPL⁺; set 3, 1 μg of RNA3S⁺ and 3 μg of RNAPL⁺; set 4, 4 μg of RNAPL⁺. The other translation products (25–30 kDa) that were not due to luciferase and pre-β-lactamase were due to labeled peptidyl-tRNA resulting from translation of fragments of globin mRNA (35), because they appeared in a reaction containing no added mRNA. Additionally, nonspecific bands (33–62 kDa) might be due to aberrant initiation of luciferase translation and endogenous methionine-binding lysate proteins (35). Marker, prestained SDS-PAGE standards (Bio-Rad). Gel patterns (left side) and intensities (right side). B, translations with RNA3S⁺, RNA3A⁺, and RNAPL⁺. 4 μg of each RNA⁺ were added into a 20-μl translation. Intensities of luciferase and pre-β-lactamase bands in the control were not shown because of apparently higher intensities. Gel patterns (left side) and intensities (right side). C, translations with three types of
These RNA sequences are shown in Table I. The synthesized RNA's were added into the standard translation as described under "Experimental Procedures." A dose-effect test showed that 0.2 μg of RNA3S/μl of translation suppressed most efficiently the overall protein synthesis, reducing the amounts of luciferase and pre-β-lactamase by about 55 and 65%, respectively, at 30 min of incubation when compared with those with RNAPL alone (Fig. 1A). Upon translation with RNA3S, RNA3A, and RNAPL, RNA3S suppressed most significantly the translation (Fig. 1B). Compared with the translation with RNAPL, the amounts of luciferase and pre-β-lactamase synthesized with RNA3S were reduced by about 60 and 70%, respectively, at 60 min of incubation. RNA3A and RNAPL also appeared to suppress the translation when compared with RNA3S with different structures. Capped RNA3S with a poly(A) tail, uncapped RNA3S with a poly(A) tail, and uncapped RNA3S without a poly(A) tail were synthesized by using pS3S and SP6 RNA polymerase. 4 μg of each RNA3S were added into a 20-μl translation. PA indicates poly(A) tail. Gel pattern (left side) and intensities (right side) are shown. D, translations with RNA3S, RNA3SG, and RNA3SM. 5 μg of each RNA were added into a 25-μl translation. Gel patterns (left side) and intensities (right side) are shown. These translation experiments were repeated with similar results.
FIG. 2. **RNase H cleavage assays.** A, agarose gel analysis. 10 μl of lysate were incubated with ODN at the indicated amount in the presence of 20 units of RNaseH at 37 °C for 60 min. Total RNA from each sample was electrophoresed on a 1.2% agarose gel. Gels were stained with ethidium bromide and visualized. Lane 1, control; lane 2, RNaseH alone; lanes 3–5, 4, 2, and 1 μg of 3S-9nt, respectively; lane 6, 4 μg of 3S-9nt alone; lanes 7–9, 4, 2, and 1 μg of 3A-9nt, respectively; lane 10, 4 μg of 3A-9nt alone. B, nondenaturing polyacrylamide gel analysis. 10 μl of lysate were treated with 4 μg of either 3S-9nt or 3A-9nt in the presence of 20 units of RNase H at 37 °C for 60 min. Total RNA was electrophoresed on a 6%
Determination of the site of 7SL RNA in contact with 3A-9nt.

**A**, size of RNAx. Approximately 0.1 μg of the gel-purified RNAx was end-labeled and electrophoresed in a 6% sequencing gel. Marker (lane M), 32P-labeled MspI digest of pBR322.

**B**, sequencing gel of the 7SL2 cDNA and its sequence. The cloned 7SL2 DNA was sequenced in both orientations, linearizing pG7SL2 with either HindIII or PvuII. The sequencing gel is displayed in the sense direction, and the 7SL2 RNA sequence derived from the cDNA sequence is shown in the proposed secondary structure (40).

**C**, mapping of the 5' end of the RNase-protected fragment. Total RNA was recovered from 10 μl of lysate treated with 4 μg of ODN in the presence of 20 units of RNaseH. The antisense 7SL2 cRNA probe was prepared by using SP6 RNA polymerase and pG7SL2 digested with EcoRI and should be thus 185 nucleotides in length (lane 1). Lane 2, 10 μg of yeast tRNA; lanes 3 and 6, control RNA; lane 4, 3S-9nt-treated RNA; lanes 5 and 7, 3A-9nt-treated RNA. Marker (lane M), 32P-labeled MspI digest of pBR322. Sizes of the DNA fragments are indicated in nucleotides. A DNA sequencing is shown in parallel to determine the cleavage sites of 7SL RNA. The nibbling pattern is also shown for a shorter exposure in the lower side.

**D**, denaturing polyacrylamide gel analysis. 10 μl of lysate were incubated with ODN at the indicated amount in the presence of 20 units of RNaseH at 37 °C for 60 min. Total RNA was electrophoresed on a 5% denaturing gel containing 7 M urea. Lane 1, control; lane 2, RNaseH alone; lanes 3–5, 4, 2, and 1 μg of 3S-9nt, respectively; lane 6, 4 μg of 3S-9nt alone; lanes 7–9 and 11, 4, 2, 1, and 6 μg of 3A-9nt, respectively; lane 10, 4 μg of 3A-9nt alone. RNAx and RNAX indicate the same RNAs as shown in **B**. E, Northern blot analysis. RNAs shown in **D** were electroblotted onto the nitrocellulose filter. The filter was hybridized with an antisense 7SL7 cRNA probe at 10^9 cpm/ml. The intensities of RNAx bands were quantitated and found to decrease with the increased amount of 3A-9nt (data not shown). RNAx and RNAX were the same RNAs as shown in **D**.
pressed the translation (Fig. 1C) the cleavage. This suggests that 3S-9nt is specific to cleavage of 28 S rRNA in a dose-dependent manner, generating some cleavage products. This requires both the cap and poly(A) tail to exert its function.

In the previous study, RNA3SG+, in which the polypurine sequence was changed from GGAGGGAG to GGAGGGGGG, exhibited the transforming potential similar to that of RNA3S+ (13). On the other hand, RNA3SM+, which has the CCUCUCUCU in place of the polypurine sequence in RNA3S+, lost the potential completely. We also examined the effects of RNA3SG+ and RNA3SM+ (Table I) on the translation. As shown in Fig. 1D, RNA3SG+ also suppressed the pre-β-lactamase synthesis, whereas RNA3SM+ did not suppress it. We noted that RNA3S+ and RNA3SG+ preferred to affect the secretory rather than nonsecretory protein synthesis, depending upon their polypurine sequences.

The Polypurine Sequence Contacts 28 S rRNA in Ribosome, whereas Its Antisense Sequence Contacts 7SL RNA in SRP—From the above data, the polypurine sequence might contact directly an integral RNA in polysomes. We thus prepared the ODN, 3S-9nt (GGAGGGAGA), and searched the lysate for such an RNA. An ODN-directed RNase H cleavage assay has been useful for identifying the RNA sequences that are accessible for base pairing with DNA molecules. Total RNA was recovered from the lysate treated with 3S-9nt in the presence of RNase H and analyzed in gels. As shown in Fig. 2A, 3S-9nt mediated the cleavage of 28 S rRNA in a dose-dependent manner, generating some cleavage products. This suggests that 3S-9nt is specific to the cleavage. 4 μg of 3S-9nt/10 μl of lysate were required to degrade all the target RNAs. Neither 3S-9nt alone nor RNaseH alone affected 28 S rRNA. A similar assay was performed by using 3A-9nt (TTCCCTTCC) that was the antisense 3S-9nt ODN. In a nondenaturing gel analysis (Fig. 2B), 3A-9nt appeared to mediate the cleavage of an RNA species (designated RNAx in Fig. 2B) located between 18 S rRNA and 5.8 S rRNA, generating a new band RNA (RNAx in Fig. 2B). From the separating gel pattern of cytoplasmic RNA as reported previously (36), it is suggested that RNAx might be 7SL RNA and thus RNAx the cleavage product of 7SL RNA. We determined the sequence of RNAx in a manner as described under “Experimental Procedures.” Both a cap and a poly(A) tail were added to the gel-purified RNAx. After reverse transcription of the modified RNAx followed by PCR amplification of the resulting cDNAs, products were cloned into a plasmid and sequenced. As shown in Fig. 2C, RNAx was identified as 7SL RNA, exhibiting a 98.3% homology with the human 7SL DNA sequence. To confirm that RNAx is a cleavage product of 7SL RNA, we carried out a Northern blot hybridization. We constructed several plasmids containing the partial 7SL cDNA fragments by using the reverse transcription products of the gel-purified RNAx. One of those plasmids, pG7SL7 contained the cDNA fragment corresponding to nucleotides 110–183 of 7SL RNA but not the Alu sequence. Thus, pG7SL7 produces an antisense partial 7SL cRNA specific to 7SL RNA. Again, the cleavage products were analyzed in a denaturing gel (Fig. 2D). RNAx from the 3A-9nt-treated lysate appeared as a broader and lower intensive band (lanes 7–9 and 11 in Fig. 2D). RNAx appeared in the nondenaturing gel was just visible in the denaturing gels. In Northern blot analysis (Fig. 2E), both RNAx and RNAx (lanes 7–9 and 11 in Fig. 2E) indexed hybridized with the antisense 7SL7 cRNA. In addition, 3A-9nt was found to mediate the cleavage of 7SL RNA dose-dependently. Neither 3A-9nt alone nor RNaseH alone affected 7SL RNA. These results suggest that 3S-9nt and 3A-9nt contact specifically 28 S rRNA in the ribosome and 7SL RNA in SRP, respectively, which are known to be engaged in secretory protein synthesis (38).

3A-9nt Contacts the Alu Portion in 7SL RNA—The three distinct activities of SRP (signal recognition, elongation arrest, and translocation promotion) reside in separate domains (39). We thus determined the site of 7SL RNA in contact with 3A-9nt. First, the gel-eluted RNAx was, without an alkaline
phosphatase treatment, incubated with \( {\gamma}^{-32}\text{P} \)ATP in the presence of polynucleotide kinase and analyzed in a sequencing gel. As shown in Fig. 3A, RNAx accepted the radioactive phosphate, suggesting that RNAx lost the 5' site of 7SL RNA. The smear pattern (indicated by arrows) suggests that RNAx might be a mixture of the partial 7SL RNAs resulting from the RNaseH digestion and still have a structure. It was estimated to be more than at least 230 nucleotides in length. Out of the plasmids containing partial 7SL DNA fragments as described above, pG7SL2 that contains the cDNA fragment corresponding to nucleotides 44–183 of 7SL RNA was used to prepare a ribo-probe in the following RNase protection assay. Fig. 3B shows the sequencing gel of 7SL2 DNA and its sequence. The antisense 7SL2 cRNA was hybridized with total RNA from the 3A-9nt-treated lysate. After the digestion with RNase A and RNase \( T_{1} \), samples were analyzed in a sequencing gel (Fig. 3C). As expected, the contact site of 7SL RNA displayed a nibbling pattern, demonstrating the ODN-directed RNaseH digestion. Because 7SL2 RNA is 139 nucleotides in length, the site was found to be GAGG at 5–8 nucleotides from the 5' end of 7SL2 RNA when compared with a DNA sequencing shown in parallel. This site corresponds to nucleotides 48–51 of 7SL RNA. The result indicates that 3A-9nt contacts the Alu portion in 7SL RNA that is assigned to the elongation arrest activity of SRP (41).

3S-9nt and 3A-9nt Affect Secretory Protein Synthesis—To confirm that 3S-9nt and 3A-9nt affect only secretory protein synthesis, we performed the synchronized translation with 3S-9nt or 3A-9nt. In this experiment, we used a tRNA-mediated protein labeling system to detect proteins synthesized because the ability of 3S-9nt to affect the pre-\( \beta \)-lactamase synthesis was very large. After the reaction was synchronized by allowing initiation for 3 min, the RNA cap analogue was added to block further initiation. As shown in Fig. 4, the pre-\( \beta \)-lactamase polypeptide chains were observed after 5–6 min of synthesis in the control translation. The chains appeared after 8–10 min of synthesis in the translation with 3S-9nt, although they were clearly detected even after 5 min of synthesis in the translation with 3A-9nt. The amount of pre-\( \beta \)-lactamase synthesized with 3S-9nt was reduced by more than 70% after 10 min of incubation, whereas that with 3A-9nt was increased to about 120–150% at each time of incubation. On the other hand, luciferase polypeptide chains appeared after 10 min of synthesis in all the three translations. In addition, both ODNs did not change the pattern, demonstrating the ODN-directed RNaseH digestion.

**TABLE II**

| ODN sequences used for the RNaseH cleavage assay |
|-----------------------------------------------|
| 3S-9nt                                     | GGAGAGAAA |
| 2AM-9nt                                    | GGAGAGAAA |
| 3A-9nt                                     | TTCCCTCCC |
| 3SM1–9nt                                   | GAAGAGGAA |
| 3SM2–9nt                                   | GGAGAGAAA |
| 2A-9nt                                     | GAAGAGAAA |
| GA-9nt                                     | GAAGAGAG |
| G-9nt                                      | GGGGGGGG |

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![Fig. 5. Synchronized translation with membranes. Translation was carried out in a 30-\( \mu \)l reaction containing 2.4 equivalents of membranes, 2.4 \( \mu \)g of pre-\( \beta \)-lactamase mRNA, 0.6 \( \mu \)g of luciferase mRNA, and 12 \( \mu \)g of either 3S-9nt or 3A-9nt. At the indicated times, aliquots (2 \( \mu \)l) were analyzed by 3D-PAGE (left side) and scanning densitometry (right side), mm, microsome membranes. The translation was repeated with similar results.]
amount of luciferase synthesized. These results indicate that 3S-9nt markedly suppresses the secretory protein synthesis, whereas 3A-9nt modestly enhances it. It is also evident that the delay observed with 3S-9nt is not due to suppression of overall protein synthesis.

The Effect on the Secretory Protein Synthesis Occurs at the Level of Nascent Chain Elongation—To see whether the ODNs affect either initiation or elongation of translation, microsomal membranes were supplemented into the translation. Membranes that contain the SRP receptors relieve the SRP-mediated elongation arrest and process pre-secretory protein to mature protein (42, 43). 0.08 equivalents of membranes/mlof translation continued to process pre-\(\beta\)-lactamase to \(\beta\)-lactamase without losing their activity for at least 60 min of incubation, representing 88–90% of processing efficiency (data not shown). As shown in Fig. 5, pre-\(\beta\)-lactamase polypeptide chains appeared after 6 min of synthesis in all the translations. Obviously, membranes accelerated the pre-\(\beta\)-lactamase synthesis with 3S-9nt. This result indicates that the ODNs differentially affect the secretory protein synthesis at the level of elongation but not initiation.

The Suppression of Secretory Protein Synthesis Is Due to the Direct Contact between 28 S rRNA and ODN—From the results described above, it is suggested that the suppressed pre-\(\beta\)-lactamase synthesis is due to the direct contact between 28 S rRNA and 3S-9nt. To further study this, we prepared the following altered polypurine ODNs: 3SM1-9nt, 3SM2-9nt, 2A-9nt, 2AM-9nt, and GA-9nt (Table II). By using these ODNs, we carried out the RNase H cleavage and in vitro translation assays. In the former assay, 3SM2-9nt and 2AM-9nt apparently mediated the cleavage of 28 S rRNA, representing the cleavage products similar to those with 3S-9nt (Fig. 6A). 3SM1-9nt and 2A-9nt appeared to mediate the cleavage of a very small amount of 28 S rRNA. However, the cleavage pattern with GA-9nt was quite different from that with 3S-9nt. This finding indicates that the cleavage requires a common sequence GGAG in the ODNs. Thus, 3SM1-9nt, 2A-9nt, and GA-9nt still lack a nucleotide important for the cleavage. In the latter assay, 3SM2-9nt and 2AM-9nt suppressed the pre-\(\beta\)-lactamase synthesis, whereas 3SM1-9nt, 2A-9nt, and GA-9nt did not affect the synthesis (Fig. 6B). Combined with these results, it is suggested that the suppression of secretory protein synthesis results from the direct contact between 28 S rRNA and the GGAG in the ODNs. For the transforming RNA3SG, we also examined the ability of G-9nt in these assays. As shown in Fig. 6, however, G-9nt mediated destruction of 28 S rRNA.

FIG. 6. Relationship between the 28 S rRNA cleavage and the suppression of pre-\(\beta\)-lactamase synthesis. A. RNase H cleavage of 28S rRNA with various altered polypurine ODNs. 10 \(\mu\)l of lysate were incubated with 4 \(\mu\)g of ODN but with 2 \(\mu\)g of 3S-9nt in the presence of 20 units of RNase H. Lane 1, control; lane 2, 3S-9nt; lane 3, 3SM1-9nt; lane 4, 3SM2-9nt; lane 5, 2A-9nt; lane 6, 2AM-9nt; lane 7, GA-9nt; lane 8, G-9nt. B, translations with the altered ODNs. Translation was synchronized in a 15-\(\mu\)l reaction with 6 \(\mu\)g of ODN. At the indicated times, aliquots (3 \(\mu\)l) were analyzed by SDS-PAGE (left side) and scanning densitometry (right side). With the experimental results performed repeatedly, the intensities of luciferase and pre-\(\beta\)-lactamase bands with 2A-9nt, 2AM-9nt, and GA-9nt were adjusted to those with the other ODNs.
and suppressed almost completely overall protein synthesis, suggesting that G-9nt is inappropriate to see the ability of RNA3SG⁺ in these assays.

**DISCUSSION**

In this work, we examined the translation of pre-β-lactamase and luciferase mRNAs with RNA3S⁺ and found that RNA3S⁺, requiring the cap and poly(A) tail, significantly suppressed the translation. Because the uncapped RNA3S without a poly(A) tail had no effect on the translation, it is evident that the effect of RNA3S⁺ is not due to the action of a protein bound to the RNA. Also, it is suggested that the suppression of overall protein synthesis with RNA3A⁺ or RNAPL⁺, observed when compared with the control translation, is due to the association of these RNAs with ribosomes and thus nonspecific. Therefore, these findings parallel the data that the transforming activity of RNA3S was markedly enhanced when expressed with a poly(A) tail (13, 14). In addition, RNA3S⁺ and RNA3SG⁺ suppressed the secretory rather than nonsecretory protein synthesis, whereas RNA3SM⁺ did not affect the translation. This indicates that the polyuridine sequences play a crucial role in suppressing the secretory protein synthesis and also parallels the fact that the transforming activity critically depended upon the sequences. With these results, it is strongly suggested that the suppression of secretory protein synthesis is a primary action of the transforming RNA.

In the synchronized translation, 3S-9nt suppressed the pre-β-lactamase synthesis, whereas 3A-9nt enhanced it. Because these effects were abolished by the supplementation of membranes, it is evident that the ODNs do not inactivate the ability of pre-β-lactamase mRNA to be translated. Similarly, the ODNs, because they did not change the processing efficiency of membranes, do not block the ability of membranes. We concluded that these effects occurred at the level of elongation. Therefore, this suppression appears to be consistent with the observation in the canine SRP-rabbit reticulocyte lysate system that was designed to demonstrate a transient elongation arrest activity of SRP (25).

It appeared that RNA3S⁺ also suppressed the luciferase synthesis in the amount of protein synthesized. Recently, it has been reported that the pep transferase center localized at the central circle of domain V of the 23 S-like rRNAs in bacteria is the most important functional site for protein synthesis (44). We examined whether RNA3S could affect cis-actively the pre-β-lactamase synthesis. The sequence of RNA3S was introduced into the 5'- or 3'-untranslated region of pre-β-lactamase mRNA. The translations of these modified mRNAs were the same as that of the original mRNA (data not shown). Thus, RNA3S⁺ is able to affect only trans-actively the secretory protein synthesis by acting presumably at the site of 28 S rRNA different from the pep transferase center. It seems likely that the site may not only structurally but functionally link to the pep transferase center. Because the amount of RNA3S⁺ added into the translation is thought to be in excess, it is likely that a large proportion of ribosomes are associated with the RNA. This may influence nonspecifically the overall protein synthesis in vitro. However, we do not yet know whether such a situation also takes place in cells and plays an important role in transformation.

Concerning the site (nucleotides 48–51) of 7SL RNA contacting 3A-9nt, this site forms a stem structure with the 3'-terminal region (nucleotides 295–298) of the RNA in the proposed secondary structure (40). We also noticed that any amount of 3A-9nt could not cleave all of 7SL RNAs in lysate, suggesting that the cleavage might correlate with the activity of SRP. In a chemical modification analysis (45), the A⁴⁹ in nucleotides 48–51 of 7SL RNA is a sensitive nucleotide in membrane-bound SRP, while it is protected in both polysome bound SRP and soluble SRP during the SRP cycle. Taken together, it is suggested that the stem structure might be changed during the SRP cycle. The question is how the ODNs affect the pre-β-lactamase synthesis at the level of elongation. 3A-9nt contacted the GAGG of 7SL RNA, and the GGAG of 3S-9nt contacted 28 S rRNA, suggesting a possible contact sequence CUCC of the RNA. At these sites, 28 S rRNA and 7SL RNA potentially form base triplets with the basic orientation of 7SL RNA in SRP in a microsomal analysis (46) and a three-legged model in which the tRNA-like 5' domain of 7SL RNA is in direct contact with tRNA binding sites of ribosome (47), these possible base pairings may have a physiological regulatory function for the secretory protein synthesis.

In mammalian cells, most secretory and membrane proteins are synthesized through the SRP-mediated translocation system (26, 48, 49). If a transforming RNA that is an RNA polymerase II-dependent noncoding transcript containing a unique polypurine sequence with a poly(A) tail is persistently expressed, the normal cellular process would continue to be perturbed because secretory protein synthesis could be suppressed. Such a transforming RNA could be generated by a genetic mutation in an initial event of carcinogenesis. Under such a repressed cellular activity, cells may change themselves so as to adapt to the environment for the main purpose of surviving. Based on the studies of liver carcinogenesis (50), it is assumed that tumors develop by cellular adaptation to perturbations in the environment (51). By analogy with studies in bacteria, in which Escherichia coli kept in the stationary phase under selective conditions mutate to gain a selective advantage (52–54), it is assumed that multiple genetic changes in carcinogenesis are adaptive mutations (55–59). This adaptive mutation theory seems currently most attractive to understanding the transformation mechanisms with a transforming RNA.

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