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Coupled transcription and processing of mouse ribosomal RNA in a cell-free system

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An *in vitro* processing system of mouse rRNA was achieved using an RNA polymerase I-specific transcription system, (S100) and recombinant plasmids consisting of mouse rRNA gene (rDNA) segments containing the transcription initiation and 5′-terminal region of 18S (or 41S) rRNA. Pulse-chase experiments showed that a specific processing occurred with transcripts of the plasmid DNAs when the direction of transcription was the correct orientation relative to the 18S rRNA coding sequence, but not with transcripts of the DNA templates in which this coding sequence was in the opposite orientation. From the S1 nuclease protection analyses, we concluded that there are several steps of endonucleolytic cleavage including one 105 nucleotides upstream from the 5′ end of 18S rRNA. Intermediates cleaved at this site were identified in *in vivo* processing of rRNA. This result indicates that endonucleolytic cleavage takes place 105 nucleotides upstream from the 5′ terminus of 18S rRNA prior to the formation of mature 18S rRNA. Trimming or cleavage of the 105 nucleotides may be involved in the formation of the 5′ terminus of mature 18S rRNA.

Key words: mouse rRNA/RNA processing/specific endonucleolytic cleavage/transcription

Introduction

The mammalian rRNA gene (rDNA) is transcribed by RNA polymerase I as a primary transcript of 45S pre-rRNA, which is subsequently processed in several steps to yield the mature 18S, 5.8S and 28S rRNA species (Perry, 1976). The development of an RNA polymerase I-specific *in vitro* transcription system (Mishima *et al.*, 1981; Grummt, 1981; Miller and Sollner-Webb, 1981) has provided significant information on the initiation of transcription of rDNA, especially in mouse cells; determination of promoter regions (Grummt, 1982; Yamamoto *et al.*, 1984), species specificity of rDNA transcription *in vitro* (Grummt *et al.*, 1982), identification of species-dependent initiation factor(s) (Mishima *et al.*, 1982), and so on.

However, little is known about the processing sites of rRNA or about the enzyme(s) and signal sequence(s) necessary for processing. Wellauer *et al.* (1974) reported the major processing scheme of mouse L cell rRNA by an electron microscopic study. Bowman *et al.* (1981) have reported the three major rRNA cleavage pathways of mouse L cells by RNA gel transfer and blot hybridization. In addition to the major processing steps, it has been reported that some trimming processes are involved in the formation of rRNA (Nazar, 1974; Kominami and Muramatsu, 1977). Recently, using an *in vitro* transcription system, Miller and Sollner-Webb (1981) determined the early processing site located 650 nucleotides downstream from the transcription initiation site, which had been previously reported as a putative transcription initiation site (Urano *et al.*, 1980).

Here we describe a coupled transcription and processing system focusing on the processing of the 5′-terminal region of 18S rRNA, because the cleavage of this region is considered to be the first major processing step to yield 24S and 41S rRNAs (Wellauer *et al.*, 1974; Perry, 1976; Bowman *et al.*, 1983). 41S rRNA is then cleaved at the 3′ end of the 18S rRNA to generate 18S and 36S rRNAs (Wellauer *et al.*, 1974). From S1 nuclease mapping using *in vivo* pre-rRNA, Bowman *et al.* (1983) reported that an initial cleavage process of 45S pre-rRNA generated the mature 5′ terminus of 18S rRNA. Here we demonstrate that an *in vitro* processing event occurred at a position 105 nucleotides upstream from the 5′ end of 18S rRNA in a coupled transcription and processing system (consisting of a mouse S100 extract and fused plasmid DNAs as templates) in which the transcription initiation segment was joined to the 5′ end region of 18S rRNA. This endonucleolytic cleavage site was also detected during rRNA processing *in vivo*. This finding indicates that a site of 105 nucleotides upstream from the 5′ terminus of 18S rRNA is also involved in the formation of the mature 5′ terminus of 18S rRNA. This coupled transcription and processing system will provide a useful

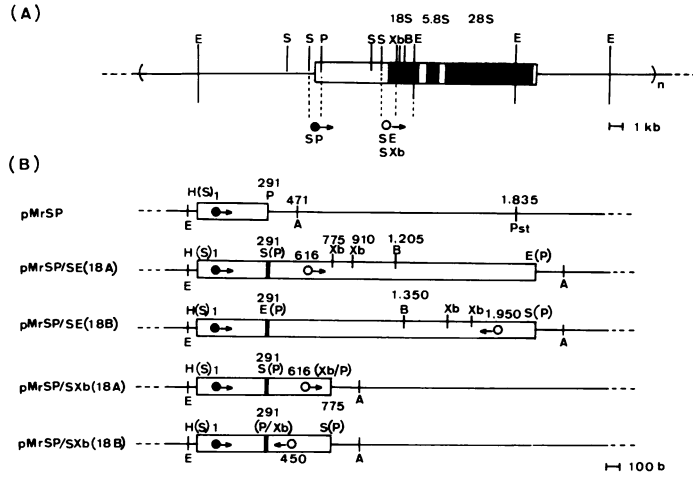

Fig. 1 (**A**) Maps of the mouse rRNA gene and (**B**) templates used for *in vitro* transcription and processing. (**A**) Partial restriction enzyme cleavage sites of mouse rDNA. The box shows the 45S pre-rRNA coding region. The open boxes show the transcribed spacer and the closed boxes show the 18S, 5.8S and 28S rRNA coding regions. The closed and open circles indicate the site of transcription initiation and 5′ end of 18S rRNA, respectively. The arrows indicate the direction of transcription. E: *Eco*RI, S: *Sal*I, P: *Pvu*II, Xb: *Xba*I, B: *Bma*HI. (**B**) DNA templates used for *in vitro* transcription and processing. Constructions of these plasmid DNAs are described in Materials and methods. The boxes containing closed circles are derived from the initiation region (SP) shown in (**A**). The boxes containing open circles are derived from the 5′ end region of 18S rRNA (SE and SXb) shown in (**A**). Arrows indicate the direction of transcription. Solid lines show pBR322 DNA. Numbers indicate the length in nucleotides from the transcription initiation site (Urano *et al.*, 1980; Mishima *et al.*, 1981; Goldman *et al.*, 1983). Restriction sites shown in a parenthesis represent the cleavage sites before construction, which are destroyed by the blunt-end ligation. H: *Hind*III, A: *Acc*I, Pst: *Pst*I.

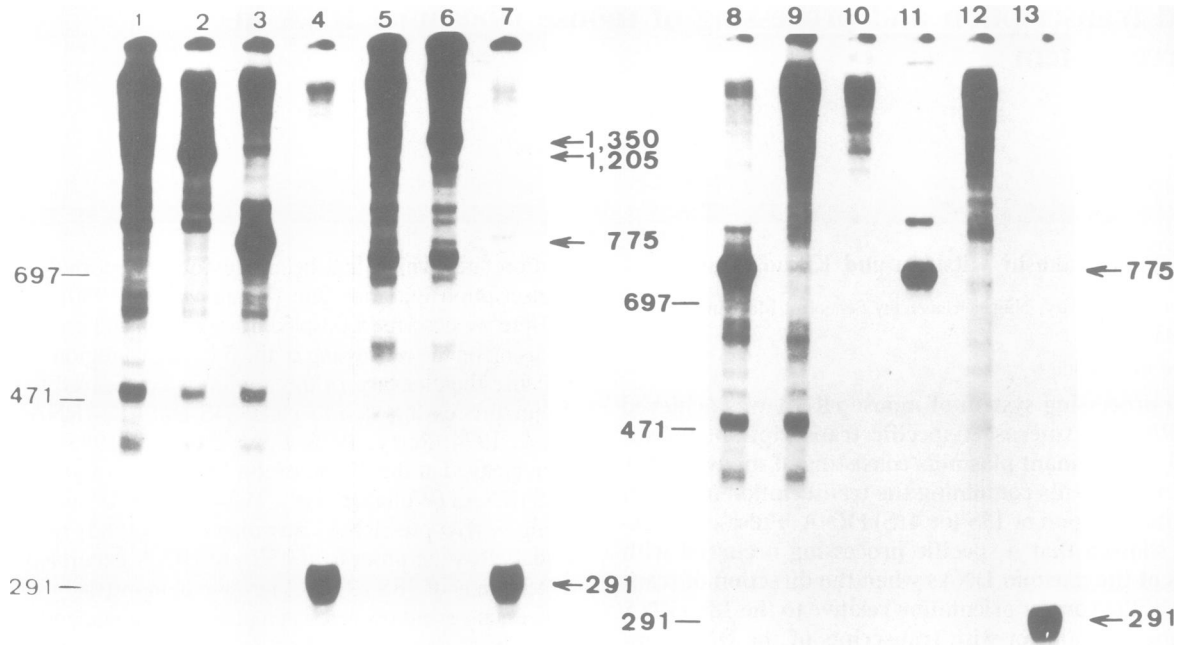

Fig. 2. Transcription of pMrSP/SE and pMrSP/SXb DNAs in extracts from mouse cells. *In vitro* RNA synthesis was carried out at 30°C for 50 min as described previously (Mishima *et al.*, 1981, 1982). [^{32}P]RNA was glyoxalated and analyzed by electrophoresis on a 4% polyacrylamide gel as described previously (Mishima *et al.*, 1981). DNA templates used are as follows: **lane 1**, non-cleaved circular pMrSP/SE(18A); **lane 2** *Bam*HI-cleaved pMrSP/SE(18A); **lane 3**, *Xba*I-cleaved pMrSP/SE(18A); **lane 4**, *Sal*I-cleaved pMrSP/SE(18A); **lane 5**, non-cleaved circular pMrSP/SE(18B); **lane 6**, *Bam*HI-cleaved pMrSP/SE(18B); **lane 7**, *Eco*RI-cleaved pMrSP/SE(18B); **lane 8**, the same as **lane 3**; **lane 9**, non-cleaved circular pMrSP/SXb(18A); **lane 10**, non-cleaved circular pMrSP/SXb(18B); **lane 11**, *Sal*I-cleaved pMrSP/SXb(18B); **lane 12**, non-cleaved circular pMrSP; **lane 13**, *Pvu*II-cleaved pMrSP. Size markers of 291 and 471 nucleotides shown on the right side of the figure are transcripts from the *Pvu*II-cut and *Acc*I-cut pMrSP DNAs, respectively. The marker of 697 nucleotides is the transcript from *Sal*I-cut human rDNA (Mishima *et al.*, 1982; Financsek *et al.*, 1982). Nucleotide lengths shown on the left side of the figure indicate the length expected from the run-off transcripts shown in Figure 1B.

approach to investigate the mechanism of rRNA processing at the molecular level.

Results

The in vitro transcription and processing system

To develop an *in vitro* transcription and processing system, we constructed several fusion genes joining the 0.45-kb segment containing the transcription initiation region of mouse rDNA to the 1.9-kb or 0.48-kb segment containing the 5′ end of 18S rRNA (or 41S rRNA) as shown in Figure 1B. About 3.3-kb of transcribed spacer was deleted in these fusion genes, as shown in Figure 1A (Mishima *et al.*, 1980, 1981).

pMrSP/SE(18A) and pMrSP/SE(18B) plasmid DNAs contained the initiation region (0.45 kb in length) and about two thirds of 18S rRNA coding sequences (1.9 kb in length) in the same and opposite orientation of transcription, respectively (Figure 1B). We examined the transcripts from both DNA templates using an RNA polymerase I-specific *in vitro* transcription system (Mishima *et al.*, 1981; Grummt, 1981; Miller and Sollner-Webb, 1981). Figure 2, lanes 1 – 4, shows the [^{32}P]RNA products from closed circular, *Bam*HI-cut, *Xba*I-cut and *Sal*I-cut pMrSP/SE-(18A) DNAs, respectively. The 1205-bp (lane 2) and 775-bp (lane 3) RNAs were run-off transcripts from the *Bam*HI-cleaved and *Xba*I-cleaved templates, respectively. At least three discrete bands of 500, 470 and 420 bp, in addition to several bands around 600 bp, were synthesized from the DNA templates with and without truncation (lanes 1 – 3). These RNAs were transcribed from the initiation site at nucleotide 1 shown in Figures 1B and 4D, because only 291-bp RNA was generated from the *Sal*I-cut pMrSP/SE(18A) DNA (lane 4).

The transcripts from the non-cleaved circular, *Bam*HI-cut and *Eco*RI-cut pMrSP/SE(18B) DNA templates are shown in Figure 2, lanes 5 – 7. The run-off transcript of 1350-bp was generated from the *Bam*HI-cut template (lane 6). A discrete band of 550 bp and several bands in the 800-bp region were synthesized from the circular (lane 5) and *Bam*HI-cleaved (lane 6) pMrSP/SE(18B) DNA templates. These bands were also transcribed from the initiation site at nucleotide 1 shown in Figures 1B and 4D, because only 291-bp RNA was generated from the *Eco*RI-cut pMrSP/SE(18B) template (lane 7).

Next we examined the products from the templates of pMrSP/SXb(18A) and pMrSP/SXb(18B), which contain the 0.45-kb initiation segment joined to the 0.48-kb fragment containing the 5′ end of the 18S rRNA coding region in the same and opposite directions, as shown in Figure 1B. 500-, 470- and 420-bp products were observed from the circular pMrSP/SXb(18A) DNA template (lane 9), as shown in the case of pMrSP/SE(18A) DNA (lanes 1 – 3 and 8). No discrete RNA products were found from the closed circular (lane 10) and *Sal*I-cut (lane 11) pMrSP/SXb(18B) DNAs, except for the 775-bp run-off transcript (lane 11). The same results were obtained in the case of non-cleaved (lane 12) and *Pvu*II-cleaved (lane 13) pMrSP DNAs.

In this series of experiments (Figure 2), many minor bands were observed in addition to the run-off transcripts of 1350, 1205, 775 and 291 nucleotides from the fusion DNA templates. The possibility may exist that these minor bands were processed RNAs or products terminated by RNA polymerase I.

Detection of processed RNA

To examine whether the RNAs synthesized *in vitro* shown in Figure 2 are processed products, pulse-chase experiments were

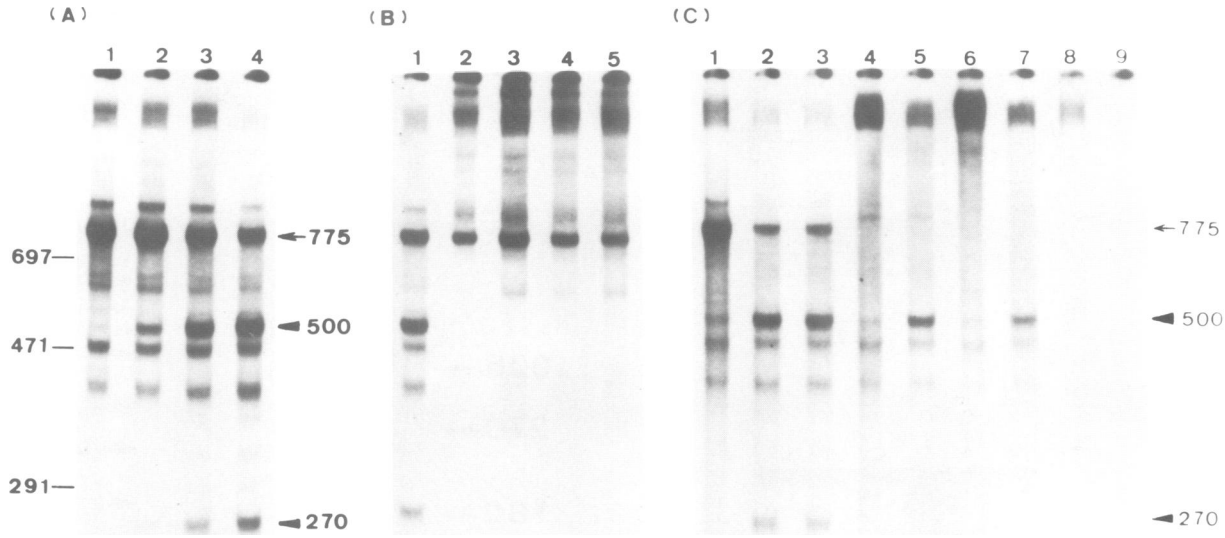

Fig. 3. Pulse-chase experiments. **(A)** Pulse-chase products from the *Xba*I-cleaved pMrSP/SE(18A) DNA template. **Lane 1** shows the products of 20 min reaction with [α-^{32}P]UTP. After this 20 min reaction, a 100-fold excess of cold UTP was added, followed by a chase for an additional 20 min **(lane 2)**, 40 min **(lane 3)** and 60 min **(lane 4)**, respectively. Size markers used are the same as described in Figure 2. The arrow at 775 nucleotides indicates the run-off transcript and arrowheads at 500 and 270 nucleotides indicate the accumulated RNA bands by the chase. **(B)** Pulse-chase products from the *Sal*I-cleaved pMrSP/SXb(18B) DNA template. **Lane 2** shows the products of the 20 min reaction. After 20 min reaction, a 100-fold excess of cold UTP was added and further incubated for 20 min **(lane 3)**, 40 min **(lane 4)** and 60 min **(lane 5)**, respectively. **Lane 1** shows the products obtained under the same conditions as **lane 4** of **(A)**, which was used as the control. **(C)** Pulse-chase products from the *Xba*I-cleaved and non-cleaved pMrSP/SE(18A), non-cleaved pMrSP/SXb(18A) and non-cleaved pMrSP/SXb(18B) DNA templates. **Lanes 1–3** show the products from the *Xba*I-cleaved pMrSP/SE(18A) DNA template. **Lane 1** shows the products of the 80 min reaction. **Lanes 2** and **3** show the products of the 60 min chase in the absence and presence of 20 μg/ml actinomycin D, respectively, after a 20 min reaction. **Lanes 4, 6** and **8** show the products of the 80 min reaction from the non-cleaved pMrSP/SE(18A), pMrSP/SXb(18A) and pMrSP/SXb(18B) DNA templates, respectively. **Lanes 5, 7** and **9** show the products obtained by a 60 min chase after a 20 min reaction from the non-cleaved pMrSP/SE(18A), pMrSP/SXb(18A) and pMrSP/SXb(18B) DNA templates, respectively. The arrow at 775 nucleotides indicates the length expected from the run-off transcript from the *Xba*I-cleaved pMrSP/SE(18A) DNA. Arrowheads at 500 and 270 nucleotides indicate the accumulated RNA bands generated by the chase.

carried out. If ^{32}P-labeled RNA transcribed *in vitro* is processed by endonucleolytic cleavage, the processed [^{32}P]RNA must accumulate during the chase, as demonstrated by Miller and Sollner-Webb (1981) who determined the early processing site of mouse rRNA.

As shown in Figure 3A, *Xba*I-cleaved pMrSP/SE(18A) DNA was transcribed *in vitro* with [α-^{32}P]UTP for 20 min (lane 1) followed by a chase with the addition of a 100-fold excess of non-radioactive UTP for an additional 20 min (lane 2), 40 min (lane 3) and 60 min (lane 4), respectively. While the 775-bp run-off transcript decreased, the 500- and 270-bp RNAs accumulated during the chase.

On the other hand, Figure 3B shows the chase experiment of the transcripts from the *Sal*I-cleaved pMrSP/SXb(18B) DNA as a template (Figure 1B). The 775-bp run-off transcript was not changed during a 60 min chase (Figure 3B, lanes 3–5), although the 775-bp run-off transcript from *Xba*I-cut pMrSP/SE(18A) DNA was processed into 500- and 270-bp RNAs (lane 1) (shown as a control). This result strongly suggests that the accumulation of the 500- and 270-bp RNAs requires the correct orientation of transcription.

To confirm the results obtained above, further chase experiments were carried out (Figure 3C). Lane 1 shows the transcripts obtained by 80 min incubation of the *Xba*I-cut pMrSP/SE(18A) DNA. Lanes 2 and 3 show the products of 20 min incubation of the same DNA template followed by a 60 min chase with a 100-fold excess of cold UTP in the absence and presence of 20 μg/ml actinomycin D, respectively. 500- and 270-bp RNAs were generated from the 775-bp run-off transcript by the chase. No difference was observed in the absence or presence of actinomycin D during the chase (lanes 2 and 3), which completely inhibited the *in vitro* RNA synthesis, as demonstrated previously (Mishima *et al.*, 1981).

Figure 3C, lanes 4–9, shows the same chase experiments with the transcripts from the circular DNAs of pMrSP/SE(18A) (lanes 4 and 5), pMrSP/SXb(18A) (lanes 6 and 7) and pMrSP/SXb(18B) (lanes 8 and 9). From the transcripts of the circular DNA templates of the same orientation of transcription (A series templates), only the 500-bp RNA accumulated during the chase (lanes 5 and 7). From the transcripts of the circular DNA template of opposite orientation (B series templates), no RNA bands accumulated (lane 9).

These results indicate that the 500-bp RNA is the processed product by the endonucleolytic cleavage of the transcripts from both truncated and circular DNA templates in this system.

Determination of the endonucleolytic cleavage site of 500-bp RNA

To locate the endonucleolytic cleavage site of the 500-bp RNA accumulated by the chase, S1 nuclease protection mapping experiments were carried out (Berk and Sharp, 1977). Non-radioactive RNAs were synthesized *in vitro* from closed circular pMrSP/SE(18A) DNA with and without a 60 min chase after a 20 min pulse. These RNAs were treated with RNase-free DNase I, and then hybridized with the 3′ end-labeled coding strand of the 0.48-kb *Sal*I/*Xba*I fragment as shown in Figure 4A. The hybrids were subjected to S1 nuclease digestion and analyzed by the polyacrylamide-urea gel electrophoresis.

As shown in Figures 4A and B, eight protected fragments of 485, 420, 390, 360, 230, 190, 150 and 120 nucleotides in length (measured by a comparison with size markers) were generated

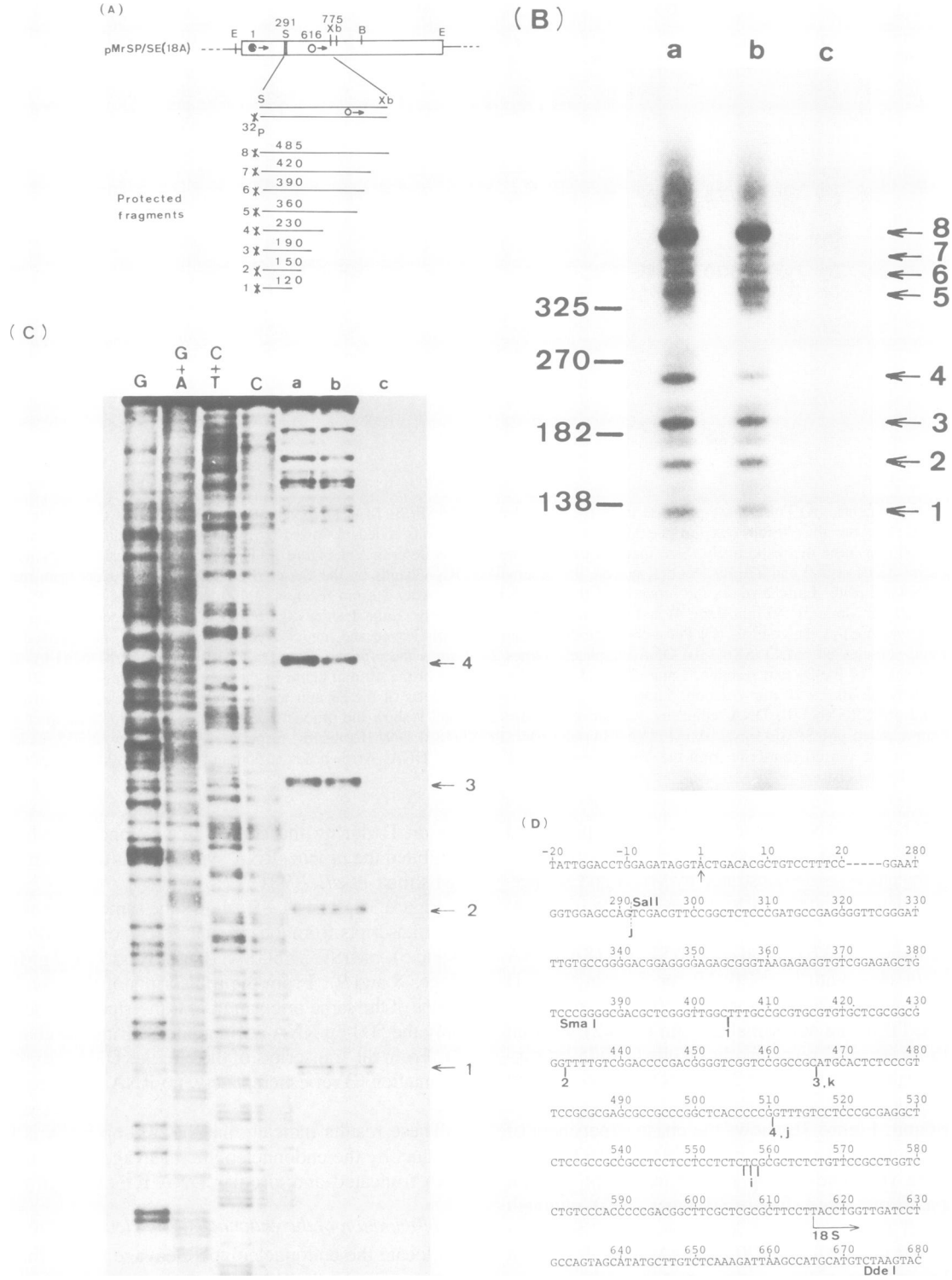

Fig. 4. S1 nuclease protection experiments. **(A)** Schematic drawing of the ^{32}P-labeled DNA probe used for the S1 nuclease analysis of the 3′ termini of RNA transcripts together with the length of the protected DNA fragments shown in **B. (B)** Radioautogram of S1 nuclease-protected fragments. The RNAs used for hybridization were synthesized from non-cleaved pMrSP/SE(18A) DNA with **(lane a)** and without **(lane b)** chase for 60 min after a 20 min reaction, or synthesized without DNA template **(lane c)**. The RNAs were hybridized with 3′ end-labeled DNA probe shown in **A** as described in Materials and methods. The hybrids were treated with S1 nuclease and electrophoresed on a 5% polyacrylamide/7 M urea gel. Size markers used were ^{32}P-labeled DNA fragments. The nucleotide length of S1 nuclease-protected fragments (numbers 1−8 in **B**) are represented in **A. (C)** Localization of the 3′ end of protected fragments. The S1 nuclease-protected fragments obtained by the same conditions as described in **B** were electrophoresed on an 8% polyacrylamide/7 M urea gel along with the same labeled fragments cleaved by base-specific chemical modifications (Maxam and Gilbert, 1980). **Lanes a−c** and **bands 1−4** correspond to those shown in **B. (D)** Nucleotide sequence of non-coding strand. The nucleotide sequence of the transcription initiation region (−20 to +291) was reported previously (Urano *et al.*, 1980; Mishima *et al.*, 1981) and that of 5′ region of mouse 18S rRNA (+292 and +690) was determined in this experiment, which has been reported by Goldman *et al.* (1983). Six nucleotide sequences located at 489, 535, 544, 577, 597 and 602 were different. The arrow at nucleotide number 1 indicates the transcription initiation site. J indicates the site of the junction of the DNA segments between the transcription initiation region and the 5′-terminal region of 18S rRNA. Numbers 1−4 correspond to those shown in **A, B** and **C**. Bands i−k correspond to those shown in Figure 6C and D.

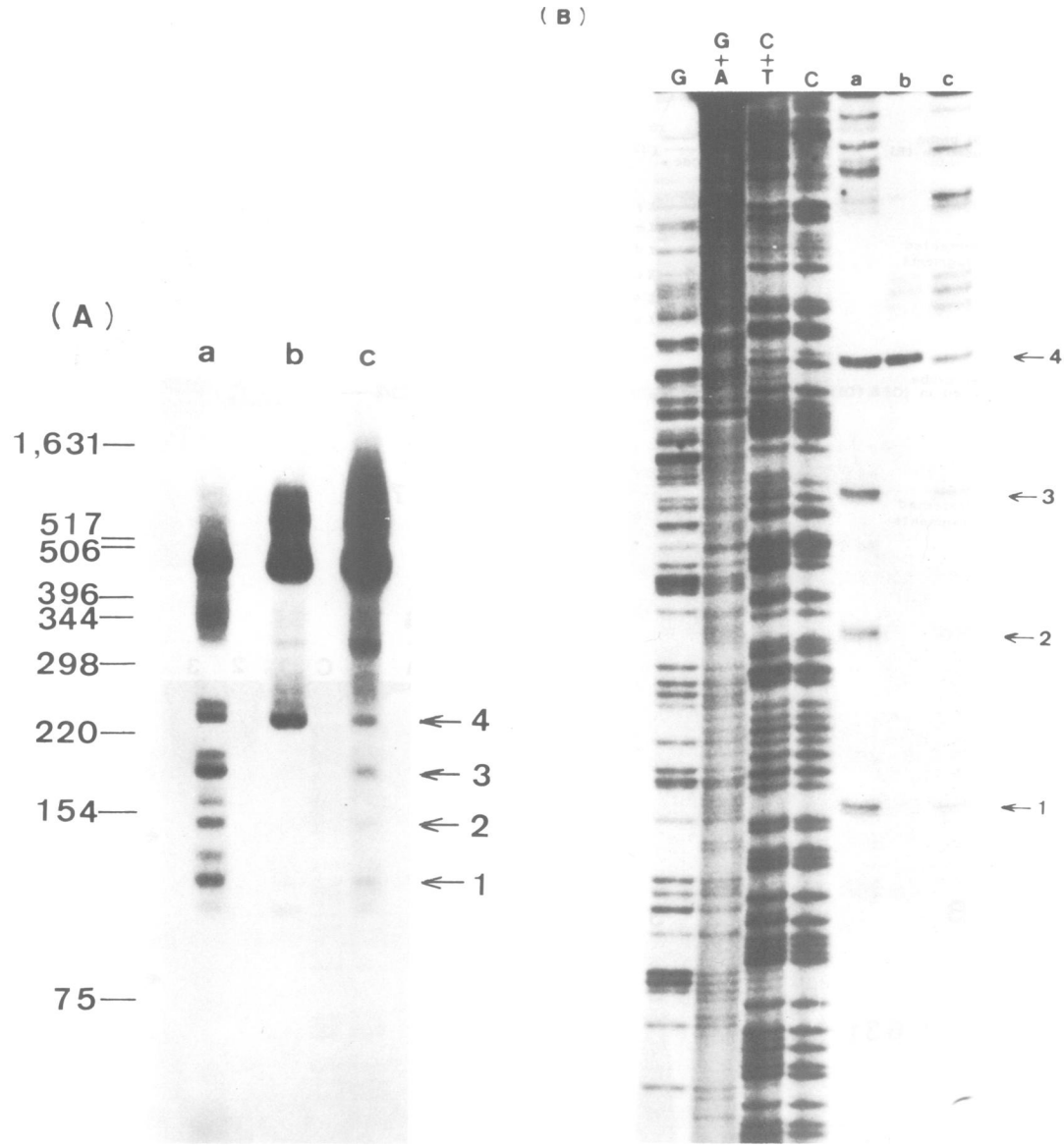

Fig. 5. S1 nuclease mapping of *in vivo* rRNA with 3′ end-labeled probe. **(A)** Radioautograph of S1 nuclease-protected fragments. The RNAs used for hybridization were *in vitro* synthesized RNA **(lane a)** as described in Figure 4B lane a, and 20 μg of whole cell RNAs, which were isolated from FM3A cells at a density of 1.2×10^6 cells/ml **(lane b)** and 7×10^5 cells/ml **(lane c)**, respectively. The RNAs were hybridized with 3′ end-labeled DNA probe shown in Figure 4A as described in Materials and methods. The hybrids were treated with S1 nuclease and electrophoresed on a normal 5% polyacrylamide gel. Size markers used were *Hinf*I-cleaved pBR322 DNA. **Numbers 1–4** correspond to those shown in Figure 4. **(B)** Localization of the 3′ end of the protected fragments (see Figure 4C). **Lanes a–c** and protected fragments **1–4** correspond to those shown in **A**.

from the hybrids between [^{32}P]DNA and RNAs obtained with chase (Figure 4B, lane a) or without chase (Figure 4B, lane b). No protected fragments, however, were obtained from the hybrids between [^{32}P]DNA and RNA transcribed in the absence of DNA template (Figure 4B, lane c). Among these protected fragments, the intensity of the 230-bp fragment was significantly increased by the chase (Figure 4, number 4). The 230-bp fragment was considered to correspond to the accumulation 500-bp RNA described in the previous section.

To locate the endonucleolytic cleavage site of the accumulated 500-bp RNA at the nucleotide level, protected ^{32}P-labeled fragments were electrophoresed in a sequencing gel in parallel with the same labeled 0.48-kb *Sal*I/*Xba*I fragment cleaved by the method of Maxam and Gilbert (1980). The typical result is shown in Figure 4C. The 3′ end points of protected fragments (numbers 1–4) resulting from Figure 4C are shown in Figure 4D, which was corrected according to Sollner-Webb and Reeder (1979). The cleavage site of the accumulated 500-bp RNA was located 105 nucleotides upstream from the 5′ end of 18S rRNA (Figure 4D, number 4).

Confirmation of in vitro endonucleolytic cleavage site by in vivo rRNA

To examine whether the endonucleolytic cleavage site observed in the *in vitro* system is related to an *in vivo* rRNA processing event, we carried out the S1 nuclease protection experiments with whole cell RNA and the 3′ end-labeled 0.48-kb *Sal*I/*Xba*I fragment (Figure 4A) or the 5′ end-labeled 0.48-kb *Sal*I/*Xba*I and 0.29-kb *Sma*I/*Dde*I fragments (Figure 6A) as probes.

As shown in Figure 5A, the same protected fragments of 230, 190, 150 and 120 nucleotides in length (numbers 1–4) were observed from the hybrids between 3′ end-labeled 0.48-kb

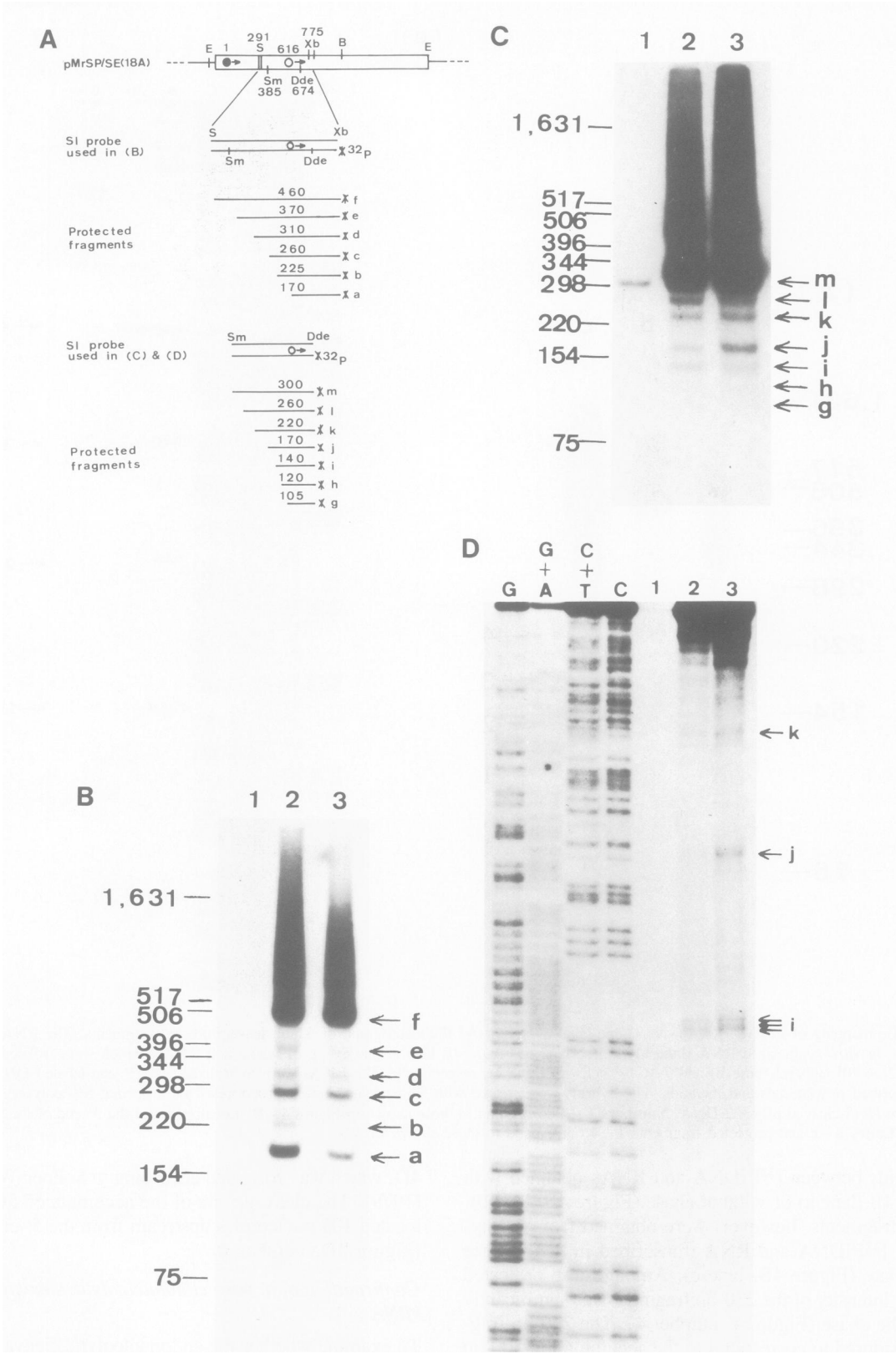

Fig. 6. S1 nuclease mapping of *in vivo* rRNA with 5′ end-labeled probes. **(A)** Schematic drawing of ^{32}P-labeled DNA probes used for S1 nuclease analyses of 5′ termini of *in vivo* RNA together with the protected DNA fragments shown in **B** and **C**. **(B)** Radioautograph of protected fragments. The 5′ end-labeled 0.48-kb *SalI*/*XbaI* fragment shown in **A** was hybridized with 50 μg yeast RNA **(lane 1)** and 50 μg of whole cell RNAs, which were isolated from FM3A cells at a density of 1.2×10^6 cells/ml **(lane 2)** and 5×10^5 cells/ml **(lane 3)**, respectively. S1 nuclease-protected hybrids were analyzed as described in Figure 5A. **(C)** Radioautograph of S1 nuclease-protected fragments obtained by the same conditions as described in **B** except that the 5′ end-labeled 0.29-kb *SmaI*/*DdeI* fragment was used as a probe. **(D)** Localization of the 5′ end of protected fragments obtained by the same conditions as described in **C**. The S1 nuclease-protected fragments were electrophoresed on a sequence gel along with the same labeled 0.29-kb *SmaI*/*DdeI* fragment cleaved by the method of Maxam and Gilbert (1980). **Lanes 1–3** and bands **i–k** correspond to those shown in **A** and **C**.

*Sal*I/*Xba*I DNA and RNAs obtained *in vitro* (lane a) and *in vivo* (lanes b and c). The end points of these protected fragments (numbers 1 – 4) generated from *in vitro* RNA (lane a) and *in vivo* rRNA (lanes b and c) were identical at the nucleotide level as shown in Figure 5B. It is interesting to note that the intensities of protected fragments obtained from *in vivo* RNAs, which were isolated from late log phase (lane b) and middle log phase (lane c), were different. In the latter case (lane c), four distinct bands (numbers 1 – 4) are detected, as also shown in the *in vitro* system, (lane a) in which S100 lysate was prepared from cells in the same phase. On the other hand, in the former case (lane b), the major band (number 4) is distinct but the other three bands (numbers 1 – 3) are very faint. We do not yet have an explanation for these differences related to cell density.

Next we analyzed further *in vivo* RNA using the two different kinds of 5′ end-labeled DNA probe shown in Figure 6A. As shown in Figure 6B, six protected fragments of 460, 370, 310, 260, 225 and 170 nucleotides were generated from the hybrid between ^{32}P-labeled 0.48-kb *Sal*I/*Xba*I DNA fragment and RNAs isolated from late log phase (lane 2) and middle log phase (lane 3). No protected fragments were observed from the hybrid between [^{32}P]DNA and yeast RNA (lane 1). Although the end points of the protected fragment were not determined, it is reasonable to consider that the 170-bp protected fragment (termed a) is derived from the hybrid between [^{32}P]DNA and mature 18S rRNA and that the protected fragments (b – e) represent the existence of longer precursors extending from the 5′ end of mature 41S or 18S rRNA.

To locate the 5′ end of these protected fragments, a 5′ end-labeled 0.29-kb *Sma*I/*Dde*I fragment was used as a S1 probe. Seven protected fragments of 300, 260, 220, 170, 140, 120 and 105 nucleotides were obtained as shown in Figure 6A and C. However, the 60-bp protected fragment, which is expected to arise from the mature 18S rRNA, was not detected. Further studies are needed to clarify this point. The 5′ end points of protected fragments i, j and k were determined at the nucleotides level as shown in Figures 6D and 4D. The 5′ end of protected fragment j was located 105 nucleotides upstream from the 5′ end of 18S rRNA.

The results of Figures 5 and 6 indicate that the endonucleolytic cleavage site located 105 bp upstream from the 5′ end of 18S rRNA in this *in vitro* system is also detected in *in vivo* rRNA, demonstrating that an *in vitro* processing event in this system faithfully reproduces an *in vivo* processing event.

Discussion

To study the processing of mouse rRNA at the molecular level, we tried to develop a coupled transcription and processing system using mouse S100 extract and recombinant DNAs as templates. In this *in vitro* system, we found that a specific endonucleolytic cleavage takes place 105 nucleotides upstream from the 5′ end of 18S rRNA, as shown in Figure 4. This cleavage site, as well as some other sites, was identified in *in vivo* rRNA processing by S1 nuclease protection experiments using 3′ end or 5′ end-labeled 0.48-kb *Sal*I/*Xba*I fragments and a 5′ end-labeled 0.29-kb *Sma*I/*Dde*I fragment (Figures 5 and 6). The cleavage at 105 nucleotides upstream of the 5′ end of 18S rRNA indicates that our coupled transcription and processing system reflects faithfully an *in vivo* processing step of the rRNA precursor. By using 5′ end-labeled fragments, we showed that large precursors exist in the processing of 41S or 18S rRNA. Some further processing step(s) and/or trimming processes of the 105 nucleotides may also be involved in the formation of mature 18S rRNA. This processing pathway has been excluded by Bowman *et al.* (1983) by S1 nuclease protection mapping of *in vivo* rRNA. The discrepancy may be due to the difference of mouse strain, or they might have overlooked the longer precursor found in this experiment, because they did not examine the larger fragment containing the 105 nucleotides upstream from the 5′ end of 18S rRNA (Bowman *et al.*, 1983). Judging from the S1 nuclease protection data of Figure 6B, the amount of the longer precursor is ~25 – 50% of mature 41S and 18S rRNAs. In this respect, Nazar (1977) showed that ~70% of 18S rRNA molecules of Novikoff ascites hepatoma begin with $_pU_p$ and the remainder begin with $_pG_p$, $_pA_p$ and $_pC_p$. From this he concluded that a trimming process was involved in the formation of 18S rRNA.

We do not yet know why the 5′ end of 18S rRNA was not created in this system. Some further sequences or factor(s) may be necessary for the *in vitro* processing of the 5′ end of 18S rRNA. However, in the present *in vitro* system, RNA products obtained from both truncated and circular DNA templates can be processed into 500-bp RNA by endonucleolytic cleavage as shown in Figure 3. This processed RNA was generated from the DNA templates with the correct orientation of transcription but not from the DNA templates with the opposite orientation (Figure 3). This finding indicates that some specific signal sequence(s) is required for the processing. This will be elucidated by the assay of the deletion mutant of the 5′ end region of 18S rRNA.

We observed some minor RNA bands other than 500- and 270-bp RNAs (Figure 2), which were not accumulated during the chase (Figure 3). These RNAs were generated from both truncated and circular DNA templates (Figures 2 and 3). These cleavage or termination sites *in vitro* were also detected in *in vivo* rRNA (Figures 5 and 6), suggesting that these RNAs are alternative rapid processing intermediates that are subsequently cleaved *in vivo* regardless of their accumulation in this *in vitro* system. Another possibility is that these RNAs represent the premature termination of transcription on the rRNA gene by RNA polymerase I *in vitro*. Maderious and Chen-Kiang (1984) have reported evidence of premature termination of transcription of adenovirus DNA by RNA polymerase II.

In the course of this work it was observed that processing efficiency was lost gradually during several months of storage of extracts even at −80°C, although the efficiency of transcription initiation was not significantly diminished. This suggests that the processing of rRNA in mouse cell extracts is an enzymatic process, although the processing of *Tetrahymena* rRNA was reported to be non-enzymatic (Zaug and Cech, 1982). Whether a single or multiple enzymes are involved in the processing steps remains to be examined. For this purpose, it will be useful to utilize an uncoupled transcription-processing system, but it is not possible at present because RNA products synthesized *in vitro* were degraded during the incubation for processing. We also have not succeeded in showing transcription-processing with the reconstituted system reported previously (Mishima *et al.*, 1982), although transcription initiation occurred. This finding may suggest that the machineries of transcription and processing are different.

Grummt *et al.* (1982) reported that rDNA transcription *in vitro* is species specific, and Mishima *et al.* (1982) identified species-dependent initiation factor(s). In this connection, it would be interesting to know whether the processing of rRNA is species specific. We would like to point out that the 19-nucleotide sequence of the early processing site of mouse rRNA (Miller and Sollner-Webb, 1981) (located at +2 to +20 published by Urano *et al.*, 1980) is identical to that of human rRNA (located at +414 to

+432 published by Financsek *et al.*, 1982) except for one nucleotide. A mouse-human hybrid 'processing gene' will help to answer the question of species specificity of rRNA processing.

Our experiment is the first to achieve specific processing at the site 105 bp upstream from the 5′ end region of 18S rRNA, which may create the precursor of 41S or 18S rRNA. We used recombinant DNAs containing the transcription initiation site and the specific region of the 5′ end of 18S rRNA. Because such recombinant DNAs may be used for the processing of the 5.8S and 28S rRNAs, the coupled transcription and processing system will be generally useful for the understanding of the mechanisms of rRNA processing.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase and bacterial alkaline phosphatase were purchased from Takara Shuzo. RNase-free DNase I was obtained from Sigma. S1 nuclease was from P-L Biochemicals. Nucleoside triphosphates were purchased from Yamasa Shoyu. [α-^{32}P]UPT, [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Amersham.

Construction of recombinant plasmids

Plasmid diagrams are shown in Figure 1B. pMrSP contained the transcription initiation region of a mouse rDNA (−167 to +291; +1 is the first nucleotide of the rDNA transcript) (Mishima *et al.*, 1981; Yamamoto *et al.*, 1984), which was inserted by ligation into the *Hind*III/*Pvu*II site of the plasmid pBR322.

To construct pMrSP/SE(18A) and pMrSP/SE(18B) plasmid DNAs, the 1.9-kb *Sal*I/*Eco*RI fragment carrying the 5′ end and ~2/3 parts of the 18S rRNA coding region (Mishima *et al.*, 1980) was isolated. The cleavage sites of *Sal*I and *Eco*RI were filled in with DNA polymerase I Klenow fragment. This repaired fragment was ligated to *Pvu*II-cleaved pMrSP DNA by T4 DNA ligase and transformed *Escherichia coli* HB 101, followed by screening with ^{32}P-labeled 18S rRNA as a probe. pMrSP/SE(18A), the same orientation of transcription, and pMrSP/SE(18B), the opposite orientation, were selected on the basis of the patterns of restriction enzyme cleavage.

To construct pMrSP/SXb(18A) and pMrSP/SXb(18B) plasmid DNAs, the 0.48-kb *Sal*I/*Xba*I fragment was isolated. The cleavage sites of *Sal*I and *Xba*I of the 0.48-kb *Sal*I/*Xba*I fragment were repaired by the Klenow fragment of DNA polymerase I followed by blunt-end ligation to *Pvu*II-cleaved pMrSP DNA. Plasmid DNAs were screened with [^{32}P]18S rRNA, and pMrSP/SXb(18A) and pMrSP/SXb(18B) were recovered as shown in Figure 1B.

All recombinant DNA experiments were performed according to the Japanese Guidelines for recombinant DNA research.

In vitro transcription

0.7 pmol of DNA template was incubated at 30°C for indicated time periods in a 50 μl reaction mixture containing 10 mM Hepes (pH 7.9), 0.33 mM dithiothreitol, 0.67 mM each of ATP, GTP and CTP, 0.026 mM UTP and 5 μCi of [α-^{32}P]UTP, 80 mM KCl, 2 mM Mg-acetate, 100 μg/ml α-amanitin and 15 μl (300 μg protein equivalent) of S100. The preparation of S100 from mouse FM3A cells was described previously (Mishima *et al.*, 1981). *In vitro* transcription products were glyoxalated and analyzed by 4% polyacrylamide gel electrophoresis (Mishima *et al.*, 1981, 1982).

DNA sequencing

pMrSP/SE(18A) DNA was digested by *Sal*I, which is located at position 291 shown in Figure 4D, and labeled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase or at the 3′ end using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. After digestion by *Xba*I, the ^{32}P-labeled 0.48-kb *Sal*I/*Xba*I fragment was separated on a 4% polyacrylamide gel. The ^{32}P-labeled 0.29-kb *Dde*I/*Sma*I fragment was isolated in the same way. DNA sequencing was carried out by the method of Maxam and Gilbert (1980).

S1 nuclease protection mapping analysis

In vitro synthesized RNAs were treated with 10 μg/ml of RNase-free DNase I, which was then hybridized with 3′ end-labeled 0.48-kb *Sal*I/*Xba*I or 5′ end-labeled 0.48-kb *Sal*I/*Xba*I and 0.29-kb *Sma*I/*Dde*I DNA fragments described above in 50 μl of 70% formamide, 0.5 M NaCl, 1 mM EDTA, 40 mM Pipes (pH 6.8) at 56°C for 2 h after heating at 90°C for 3 min. The reaction mixture was diluted 10-fold with 30 mM sodium acetate (pH 4.5), 1 mM $ZnSO_4$, 0.2 M NaCl and treated with 500 units of S1 nuclease at 37°C for 60 min followed by ethanol precipitation (Berk and Sharp, 1977). The samples were electrophoresed on an 8% polyacrylamide/7 M urea gel along with the same labeled fragments cleaved by base-specific chemical modifications (Maxam and Gilbert, 1980).

Isolation of RNA

Whole cell RNA was extracted from cultured FM3A cells by a modification of the hot SDS-phenol method as described by Muramatsu (1973).

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