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Location of the Initial Cleavage Sites in Mouse Pre-rRNA

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The locations of three cleavages that can occur in mouse 45S pre-rRNA were determined by Northern blot hybridization and S1 nuclease mapping techniques. These experiments indicate that an initial cleavage of 45S pre-rRNA can directly generate the mature 5′ terminus of 18S rRNA. Initial cleavage of 45S pre-rRNA can also generate the mature 5′ terminus of 5.8S rRNA, but in this case cleavage can occur at two different locations, one at the known 5′ terminus of 5.8S rRNA and another 6 or 7 nucleotides upstream. This pattern of cleavage results in the formation of cytoplasmic 5.8S rRNA with heterogeneous 5′ termini. Further, our results indicate that one pathway for the formation of the mature 5′ terminus of 28S rRNA involves initial cleavages within spacer sequences followed by cleavages which generate the mature 5′ terminus of 28S rRNA. Comparison of these different patterns of cleavage for mouse pre-rRNA with that for *Escherichia coli* pre-rRNA implies that there are fundamental differences in the two processing mechanisms. Further, several possible cleavage signals have been identified by comparing the cleavage sites with the primary and secondary structure of mouse rRNA (see W. E. Goldman, G. Goldberg, L. H. Bowman, D. Steinmetz, and D. Schlessinger, Mol. Cell. Biol. 3:1488–1500, 1983).

Small and large subunit rRNAs are cotranscribed in both procaryotic and eucaryotic cells in long precursors which are then processed to produce mature rRNAs (for reviews, see references 1, 12, 25). Since the initial rRNA precursors and mature rRNAs are analogous in all cells, it is reasonable to ask whether the mechanism of processing is also universal.

The initial steps in rRNA processing are especially well characterized in *E. coli*: primary cleavages are made by RNase III at very stable double-stranded stems enclosing large loops containing the 16S or 23S rRNA sequences (8, 34). The resultant RNAs bear 7 to 150 extra nucleotides at their termini; the mature 5′ and 3′ termini are formed by secondary cleavages, which occur after the intermediates have bound ribosomal proteins.

Although the mechanisms of mammalian pre-rRNA processing are largely unknown, previous studies have suggested some differences between the pre-rRNA processing in mammalian cells and *Escherichia coli*. The spacers flanking the small and large subunit rRNAs in yeast (31), *Xenopus laevis* (13, 19), and mouse (11) cannot form long stable stems as in *E. coli*. Nevertheless, stems of much lower stability are possible, and it has been suggested (at least for yeast) that these may be analogous to the *E. coli* stems. The initial cleavages of pre-rRNA in *E. coli* occur while the pre-rRNA is being transcribed, whereas the initial cleavages of mammalian pre-rRNA occur well after the completion of transcription. Also, the order of pre-rRNA cleavages is sequential in bacteria, whereas there is considerable flexibility in the order of cleavages in mammalian cells (7, 9, 33). The change from a sequential to a more stochastic cleavage order may simply result from the slower pre-rRNA processing in mammalian cells.

In this paper, we report mapping mouse L cell pre-rRNA cleavage sites precisely to permit a more conclusive comparison with the bacterial paradigm and to elucidate further the mechanisms of mammalian pre-rRNA processing. Seven cleavage sites of mouse pre-rRNA have previously been identified (Fig. 1) (7, 17, 22, 25). Although six of these sites have been located approximately near the ends of mature rRNA species, the exact locations of five of the six sites has not been determined (Fig. 1, sites 1 through 5). Of particular interest are the exact sites of the three cleavages which can occur in

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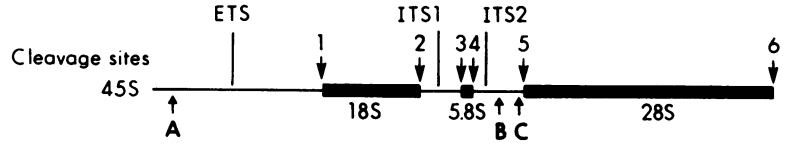


FIG. 1. 45S pre-rRNA cleavage sites. The numbered cleavage sites are those inferred to occur at or near the ends of mature rRNA. The lettered cleavage sites occur within spacer sequences. Sites B and C are inferred from data presented in this paper. ETS, External transcribed spacer; ITS 1, internal transcribed spacer 1; ITS 2, internal transcribed spacer 2.

45S pre-rRNA (Fig. 1, sites 1, 3, and 5) since these cleavages might be expected to be the most analogous to the initial cleavages of bacterial pre-rRNA. We have determined the precise locations of these initial cleavage sites by using Northern blot hybridization and S1 nuclease mapping techniques. These results, together with the sequencing of mouse rDNA (11), suggest fundamental differences in the processing of *E. coli* and mouse pre-rRNA and identify possible cleavage signals in mouse pre-rRNA.

MATERIALS AND METHODS

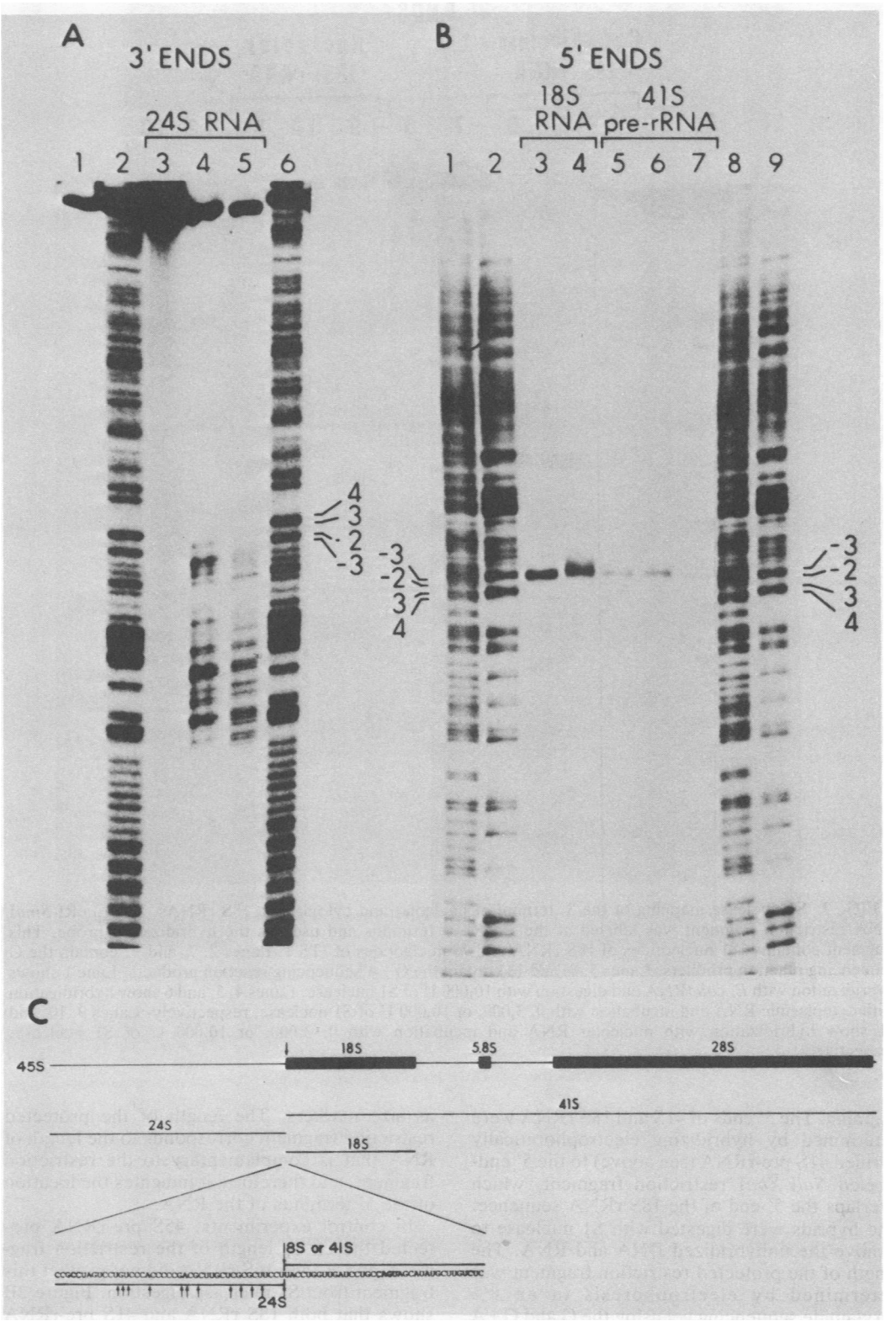
DNA sequencing and labeling of restriction fragments. DNA sequencing was performed as described by Maxam and Gilbert with 8 and 6% sequencing gels. Restriction fragments isolated from plasmid pBR322-rDNA subclones of λgtWES-rDNA phages (3, 7, 29) and were 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP (20) or 3′ end labeled with reverse transcriptase and the appropriate [γ-^{32}P]NTP (26). Only one nucleotide was added during the 3′ end labeling.

S1 nuclease mapping and Northern blot hybridization. Nuclei and nuclear RNA were isolated from dividing mouse L cells as described previously (7). The medium of nonconfluent cultures was changed 24 h before harvest to ensure that cells were rapidly dividing. Nucleoli were isolated by sonication of nuclei in 0.34 M sucrose containing 0.05 mM $MgCl_2$ followed by centrifugation through 0.88 M sucrose containing 0.05 mM $MgCl_2$ (15). Northern blot hybridizations were performed exactly as described previously (2, 7), except that nitrocellulose paper was used (28). For S1 nuclease analysis, 41S, 32S, 19-27S, 18S, and 10-14S rRNA were isolated from a urea-agarose gel (18) and judged pure by subsequent electrophoresis. The gel profile of RNA isolated from these dividing cells was exactly as previously described (7). The RNAs were hybridized to the appropriate end-labeled restriction fragment in 80% formamide–0.4 M NaCl–0.02 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.8]–0.001 M EDTA–20 μg of *E. coli* 16S rRNA or tRNA per ml. The optimum temperature for each hybridization was determined in pilot experiments. The temperature which gave the least hybridization of DNA to DNA and significant RNA-DNA hybridization was chosen. In some experiments, the strands of the restriction fragment were separated (20) and hybridized to RNA at 65°C in 0.4 M NaCl–0.02 M PIPES (pH 6.8)–0.001 M EDTA–200 μg of *E. coli* RNA per ml for 2 h. The hybrids were diluted 10-fold with ice-cold 0.25 M NaCl–0.03 M sodium acetate (pH 4.75)–0.001 M $ZnSO_4$–5% glycerol and were then digested with various concentrations of S1 nuclease (5). The resulting hybrids were extracted with phenol-chloroform, precipitated with ethanol, and fractionated on a 6 or 8% acrylamide sequencing gel.

RESULTS

Cleavage at the 5′ end of 18S rRNA (site 1). Cleavage of mouse L cell 45S pre-rRNA near the 5′ end of 45S rRNA (Fig. 1, site 1) produces 41S pre-rRNA and the 24S external transcribed spacer (ETS; Fig. 2). We have previously shown that this cleavage occurs near the 5′ terminus of the 18S rRNA sequence (7). The precise location of this cleavage was determined by mapping the 5′ end of 41S pre-rRNA and the 3′ end of the 24S ETS along the rDNA sequence by S1 nuclease

FIG. 2. S1 nuclease mapping of the cleavage sites located near the 5′ end of 18S rRNA. (A) S1 nuclease mapping of the 3′ terminus of the 24S external transcribed spacer. The *AvaI-XbaI* restriction fragment was 3′ end labeled at the *AvaI* site and used as the hybridization probe. This fragment contains 220 nucleotides of the ETS and 160 nucleotides of 18S rRNA. Lanes 2 and 6 display the G-specific sequencing reaction of the *AvaI-XbaI* fragment. The 24S RNA (*AvaI-XbaI*) hybrid was incubated without enzyme (lane 3) or incubated with 3,000 U (lane 4) or 10,000 U (lane 5) of S1 nuclease. In lane 1, the *AvaI-XbaI* fragment was hybridized to 16S *E. coli* rRNA and then digested with 10,000 U of S1 nuclease. For the 3′ end-labeled fragments, the fragments in the sequencing reaction lanes migrate one-half of a nucleotide slower than the equivalent S1 nuclease-treated DNA. (B) S1 nuclease mapping of the 5′ terminus of 41S pre-rRNA and 18S rRNA. The *SalI-SbaI* restriction fragment was 5′ end labeled at the *XbaI* site and used as the hybridization probe. This fragment contains 323 nucleotides of the ETS and 160 nucleotides of 18S rRNA. Lanes 1 and 8 show fragments after G+A sequencing reactions with the *SalI-XbaI* fragment. Lanes 2 and 9 show the G-specific reactions with the same fragment. The 18S-*SalI-XbaI* hybrid was treated with 8,000 U (lane 3) or 2,000 U (lane 4) of S1 nuclease. The 41S RNA (*SalI-XbaI*) hybrid was treated with 8,000 U (lane 5), 2,000 U (lane 6), or 1,000 U (lane 7) of S1 nuclease. For 5′ end-labeled fragments, the products of sequencing reactions migrate 1.5 nucleotides faster than the equivalent S1 nuclease-treated DNA. (C) The location of the termini of 41S, 24S, and 18S rRNA. The arrows indicate the termini of the various rRNA species.


A
3' ENDS
24S RNA
1 2 3 4 5 6
4
3
2
-3
B
5' ENDS
18S RNA
41S pre-rRNA
1 2 3 4 5 6 7 8 9
-3
-2
3
4
C
45S
18S
5.8S
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41S
24S
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18S or 41S
24S

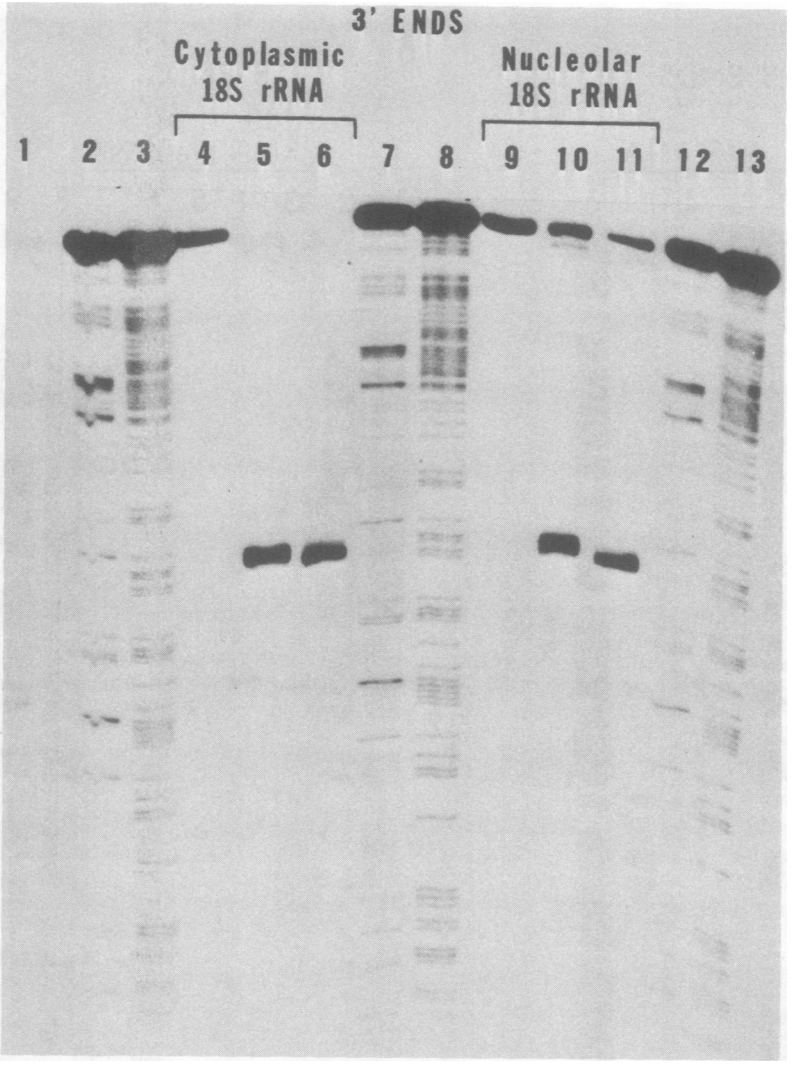

FIG. 3. S1 nuclease mapping of the 3' termini of nucleolar and cytoplasmic 18S rRNAs. The *Eco*RI-*Sma*I DNA restriction fragment was labeled at the *Eco*RI 3' terminus and used as the hybridization probe. This fragment contains 181 nucleotides of 18S rRNA and 78 nucleotides of ITS 1. Lanes 2, 7, and 12 contain the G sequencing reaction products. Lanes 3, 8, and 13 contain the G+A sequencing reaction products. Lane 1 shows hybridization with *E. coli* tRNA and digestion with 10,000 U of S1 nuclease. Lanes 4, 5, and 6 show hybridization with cytoplasmic RNA and incubation with 0, 3,000, or 10,000 U of S1 nuclease, respectively. Lanes 9, 10, and 11 show hybridization with nucleolar RNA and incubation with 0, 3,000, or 10,000 U of S1 nuclease, respectively.

mapping. The 5' ends of 41S and 18S rRNA were determined by hybridizing electrophoretically purified 41S pre-rRNA (see above) to the 5' end-labeled *Sal*I-*Xba*I restriction fragment, which overlaps the 5' end of the 18S rRNA sequence. The hybrids were digested with S1 nuclease to remove the unhybridized DNA and RNA. The length of the protected restriction fragment was determined by electrophoresis in an 8% acrylamide sequencing gel using the G and G+A sequencing reactions of the *Sal*I-*Xba*I fragment as size markers. The length of the protected restriction fragment corresponds to the length of RNA that is complementary to the restriction fragment, and therefore, it indicates the location of the 5' terminus of the RNA.

In control experiments, 45S pre-rRNA protected the entire length of the restriction fragment, and *E. coli* 16S rRNA did not protect this fragment from S1 nuclease digestion. Figure 2B shows that both 18S rRNA and 41S pre-rRNA protect the fragment up to the known 5' termi-

nus of 18S rRNA. This indicates that 18S rRNA and 41S pre-rRNA have the same 5′ terminus and suggests that this initial cleavage occurs at the 5′ end of the 18S rRNA sequence.

It was still possible, however, that an initial cleavage of the rRNA could have occurred 5, 10, or even 200 nucleotides more 5′ proximal but was rapidly followed by an accurate secondary cleavage to produce the mature 5′ end of 18S rRNA. If, on the other hand, the primary cleavage directly generated the 5′ terminus, it would also generate a 24S rRNA with a 3′ terminus that abuts the 5′ end of 18S rRNA. To test this possibility, the 3′ end of the 24S external transcribed spacer was located by hybridizing 19S-27S nuclear RNA to the *AvaI-XbaI* restriction fragment labeled at its *AvaI* 3′ end. The resulting hybrids were digested with S1 nuclease, and the protected DNA was fractioned on a 6% acrylamide sequencing gel along with the G sequencing reaction of the fragment.

The result of this experiment (Fig. 2A) indicates that the 3′ end of 24S rRNA is heterogeneous. Twelve major bands are detected which span a length of 31 nucleotides. This heterogeneity was reproducibly detected even when different lots of S1 nuclease and different preparations of RNA were employed. The largest band corresponds to a 24S molecule whose 3′ end precisely abuts the 5′ end of 18S rRNA. The simplest interpretation of these results is that the initial cleavage actually occurs at the 5′ end of the 18S rRNA sequence and that heterogeneity at the 3′ end of the 24S molecule is due to subsequent nuclease digestion.

Cleavage at the 3′ end of 18S rRNA (site 2). The 3′ termini of cytoplasmic and nuclear 18S rRNAs were located in S1 nuclease mapping experiments. Figure 3 shows that the 3′ termini of cytoplasmic and nuclear 18S rRNAs are identical and relatively homogeneous. This indicates that terminal processing of nuclear 18S rRNA is not required to produce the mature 3′ terminus of 18S rRNA. This result also suggests that cleavage site 2 is located directly at the 3′ terminus of 18S rRNA sequence. Confirmatory evidence from the 5′ end of the site 2 cleavage product could not be obtained because the cellular concentrations of these molecules are extremely low (7).

Cleavage at the 5′ end of 5.8S rRNA (site 3). Cleavage of 45S pre-rRNA near the 5′ end of the 5.8S rRNA sequence produces two species of pre-rRNA, 34S and 32S (Fig. 3). Furthermore, the 5′ end of 12S pre-rRNA and the 3′ end of 20S pre-rRNA are also formed by cleavage at this site. The 5′ and 3′ ends of molecules terminating in this region were located by S1 nuclease mapping techniques.

Figure 4A shows that 32S and 12S pre-rRNAs have the same 5′ end as the mature 5.8S rRNA. However, all of these molecules have two types of 5′ ends. The most abundant end corresponds to RNAs whose 5′ terminus coincides with the known 5′ terminus of 5.8S rRNA. The minor species terminate within the spacer 6 or 7 nucleotides upstream. Both species terminate at identical locations within the members of an imperfect 6-base-pair repeat (see below).

Figure 4 also shows that the 3′ end of 34S RNA is heterogeneous and exhibits a range of 3′ termini similar to that at the 3′ end of the 24S pre-rRNA which terminates at the 5′ end of 18S rRNA. 20S pre-rRNA also shows equivalent heterogeneity at its 3′ end (data not shown).

The longest of these 34S pre-rRNA molecules actually abuts the major 5′ end of 5.8S rRNA, and some also abut the 5′ ends of the minor species. The simplest explanation of these results is that the predominant cleavage occurs at the mature 5′ end of 5.8S rRNA. Less frequently the cleavage occurs 6 or 7 nucleotides upstream, and the 5′ termini of the resulting molecules are not cleaved further.

Cleavages between 5.8S and 28S rRNA. Cleavage of 45S pre-rRNA near the 5′ end of 28S rRNA generates 37S pre-rRNA and 28S rRNA, and cleavage of the abundant 32S pre-rRNA at this site produces 28S rRNA and a 12S precursor to 5.8S rRNA (7). Because 37S pre-rRNA is not very abundant, the location of this cleavage site was determined by mapping the 3′ end of the relatively abundant, well-characterized 12S pre-rRNA and the 5′ end of nuclear 28S rRNA.

The approximate location of these termini was determined by Northern blot hybridization. Nuclear RNA was denatured with glyoxal, fractionated by agarose gel electrophoresis (21), transferred to nitrocellulose paper, and hybridized to the two DNA restriction fragments indicated in Fig. 5. 12S pre-rRNA hybridized strongly to fragment A, but not to restriction fragment B, even after an exposure three times that shown in Fig. 5. Because fragment B begins 308 nucleotides from the 5′ end of the 28S rRNA sequence, the 3′ end of 12S rRNA is located more than about 300 nucleotides from the 5′ end of 28S rRNA. This inference is not affected by the appearance of an additional species at about 25S which hybridized to both fragments A and B. This RNA is probably a mixture of 26S pre-rRNA and an incompletely characterized 24S rRNA (7). The analysis of this latter RNA is incomplete, partly because its significance is dubious: the amount varies from one RNA preparation to another, suggesting that much of it is a breakdown product formed during RNA isolation.

The location of the 3′ end of 12S pre-rRNA was determined more precisely by S1 nuclease

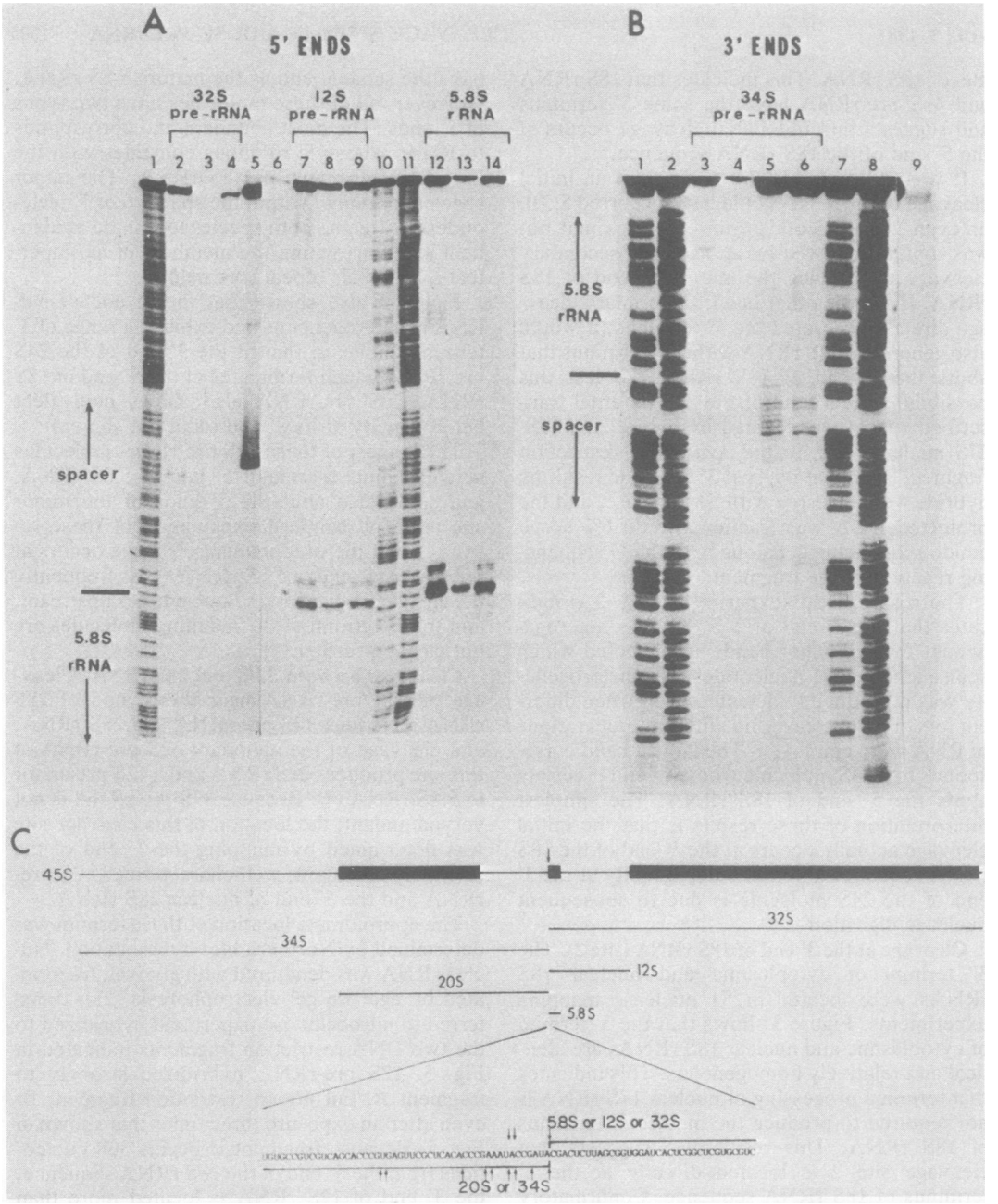

FIG. 4. S1 nuclease mapping of the cleavage sites located near the 5′ end of 5.8S rRNA. (A) S1 nuclease mapping of the 5′ ends of 32S, 12S, and 5.8S rRNAs. The *Hae*II-*Sma*I DNA restriction fragment was labeled at the *Sma*I 5′ end and used as the hybridization probe. This fragment contains 284 nucleotides of the ITS 1 and 117 nucleotides of 5.8S rRNA. Lanes 1 and 11 show the products of the G+A sequencing reaction of the restriction fragment. Lane 10 shows the fragments after the G-specific sequencing reaction. Lanes 2 and 4 show hybridization with 32S and digestion with 7,000 or 25,000 U of S1 nuclease, respectively. Lane 5 shows hybridization to 5.8S rRNA with no subsequent digestion with S1 nuclease. Lane 6 shows hybridization to *E. coli* 16S rRNA and digestion with 12,000 U of S1 nuclease. Lanes 7, 8, and 9 show hybridization with 12S pre-rRNA and digestion with 7,000, 12,000, or 25,000 U of S1 nuclease, respectively. Lanes 12, 13, and 14 show hybridization with 5.8S rRNA and digestion with 7,000, 12,000, or 25,000 U of S1 nuclease, respectively. (B) S1 nuclease mapping of the 3′ terminus of 34S pre-rRNA. The *Ava*II-*Alu*I restriction fragment that overlaps the 5′ end of 5.8S was labeled at the *Ava*II 3′ end and used as the hybridization probe. This fragment contains 104 nucleotides of the ITS 1 and 48 nucleotides of the 5.8S rRNA sequence. Lanes 1 and 7 show the fragments after the G-specific sequencing reaction with the fragment. Lanes 2 and 8 show products of the G+A sequencing reactions. Lanes 3, 5, and 6 show hybridization to 29S-36S nuclear RNA and incubation with 0, 7,000, or 12,000 U of S1 nuclease, respectively. Lanes 4 and 9 show hybridization to *E. coli* 16S rRNA and digestion with 12,000 U of S1 nuclease. (C) Locations of the relevant termini of 34S, 32S, 12S, and 5.8S rRNA, indicated with arrows.

mapping. The *SalI-BamHI* restriction fragment was 3′ end labeled at the *SalI* site and hybridized to nuclear RNA. The hybrids were digested with S1 nuclease and fractionated by acrylamide gel electrophoresis. A major protected species (Fig. 6) corresponds to a molecule that terminates 295 nucleotides from the labeled 3′ end of the restriction fragment, with its 3′ end 295 nucleotides from the 5′ end of 28S rRNA. Other minor species were also detected, but most of these were also present in control hybridizations with *E. coli* tRNA. Thus, these experiments suggest that there is a potential cleavage site within the internal transcribed spacer 2 (ITS 2) approximately 295 nucleotides from the 5′ terminus of the 28S rRNA sequence. The suggestion cannot as yet be proven because we were unable to detect any relatively abundant pre-rRNA whose 5′ end abuts the 3′ end of these molecules; neither S1 nuclease mapping nor Northern blot hybridization detected such a species.

A nuclear 28S rRNA containing 4 to 6 extra nucleotides at its 5′ end was also detected by S1 nuclease mapping of nuclear compared with cytoplasmic 28S rRNA (Fig. 7). Although this pre-28S rRNA is only a small fraction of total nuclear 28S rRNA, its existence suggests that cleavage can occur 5 to 6 nucleotides from the 5′ terminus (see below). Thus, our results to date suggest rapid successive cleavages in ITS 2 since the 5′ and 3′ ends of mature rRNAs and pre-rRNAs terminating in this region do not abut.

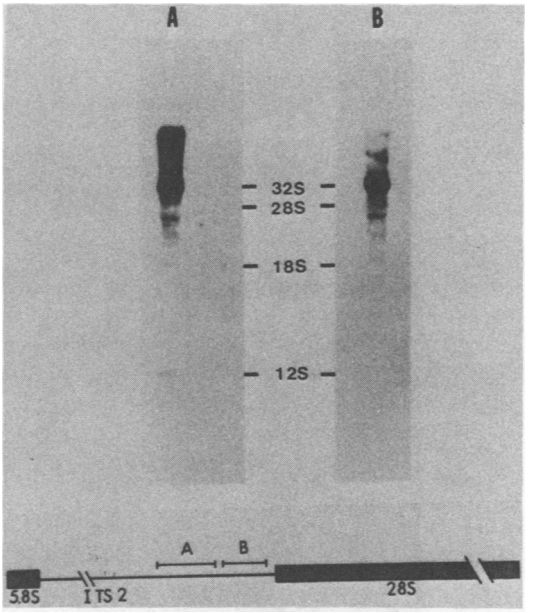

FIG. 5. Northern blot hybridization analysis of 12S pre-rRNA. Hybridization with the restriction fragments A and B. Below is shown the location along the DNA of the restriction fragments used for the Northern blot hybridization. Fragment A is the *SalI-AvaI* fragment from nucleotides 2590 to 2833, and fragment B is the *SacII-SacII* fragment whose coordinates are 2871 to 3147 in reference 11.

DISCUSSION

Our analysis of the termini of pre-rRNAs and mature rRNAs specifies three different cleavage patterns in mouse pre-rRNA. In the first type, the initial cleavage of 45S pre-rRNA directly generates mature, homogenous termini of rRNAs. This type is best documented for the cleavage occurring at the 5′ terminus of 18S rRNA, and it also probably occurs at the 3′ termini of 18S and 28S (17) rRNA. This pattern is inferred from our results showing that the 41S precursor to 18S rRNA has the same 5′ terminus as 18S rRNA and that some 3′ termini of 24S pre-rRNA actually abut the 5′ terminus of 18S rRNA. Because the 3′ terminus of 24S pre-rRNA is heterogeneous, it is formally possible that the initial cleavage in this region can occur at many different sites within about 30 nucleotides of the 5′ terminus of 18S. Rapid nuclease action would then generate the mature 5′ terminus of 18S rRNA. However, the simpler explanation for the data is that there is only one initial cleavage site and that heterogeneity at the 3′ border is generated by subsequent exonucleolytic cleavage of 3′ spacer sequences which are destined to be totally degraded.

A second, different type of cleavage pattern occurs at the 5′ terminus of the 5.8S rRNA sequence. In this case, the initial cleavage can occur at two different locations. The predominant cleavage occurs at the 5′ terminus of the known 5.8S rRNA sequence, and a less predominant cleavage occurs 5 to 7 nucleotides further upstream. The extra five to seven nucleotides are not trimmed from the 5.8S rRNA, and mature cytoplasmic 5.8S rRNA is therefore heterogeneous. As in the case of cleavages at the 5′ terminus of 18S, other interpretations of the data are formally possible but are more complex.

It was previously suggested that the 5′ ends of 41S and 32S pre-rRNAs are more heterogeneous than the 5′ ends of 18S rRNA and 5.8S mature rRNAs, from analyses of the terminal nucleotides of these RNAs (23). However, our results clearly indicate that the ends of the mature rRNAs and their precursors do not differ by more than 1 or 2 nucleotides in length. Further, it has also been demonstrated in yeasts that the precursors to 18S and 5.8S rRNA have the same 5′ termini as the mature rRNA (4, 30). In sum, there is little or no 5′ terminal processing of these molecules in eucaryotic cells.

A third cleavage pattern was detected for the initial cleavage of 45S rRNA that occurs near the

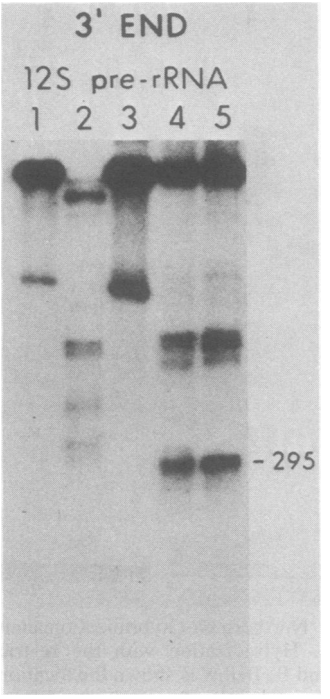

FIG. 6. S1 nuclease mapping of the 3' end of 12S pre-rRNA. The *SalI-Bam*HI restriction fragment was 3' end labeled at the *SalI* end and used as the hybridization probe. This fragment contains 588 nucleotides of ITS 2 and 1,242 nucleotides of 28S rRNA. Lanes 1 and 2 show hybridization to *E. coli* tRNA and incubation with 0 or 12,000 U of S1 nuclease, respectively. Lanes 3, 4, and 5 show hybridization with nuclear RNA and incubation with 0, 5,000, or 12,000 U of S1 nuclease, respectively.

5' terminus of 28S rRNA. In contrast to the previous patterns, the initial cleavage may occur well within spacer sequences, and further trimming is required to produce the mature termini of rRNAs. This is based on the finding that the 3' terminus of 12S pre-rRNA is located approximately 295 nucleotides from the 5' terminus of 28S rRNA and on the detection of a 28S pre-rRNA that extends 5 or 6 nucleotides into ITS 2. These results suggest that several cleavages occur within ITS 2. The exact location of these sites requires further analysis.

An alternate interpretation of these results is that the predominant initial cleavage occurring in this region actually directly generates the mature terminus of 28S rRNA and only occasionally occurs at sites within ITS 2. In this view, cleavages within ITS 2 would usually just initiate the turnover of the metabolically unstable spacer sequence. In fact, some of the precursors detected could simply be relatively stable breakdown products of the ITS, resulting from exonucleolytic or endonucleolytic cleavages which are unimportant for the formation of mature rRNAs. However, the fact that the 5' terminus of 28S and the 3' terminus of 5.8S rRNAs are generated more slowly than other termini suggests that additional cleavages may be important. Speculatively, this more complicated processing could be linked to the requirement that 5.8S and 28S rRNAs be brought together for ribosome formation.

Possible cleavage signals in pre-rRNA. One signal for initial cleavage could be the primary structure of the transcribed spacer sequences flanking cleavage sites. Goldman et al. (11) have shown that there is virtually no conservation of the spacer sequences adjacent to the 5' ends of 18S, 5.8S, and 28S rRNAs, but the imperfect repeats located near the 5' end of 18S, 5.8S, and

FIG. 7. S1 nuclease mapping of the 5' termini of nuclear and cytoplasmic 28S rRNA. The *RsaI-AvaII* restriction fragment was 5' end labeled at the *RsaI* site and used as the hybridization probe. This fragment contains 117 nucleotides of the ITS 2 and 122 nucleotides of the 28S rRNA sequence. Lane 1 shows hybridization with *E. coli* tRNA and digestion with 10,000 U of S1 nuclease. Lane 2 shows the G-specific sequencing reaction products. Lanes 3 and 8 show the G+A sequencing reaction products. Lanes 4, 5, and 6 show hybridization with cytoplasmic RNA and digestion with 3,000, 7,000, or 12,000 U of S1 nuclease, respectively. Lanes 7, 9, 10, and 11 show hybridization with nuclear 28S rRNA and incubation with 0, 3,000, 7,000, or 12,000 U of S1 nuclease, respectively.

28S rRNAs (11) are suggestive. In mouse and in yeast (30), both members of the repeat at the 5′ end of 5.8S rRNA are cleaved at similar locations, generating a population of mature 5.8S rRNA molecules with correspondingly heterogeneous 5′ termini. Heterogeneity has also been observed at the 5′ ends of 5.8S rRNA from *X. laevis* (6, 10) and HeLa cells (16) and in the transient 28S rRNA precursors 5 or 6 nucleotides longer at their 5′ end (Fig. 7).

Potential double-stranded segments (11) are unlikely to be alternative signals for procesing: none fall near the points of cleavage observed in the ITS 2, and virtually no strongly base-paired structures are possible near cleavage sites 1, 2, 3, 4, or 5 of Fig. 1. Not even the less stable structures that could involve spacer sequences base pairing to surround cleavage sites 1 and 3 are evolutionarily conserved. Rather, the regions adjacent to the termini of mature RNAs show little potential for the formation of stable base-paired stems (11). Perhaps the very openness of these regions may help to guide processing enzymes to their sites of action.

The relatively high sequence conservation of mature rRNAs in eucaryotes raises the possibility that instead of spacer sequences, features of the mature rRNA sequence may help to signal cleavage sites. The mature RNA sequence or its association with specific proteins has been shown to promote cleavages that form the mature termini of *Bacillus subtilis* 5S (27) and *E. coli* 16S rRNAs (14). Consistent with this possibility is the observation that the processing of 45S pre-rRNA in eucaryotic cells occurs after it is completely transcribed and has associated with proteins.

Evolution of rRNA processing. The mode of processing of pre-rRNA has thus changed drastically during evolution. The accompanying paper (11) indicates that the spacers of mouse pre-rRNA cannot form base-paired stems similar to those found in bacteria (8, 34). In this paper, we show that some initial cleavages of 45S pre-rRNA do not occur within spacer sequences as in *E. coli* but rather directly generate the mature 5′ ends of 18S and 5.8S rRNAs. Instead of primary processing steps at giant double-stranded stems, the initial cleavages of mouse pre-rRNA may then be analogous to the secondary or final cleavages in bacterial pre-rRNA. These cleavages are formally similar in that they occur relatively slowly and only after the ribonucleoprotein particles have formed.

During evolution, the 23S rRNA sequence split and developed into 5.8S rRNA and 28S rRNA, with 5.8S rRNA corresponding to the 5′ sequences of 23S rRNA (24, 32). In this respect, ITS 2 is similar to an intervening sequence in genes coding for mRNA. However, a search for possible remnants of a splice signal within this region has been negative. At any rate, the mechanisms for generating the mature 5′ end of 28S rRNA and the 3′ end of 5.8S rRNA are considerably more complex than those generating the mature 5′ end of 18S and 5.8S rRNAs. Further comparative evolutionary studies and direct biochemical analyses of pre-rRNA cleavages are required to complete the analysis.

ACKNOWLEDGMENTS

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