Nucleic Acids Research, Vol. 20, No. 14 3737–3742

# Characterization of the spermidine-dependent, sequence-specific endoribonuclease that requires transfer RNA for its activity

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Received March 6, 1992; Revised and Accepted June 3, 1992

## ABSTRACT

**The spermidine-dependent, sequence-specific endoribonuclease (RNase 65) in mouse FM3A cells consists of protein and transfer RNA lacking its 3′ terminus. *In vitro* properties of this enzyme were characterized using partially purified enzyme. The RNase 65 activity requires spermidine, which is not replaceable with spermine or $Mg^{++}$. The enzyme cleaves an RNA substrate on the 3′ side of the phosphodiester bond. The cleavage reaction has a temperature optimum around 50°C and a pH optimum around 7.0. The optimum KCl concentration for the activity is around 10 mM. Relative cleavage efficiency of two differently folded RNA substrates with the common target sequence was analyzed at 37°C and 50°C. The results of this analysis suggest that unfolding of the target sequence is critical for recognition by RNase 65. Furthermore, in experiments using several point-mutated RNA substrates designed to form basically the same secondary structure as the wild type, one to three nucleotide substitutions in the target sequence all reduced cleavage efficiency. The RNase 65 activity is found only in cytosolic extracts, not in nuclear ones. Gel filtration analysis suggests that the native size of the endoribonuclease is approximately 150 kDa.**

## INTRODUCTION

RNA can act as an enzyme besides storing genetic information (1). It is now widely accepted that RNA may have been the primordial living molecule and functioned as the sole catalytic molecule in the RNA world (1). While the RNA world may have shifted to the present-day living system (the DNA world) through the ribonucleoprotein (RNP) world, proteins may have subsumed RNA's biochemical functions and DNA may have taken the place of RNA for more stable genetic material (1). Even today self-splicing RNAs (2) and the RNA component of RNase P (3) have catalytic functions *in vitro*. For events such as pre-mRNA splicing (4), first pre-rRNA processing (5), histone pre-mRNA 3′-end formation (6) and mitochondrial RNA processing (7), *in vitro* catalysis requires both RNA and protein components. It is thought that these molecules may be fossils of the RNA or RNP world.

Transfer RNA, ancestry of which may have emerged to give birth to the RNP world, plays a crucial intermediate role in the translation of genetic information from nucleic acid to protein. Several systems make use of tRNA for functions other than translation. Certain tRNAs are used as primers for reverse transcription of retrovirus genomic RNA (8, 9). A chloroplast $tRNA^{Glu}$ is involved in chlorophyll biosynthesis (10), and a $tRNA^{His}$ is an essential component of the ubiquitin- and ATP-dependent proteolytic system in mammalian cells (11).

We have found a novel spermidine-dependent endoribonuclease in mouse FM3A cell extracts and designated it as RNase 65 (12). This endoribonuclease cleaves RNA substrates containing a target sequence CCCCCGGUUUGU in its middle. The target sequence was first derived from a transcribed spacer region of the mouse pre-rRNA (12, 13). However, we concluded that the original spacer region is not a genuine target of the RNase 65 activity from circumstantial evidence (12). Recently, we have shown that in addition to a protein component, the RNase 65 activity requires tRNA lacking its 3′ terminus, which is identified as either $tRNA^{Gln}$, $tRNA^{Gly}$ or $tRNA^{Met}$ (14). This is the first example showing that tRNA is involved in an endoribonuclease activity. RNase 65 is of great interest in both physiological and evolutionary aspects. Although little is known about a physiological role of the sequence-specific RNA cleavages by RNase 65, the activity might be involved in specific mRNA degradation (14). We also speculate that the tRNA may have evolved from a ribozyme which had a ribonucleolytic cleavage activity. In this paper, we describe some of the *in vitro* properties of RNase 65 partially purified from mouse FM3A cell extracts.

## MATERIALS AND METHODS

### Preparation of mouse FM3A cell extracts

Mouse FM3A cells were cultured in ES medium (Nissui) containing 3% fetal calf serum (GIBCO) and harvested at a density of $5 \times 10^5$ cells/ml. Total S100 extracts were prepared as described by Weil *et al.* (15). Briefly, the cells were incubated at 4°C in twice packed cell volume of buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 10 min and homogenized with 15 strokes in a Dounce homogenizer with the tight fitting pestle. After addition of one-tenth volume of buffer B (0.3 M Hepes (pH 7.9), 1.4 M KCl, 0.03 M $MgCl_2$), the homogenate was centrifuged at 100,000 g for 1 h and the supernatant was dialysed for 5 h against buffer D

(20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol) before storage in aliquots at −80°C. The protein concentration of the total S100 extracts was between 10 and 15 mg/ml.

Cytosolic S100 extracts and nuclear extracts of the mouse FM3A cells were prepared according to the method of Dignam *et al.* (16). Briefly, to obtain the cytosolic S100 extracts, centrifugation at 100,000 g for 1 h was performed after homogenization of the cells and subsequent removal of nuclei by low speed centrifugation. For the nuclear extracts, the precipitated nuclei were further centrifuged at 25,000 g for 20 min, and the pellet was resuspended in buffer C (20 mM Hepes (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) with a Dounce homogenizer, mixed for 30 min, and centrifuged at 25,000 g for 30 min. Both supernatants were dialysed for 5 h against the buffer D. These extracts were separated into aliquots and stored at −80°C. The protein concentrations of the cytosolic S100 extracts and the nuclear extracts were 10 to 15 mg/ml and 5 to 6 mg/ml, respectively.

### Partial purification of RNase 65

*Chromatography on phosphocellulose.* 1 ml of mouse FM3A cell extracts (5−15 mg protein per ml) in the buffer D (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol) was applied to a 2 ml phosphocellulose column (Whatman) equilibrated with the buffer D. A flow-through fraction with the buffer D (designated as PA fraction) was assayed for RNase 65 activity.

*Chromatography on Superose 12.* 200 μl of the PA fraction of the total S100 extracts was applied to Superose 12 (Pharmacia), column size 30 cm×1 cm, equilibrated with the buffer D, at a flow rate of 0.3 ml/min. Fractions of 0.6 ml were collected and tested for cleavage activity.

### Mutated transcription templates

The following five pairs of oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer:

oligo M1 5′-TCGACCCCCCGTTTGTCCCCT-3′
oligo M1′ 3′-GGGGGGCAAACAGGGGAGATC-5′
oligo M2 5′-TCGACCCCCCCTTTGTCCCCT-3′
oligo M2′ 3′-GGGGGGGAAACAGGGGAGATC-5′
oligo M3 5′-TCGAACCATGGTTTGTCCCCT-3′
oligo M3′ 3′-TGGTACCAAACAGGGGAGATC-5′
oligo M5 5′-TCGAAGCCCGGTTTGTCCCCT-3′
oligo M5′ 3′-TCGGGCCAAACAGGGGAGATC-5′
oligo M6 5′-TCGACGCCCGGTTTGTCCCCT-3′
oligo M6′ 3′-GCGGGCCAAACAGGGGAGATC-5′.

Each pair of the oligonucleotides, which have one to three nucleotide substitutions (indicated by underlines) relative to the wild-type pair oligo C and oligo C′ (12), was cloned into a transcription vector pSPMr (12) digested by SAlI and XbaI (Takara Shuzo). The resultant plasmids for mutated templates were designated as pSPCM1, pSPCM2, pSPCM3, pSPCM5, and pSPCM6, respectively.

### RNA substrates

The above five variant plasmids and the variant plasmid pSPCM4 (12) digested with XbaI, and the wild-type plasmid pSPC (12) digested with either XbaI or PvuI (Takara Shuzo) were used as templates for *in vitro* transcription with SP6 RNA polymerase (Takara Shuzo). The transcription reaction was performed under the condition specified by the manufacturer using [α-$^{32}$P]UTP (Amersham Japan). Reaction products were separated on a denaturing polyacrylamide gel, and 50 nt RNAs from the XbaI-cut plasmids and a 64 nt RNA from the PvuI-cut plasmid were eluted, ethanol precipitated and dissolved in water.

### RNase 65 assay

A standard RNase 65 assay was performed under conditions as follows. The standard reaction mixture (25 μl) contained 5 μl of the PA fraction of mouse FM3A cell extracts in a solution containing 10 mM Hepes (pH 7.9), 0.33 mM DTT, 3.2 mM spermidine, 0.002−0.005 pmol RNA substrates. After incubation at 37°C for 40 min, RNAs were extracted with phenol, precipitated with ethanol and analyzed on a 10% polyacrylamide−8 M urea gel.

### 3′-end labeling

A cold 50 nt wild-type RNA substrate was synthesized from the XbaI-cut pSPC by SP6 RNA polymerase under the condition mentioned above without [α-$^{32}$P]UTP. The substrate was incubated under the standard RNase 65 assay conditions, and products were extracted with phenol and ethanol precipitated. The redissolved RNA products were labeled with [5′-$^{32}$P]pCp using T4 RNA ligase (Takara Shuzo) as described by England *et al.* (17).

## RESULTS

### Spermidine essential for the RNase 65 activity is not replaceable with spermine or $Mg^{++}$

A 50 nt RNA substrate containing the RNase 65 target sequence (Figure 2A) was synthesized from the XbaI-cut pSPC plasmid template by SP6 RNA polymerase. The substrate was incubated with the 0.1 M KCl flow-through fraction in phosphocellulose column chromatography (PA fraction) of total S100 extracts and products were analyzed by denaturing polyacrylamide gel electrophoresis. Activation of RNase 65 was observed in the presence of spermidine, but neither spermine nor $Mg^{++}$ activated the enzyme (Table I). Spermine and $Mg^{++}$ had no effect up to 6.4 mM and 100 mM, respectively (data not shown). The substrate was also not cleaved without the PA fraction even in the presence of spermidine.

### RNase 65 cleaves on the 3′ side of the phosphodiester bond

To determine which side of the phosphodiester bond RNase 65 cleaves on, a cold 50 nt wild-type RNA substrate was synthesized by SP6 RNA polymerase without [α-$^{32}$P]UTP and used for the standard assay. After the cleavage reaction, RNAs were extracted with phenol, precipitated with ethanol and redissolved in water. The RNAs with a free 3′ hydroxyl group were labeled with [5′-$^{32}$ P]pCp. The [5′-$^{32}$P]pCp-labeled products were analyzed on a denaturing polyacrylamide gel with the cleavage products uniformly labeled with [α-$^{32}$P]UTP (Figure 1). Besides tRNAs in the PA fraction of total S100 extracts and the 50 nt RNA substrate, a 35 nt cleavage product was labeled with [5′-$^{32}$P]pCp (Figure 1, lane 2). This result indicates that the 35 nt product has a free 3′ hydroxyl group and that the cleavage occurs on the 3′ side of the phosphodiester bond (Figure 2A). Although a 15 nt counterpart product ought to have been also labeled, it was not resolved on the gel due to [5′-$^{32}$P]pCp-labeled unknown RNA of a similar size in the PA fraction (Figure 1).

**Table I.** Optimization of the RNase 65 activity

| | | | | | | |
|---|---|---|---|---|---|---|
| PA fraction | − | − | + | + | + | + |
| Cofactor[a] | − | spermidine | − | spermidine | spermine | $Mg^{++}$ |
| Cleavage (%) | 0 | 0 | 0 | 12.9 | 0 | 0 |
| Time (min) | 0 | 10 | 20 | 40 | 60 | 120 |
| Cleavage (%) | 0 | 3.0 | 8.3 | 15.0 | 19.6 | 29.4 |
| Temp. (°C) | 20 | 30 | 37 | 50 | 60 | 80 |
| Cleavage (%) | 7.7 | 18.4 | 33.5 | 51.9 | 46.7 | 15.2 |
| pH[b] | 6.5 | 7.0 | 7.5 | 7.9 | 8.5 | |
| Cleavage (%) | 13.0 | 30.1 | 24.1 | 15.1 | 11.4 | |
| KCl (mM) | 0 | 10 | 50 | 100 | 200 | |
| Cleavage (%) | 13.0 | 14.8 | 12.6 | 2.2 | 0 | |

The influence of cofactor, time, temperature, pH and KCl concentration on the cleavage efficiency of 50 nt wild-type RNA substrate was studied. The substrate synthesized from the XbaI-cut pSPC plasmid by SP6 RNA polymerase with [$\alpha$-$^{32}$P]UTP was incubated with the PA fraction of total S100 extracts under the standard assay conditions (see Materials and Methods) except a varying parameter. After resolution of the reaction products on a denaturing polyacrylamide gel and subsequent autoradiography, 35 nt and 15 nt cleaved RNAs and a 50 nt not cleaved RNA were quantitated by densitometric scanning. The cleavage efficiency is defined as 100×(35 nt and 15 nt cleaved RNAs)/(35 nt and 15 nt cleaved RNAs and 50 nt not cleaved RNA).

[a]The concentrations of spermidine, spermine and $MgCl_2$ were 3.2, 3.2 and 50 mM, respectively.

[b]The buffering substances used at pH of 6.5, 7.0 to 7.9, and 8.5 were bis−Tris, Hepes, and Tricine, respectively.

## Optimization of conditions for the RNase 65 activity

Optimum conditions for the RNase 65 activity were investigated, and the results are shown in Table I. After RNase 65 assays using a 50 nt wild-type RNA substrate under various conditions, products were resolved on a denaturing polyacrylamide gel, and cleaved and not cleaved RNAs were quantitated by desitometric scanning of its autoradiogram. A time course of RNA cleavage reaction was performed at 37°C at a pH of 7.9. The RNase 65 activity was linear in time up to 40 min. The cleavage reaction had a temperature optimum around 50°C. The pH-optimum for the RNase 65 reaction was around 7.0. The optimum KCl concentration for the activity was around 10 mM; at 200 mM no cleavage was detected.

## Secondary structure of RNA substrate affects cleavage efficiency

To investigate the effect of folding of RNA substrate on cleavage efficiency, two differently folded RNA substrates containing the common target sequence were used for the RNase 65 assay. 50 nt and 64 nt RNAs were synthesized by SP6 RNA polymerase from the XbaI-cut pSPC plasmid and the PvuI-cut pSPC plasmid, respectively (12). Figure 2 shows free energy minimum secondary structures of two RNA substrates calculated by the method of Zuker and Stiegler (18) and their cleavage sites (12). The RNA substrates were incubated with the PA fraction of total S100 extracts at 37°C and 50°C. After analysis of the products on a denaturing polyacrylamide gel and subsequent autoradiography, cleaved and not cleaved RNAs were quantitated by densitometric scanning. The 50 nt RNA substrate was more efficiently cleaved than the 64 nt one at both 37°C and 50°C (Figure 3). This may be partly because both guanosine residues on the 3′ and 5′ sides of the cleavage site form base-pairing in the 64 nt RNA substrate, while the 3′ side guanosine has a pairing base and the 5′ side one does not in the 50 nt RNA substrate (Figure 2). The relative cleavage efficiency (defined in the Table II legend) of the 64 nt RNA substrate to the 50 nt one was 34% at 37°C, and increased to 61% at 50°C (averages in two experiments). This suggests that unfolding of the target sequence is critical for recognition by RNase 65.

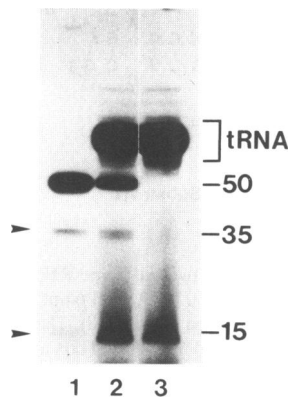


**Figure 1.** RNase 65 cleaves on the 3′ side of the phosphodiester bond. A cold 50 nt wild-type RNA substrate was synthesized from the XbaI-cut pSPC template by SP6 RNA polymerase without [$\alpha$-$^{32}$P]UTP. After incubation of the PA fraction of total S100 extracts with (lane 2) and without (lane 3) the cold RNA substrate under the standard assay conditions, RNA products were extracted and 3′-end-labeled with [5′-$^{32}$P]pCp. [5′-$^{32}$P]pCp-labeled RNAs were analyzed by denaturing polyacrylamide gel electrophoresis with the cleavage products of the [$\alpha$-$^{32}$P]UTP-prelabeled 50 nt wild-type RNA substrate (lane 1) under the standard conditions. Arrowheads indicate 35 nt and 15 nt cleavage products.

## The substrate sequence specificity of RNase 65

We then studied the substrate sequence specificity using six point-mutated RNA substrates of 50 nt. These substrates which had one to three mutated nucleotides in the 12 nt target sequence were synthesized by SP6 RNA polymerase from the XbaI-digested pSPCM series plasmids (Table II). Each variant substrate but that from the XbaI-cut pSPCM4 (12) was designed to form basically the same secondary structure as the 50 nt wild-type substrate (Figure 2A) except that some variants had slight changes at the mutated sites. In free energy minimum secondary structure of the 50 nt variant substrate from the XbaI-cut pSPCM4, only six nucleotides in a 12 nt mutated target sequence including two guanosine residues (at positions −1 and 1) at a potential cleavage site form base-pairing (data not shown). RNase 65 assays with those mutated RNA substrates and the 50 nt wild-type one were

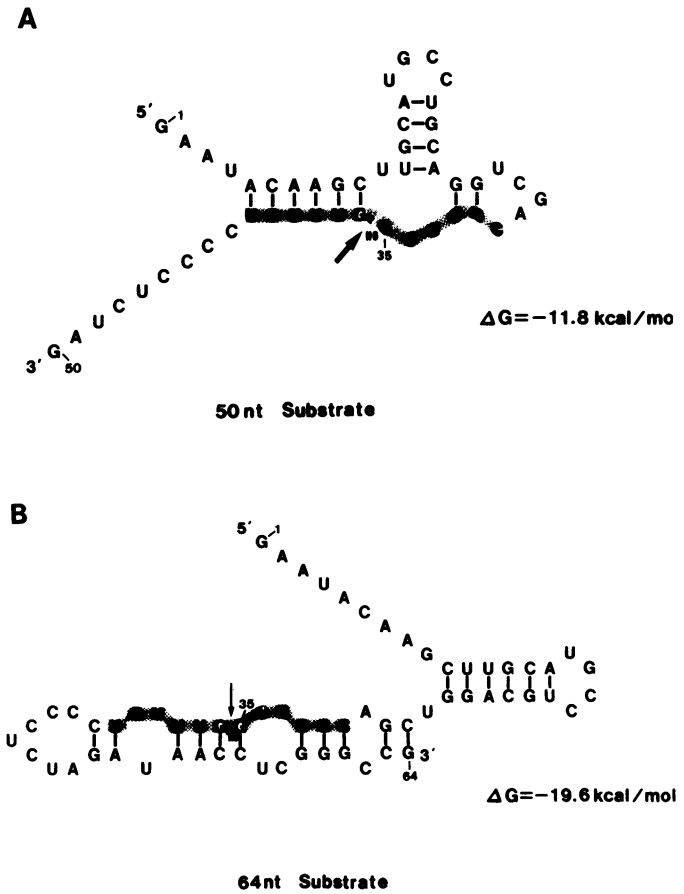


**Figure 2.** Secondary structures of two wild-type RNA substrates. Free energy minimum secondary structures of 50 nt (A) and 64 nt (B) RNA substrates were calculated by the method of Zuker and Stiegler (18). The RNase 65 target sequence and the cleavage site are represented by a shaded box and an arrow, respectively. ΔG denotes a calculated minimum free energy.

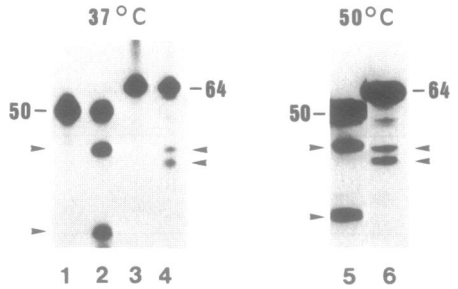


**Figure 3.** Temperature dependence of relative cleavage efficiency of two differently folded RNA substrates. Under the standard assay conditions, 50 nt (lanes 2 and 5) and 64 nt (lanes 4 and 6) wild-type RNA substrates were incubated with the PA fraction of total S100 extracts at 37°C and 50°C. Lanes 1 and 3 represent 50 nt and 64 nt input RNA substrates, respectively. Arrowheads denote cleavage products.

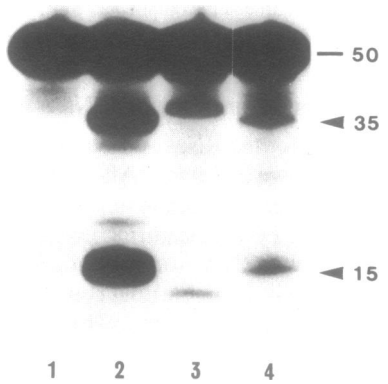


**Figure 4.** Identification of subcellular location of RNase 65. A 50 nt wild-type RNA substrate from the XbaI-cut pSPC plasmid was incubated with the PA fraction of total S100 extracts (lane 2), nuclear extracts (lane 3) and cytosolic extracts (lane 4) under the standard assay conditions. After incubation, RNA products were resolved on a denaturing polyacrylamide gel with an input 50 nt RNA substrate (lane 1). Arrowheads denote 35 nt and 15 nt cleavage products.

performed using the PA fraction of total S100 extracts under the standard conditions except at 50°C to repress the effect of RNA folding on cleavage. After resolution of the reaction products on a denaturing polyacrylamide gel and subsequent autoradiography, cleaved and not cleaved RNAs were quantitated by densitometric scanning. Table II shows relative cleavage efficiency of the variant substrates to the wild type. On the whole the relative cleavage efficiency correlates inversely with the number of nucleotide substitutions in the target sequence. However, the substrate from the XbaI-cut pSPCM5 containing two substitutions (C to G at position −5 and C to A at position −6) was more effectively cleaved than that from the XbaI-cut pSPCM6 with a single C to G substitution at position −5. This implies that A residue is preferable to C residue at position −6 for the RNase 65 target. Cleavage of the variant substrate from the XbaI-cut pSPCM3, which has C to A and C to U changes at positions −3 and −2, respectively, besides a preferable C to A change at position −6, was not detected, suggesting that two C residues at positions −3 and −2 are critical for recognition by RNase 65. The substrate from the XbaI-cut pSPCM4 containing substitutions of the above two C residues was also not cleaved at all. The wild type and the two variants from the XbaI-cut pSPCM5 and pSPCM6 were cleaved at a single site between two G residues at positions −1 and 1, while the variant from the XbaI-cut pSPCM1 with a G to C change at position −1 was cleaved equally after C residue at position −1 and G residue at position 1, and the double point-mutated (G to C changes at positions −1 and 1) substrate from the XbaI-cut pSPCM2 was cleaved faintly after C residue at position 1. These results indicate that sequential two G residues at positions −1 and 1 are involved not only in cleavage efficiency but also in determination of the cleavage site.

## The RNase 65 activity is found only in cytosolic extracts, not in nuclear ones

We have used for the RNase 65 assay the PA fraction of the total S100 extracts prepared according to the method of Weil *et al.* (15). In this preparation, the total S100 extracts contained both cytosolic and nuclear proteins. To identify subcellular location of RNase 65, we prepared cytosolic extracts and nuclear ones of mouse FM3A cells according to the method of Dignam *et al.* (16). The RNase 65 assay was performed using the PA fraction of these extracts. The activity was found only in the cytosolic extracts, not in the nuclear ones (Figure 4). A different activity from that of RNase 65 was also detected only in the nuclear extracts (Figure 4, lane 3).

**Table II.** Relative cleavage efficiency of mutated substrates

| Template | Sequence[a] | Relative cleavage efficiency (%)[b] |
|---|---|---|
| | −6 −11 6 | |
| pSPC/XbaI | CCCCCG\|G UUUGU | 100 |
| pSPCM1/XbaI | CCCCCC\|G\|UUUGU | 79.3 |
| pSPCM2/XbaI | CCCCCC C\|UUUGU | 4.8 |
| pSPCM3/XbaI | ACCAUG G UUUGU | 0 |
| pSPCM4/XbaI | CGCUAG G UUUGU | 0 |
| pSPCM5/XbaI | AGCCCG\|G UUUGU | 75.2 |
| pSPCM6/XbaI | CGCCCG\|G UUUGU | 56.4 |

[a]Vertical lines and underlines denote cleavage sites and mutated nucleotides, respectively. Sequences are listed in 5′ to 3′ direction.
[b]Relative cleavage efficiency is defined as the value of cleavage efficiency, cleaved RNAs/(cleaved and not cleaved RNAs), normalized by fixing that of the 50 nt wild-type RNA to 100%. The values are averages in two experiments.

### RNase 65 occurs as a stable complex of protein and tRNA

We estimated the native size of RNase 65 by gel filtration. Large size fractions (3 to 15) were assayed for cleavage activity under the standard conditions, because fractionation of mouse FM3A cell extracts by ultrafiltration and a subsequent assay suggested that the native size of RNase 65 is larger than 100 kDa (data not shown). The results revealed a peak of activity with a size of approximately 150 kDa (Figure 5). This indicates that RNase 65 occurs as a stable binary complex of protein and tRNA (approximately 25 kDa) even in the absence of both RNA substrate and spermidine.

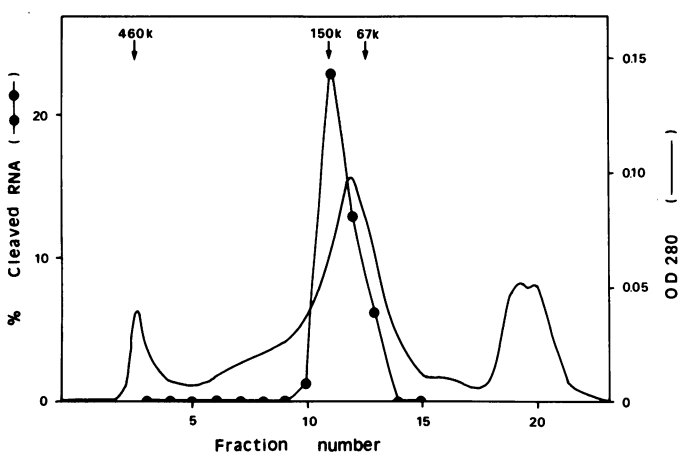


**Figure 5.** Gel filtration analysis of the RNase 65 activity. The PA fraction of total S100 extracts containing the RNase 65 activity was applied to Superose 12 (Pharmacia). Fractions 3 to 15 were incubated with an [α-$^{32}$P]UTP-labeled 50 nt wild-type RNA substrate under the standard conditions. After electrophoresis of the reaction products and subsequent autoradiography, cleaved and not cleaved RNAs were quantitated by densitometric scanning, and the values of the cleavage efficiency were plotted according to the legend of Table I. Calibration of the gel filtration was performed using apoferritin (460 kDa), immunoglobulin G (150 kDa) and bovine serum albumin (67 kDa) as size markers. The apoferritin was eluted in void volume.

## DISCUSSION

RNase 65 occurs as a stable binary complex of protein and tRNA lacking its 3′ terminus with the native size of approximately 150 kDa. The tRNA, which is identified as either $tRNA^{Gln}$, $tRNA^{Gly}$ or $tRNA^{Met}$, is an essential component for the cleavage activity of the RNase 65 (14). In both bacterial and animal cells tRNA is involved in controlled degradation processes. In *E.coli*, an idling reaction on ribosomes in the absence of charged tRNA produces guanosine 3′-diphosphate 5′-diphosphate (ppGpp) during amino acid starvation. The levels of ppGpp correlate inversely with the rate of rRNA and tRNA chain synthesis and directly with rates of protein catabolism (19). In eukaryotic cells, tRNA is required for degradation of some but not all protein substrates in the ubiquitin- and ATP-dependent proteolytic system (11, 20). Although we know little about a physiological role of the sequence-specific RNA cleavages by RNase 65 and genuine target cellular RNAs, the activity might be involved in specific mRNA degradation as discussed previously (14). This idea may be supported by the fact that RNase 65 localizes in cytosol. It has been reported that AU-rich sequences in the 3′ non-coding regions of some special mRNAs (for example, c-fos mRNA) are signals for poly(A) removal and consequent destruction of the mRNAs (21, 22). In order to find out potential target RNAs of RNase 65, we carried out computer search for sequences similar to the target sequence on DNA sequence data bases. This revealed that no other sequences than the original sequence from mouse rDNA contain a complete match to the 12 nt target sequence and that many sequences in mouse mRNAs have high homology (data not shown). For example, mouse protein kinase C-alpha mRNA (23) and mouse proto-oncogene pim-1 mRNA (24) contain sequences CCCCCAGUUUGU and CCCCCGGUUUAU, respectively, that match the original in 11 out of 12 nt. Further studies are needed to examine whether these mRNAs are indeed cleaved *in vivo* by RNase 65.

We performed analyses of the sequence specificity using several point-mutated RNA substrates. The mutations ranging from −6 to 1 all affected cleavage efficiency (Table II), indicating that at least this part of the 12 nt target sequence is important for recognition by RNase 65. Nucleotides at positions 2 to 6 were not substituted in this study to retain basically the same secondary structure as the 50 nt wild-type RNA substrate. More detailed analyses with variant substrates containing base substitutions also at positions 2 to 6 will be necessary to fully elucidate the sequence specificity of RNase 65.

Many endoribonucleases, which consist of proteins, are only specific for mononucleotides or dinucleotides (for example, RNase $T_1$ (25) cuts after guanosines), while RNA enzymes can recognize oligonucleotides in RNA substrates by Watson−Crick base-pairing. A shortened form of the *Tetrahymena* group I intron

can cleave after a sequence CUCU in RNA substrate (26). A separated hammerhead RNA can act as an endoribonuclease that cleaves RNA substrates containing partially complementary region to the ribozyme (27). Recognition of an RNA sequence by RNA via Watson–Crick base-pairing must be easier and firmer than by protein. Thus, it is very likely that some region of the tRNA component of RNase 65 recognizes the target sequence in RNA substrates by base-pairing (14). This may be supported by the results showing that unfolding of the target sequence enhances the cleavage efficiency (Figure 3). Furthermore, some other tRNA species lacking its 3′ terminus could also recognize its target sequence complementary to some region of itself and exhibit cleavage activity in the form of a binary complex with protein.

The RNase 65 activity needs spermidine as an essential cofactor, which is not replaceable with spermine or $Mg^{++}$ (Table I). Spermidine stabilizes RNA higher order structure and DNA double helical structure, activates RNA and DNA polymerases, enhances binding of aminoacyl-tRNA to ribosome and so on. A role of spermidine in the RNase 65 activity may be to help form an active conformation of the tRNA component, the binary complex of tRNA and protein, or the ternary complex of RNase 65 with an RNA substrate. Spermine and $Mg^{++}$ may be inadequate in length (longer and shorter, respectively) to make intra- or inter-molecular bridges via ionic bonds, so that the active conformation may not be formed.

We found another endoribonuclease activity in the PA fraction of nuclear extracts, which cleaved the same 50 nt wild-type RNA substrate at a different site (Figure 4, lane 3). This activity was spermidine-independent and micrococcal nuclease-insensitive (data not shown), and might be involved in the cleavages of the transcribed spacer regions of the mouse pre-rRNA (12, 13). We were not able to detect this activity in the PA fraction of total S100 extracts. This may be explained by binding of an inhibitor in cytosol to this nuclear endoribonuclease or competitive inhibition by RNase 65 for the same RNA substrate.

We tried whether the tRNA component of RNase 65 solely can cleave the target RNA like the RNA component of RNase P (3). Until now cleavage activity of the tRNA is not detected under several conditions (data not shown). Some special condition or cofactor might be required for ribozyme activity of the tRNA. Alternatively, catalytic activity of the tRNA retained in the RNA or RNP world might be replaced by the protein component during evolution.

## ACKNOWLEDGEMENTS

We thank Masami Suzuki for technical assistance and Tetsuya Komiyama for a generous gift of phosphoramidites. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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