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Multiple ribosomal RNA cleavage pathways in mammalian cells

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ABSTRACT

The sequence content of mouse L cell pre-rRNA was examined by RNA gel transfer and blot hybridization. Nuclear RNAs were separated by agarose gel electrophoresis, transferred to diazo-paper, and hybridized to twelve different restriction fragments that are complementary to various sections of 45S pre-rRNA. An abundant new 34S pre-rRNA and less abundant new 37S, 26S and 17S pre-rRNAs were detected. The presence of these new pre-rRNAs suggests the existence of at least two new pre-rRNA cleavage pathways. 34S and 26S pre-rRNAs were also detected in HeLa cells suggesting that these new cleavage pathways are characteristic of mammalian cells. Further, an abundant new 12S precursor to 5.8S rRNA was also detected and is common to all the proposed cleavage pathways. The previously identified 45S, 41S, 32S and 20S pre-rRNAs were readily detected and their general structure confirmed. The 20S pre-rRNA is characteristic of the known pathway used by HeLa and other cells, and its presence suggests that growing mouse L cells use this pre-rRNA cleavage pathway. The 36S pre-rRNA characteristic of the previously described mouse L cell cleavage pathway was not detected. In all these cleavage pathways pre-rRNA cleavage sites are apparently identical and occur at or near the termini of the mature 18S, 5.8S and 28S rRNA sequences. The pathways differ only in the temporal order of cleavage at these sites.

The position of the 5.8S rRNA sequence was located within the internal transcribed spacer. The known and conserved sequence of 5.8S rRNA from several organisms predicts a characteristic pattern of restriction enzyme sites for 5.8S rDNA. Internal transcribed spacer rDNA was mapped with restriction enzymes, and the characteristic pattern was found near the midpoint of the internal transcribed spacer. This places the 5.8S rRNA sequence at or near the 5' terminus of 32S pre-rRNA.

INTRODUCTION

Mammalian cells transcribe ribosomal DNA into a 45S pre-rRNA that contains 18S, 5.8S and 28S rRNAs as well as spacer sequences (for reviews, see 1,2). The arrangement of these sequences is 5'-external transcribed spacer-18S rRNA -internal transcribed spacer-28S rRNA. 5.8S rRNA is located at an unspecified site within the internal transcribed spacer (3). 45S pre-rRNA is cleaved at specific sites producing a series of characteristic intermediates and finally mature rRNAs. Four pre-rRNA cleavage sites have been mapped within 45S

pre-rRNA. Two of these cleavage sites are located at or near the 5' and 3' termini of 18S rRNA and another near the 5' terminus of 28S rRNA. The fourth cleavage site has been mapped to the internal transcribed spacer. Although 5.8S rRNA is distal to this cleavage site, the proximity of the 5' terminus of 5.8S rRNA to this cleavage site is unknown (4).

Pre-rRNA cleavage pathways for the formation of mature rRNAs have been described for several mammalian cells. In both HeLa and mouse L cells 45S pre-rRNA is first cleaved near the 5' end of the 18S rRNA sequence to produce 41S pre-rRNA and the external transcribed spacer. In both cells, subsequent 41S pre-rRNA cleavage occurs at apparently identical sites, but in a different order. This results in the formation of 36S pre-rRNA and 18S rRNA in mouse L cells, and of 32S and 20S pre-rRNA in HeLa cells (5,6,7). Some cells use both pathways simultaneously (8,9,10). In both pathways, the order of pre-rRNA cleavage is more or less sequential, from the 5' to the 3' end of 45S. This has led to the idea that only the 5' proximal site is initially accessible to cleavage and that cleavage at this site alters the conformation of pre-rRNA to expose the more distal cleavage sites (2).

45S pre-rRNA cleavage sites and pathways were determined from the labeling kinetics (5,11), secondary structure (6,7) and methylation patterns (12) of pre-rRNAs and mature rRNAs. However, these techniques only detect abundant pre-rRNAs whose molecular weights differ substantially. Thus, other pre-rRNA cleavage sites and pathways may have been overlooked. In fact, Dubov et al. (13) have suggested that rat liver cells process 45S pre-rRNA by additional pathways, although they were unable to detect some of the proposed pre-rRNA intermediates.

We have used the sensitive technique of RNA gel transfer and blot hybridization to detect pre-rRNAs and determine their sequence content. Pre-rRNA cleavage sites and pathways are then inferred from this information. In addition, we have located the position of the 5.8S rRNA sequence within the internal transcribed spacer, and have identified a 12S precursor of 5.8S rRNA.

MATERIALS AND METHODS

Cell Culture. Mouse L cells clone 929 were obtained from the American Type Culture Collection. Cells were grown on petri dishes in MEM Earle's medium containing 8% fetal calf serum. Culture medium was changed every third day.

Cell fractionation and RNA isolation. Non-confluent, exponentially growing cells were harvested one day after changing the medium. Culture dishes were rinsed three times with isotonic saline, harvested in cold buffer

containing 0.15 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4) and 0.3% Triton X-100, and homogenized with 4 strokes of a Type B Dounce (Kontes). Sodium deoxycholate was added to a final concentration of 0.3% and the homogenate was vortexed for 30 sec, layered over a pad of 0.5 M sucrose, 0.15 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris-HCl (pH 7.4) and centrifuged at 500 x g for 5 min. The nuclear pellet was dissolved in 0.5 M NaCl, 0.005 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4) and 0.7% SDS containing 800 µg/ml of proteinase K and incubated at 37°C for 30 min. The nuclear homogenate was then homogenized 15 times with a Type B Dounce to shear DNA, made 0.08 M in sodium acetate (pH 5.0) and 0.01 M in EDTA, and extracted twice with 1 vol of phenol and 1 vol of chloroform and once with 2 vol of chloroform. An equal vol of 4 M LiCl was added to the final aqueous phase and the solution was incubated for 20 hr at 4°C to precipitate high molecular weight RNA. DNA, transfer RNA and 5S rRNA remain in solution under these conditions. The RNA precipitate was collected by centrifugation at 10,000 x g for 20 min, rinsed with 2 M LiCl, 0.01 M EDTA (pH 7.4) and recentrifuged. The RNA pellet was dissolved in 0.005 M EDTA, 0.005 M Tris-HCl (pH 7.4) and 0.25 M sodium acetate and ethanol precipitated.

RNA gel electrophoresis, transfer to diazo-paper and hybridization. RNA was denatured with glyoxal and fractionated in 0.9% agarose gels as described (14). Alternately, RNA was fractionated in a 0.9% agarose gel in the presence of 6 M urea (15). The gels were incubated for 1 hr in 0.05 M NaOH, then for 40 min in 0.5 M sodium acetate (pH 4.0) with 2 changes of buffer and blotted to either diazobenzyloxymethyl paper or diazophenylthioether paper. Diazobenzyloxymethyl paper was prepared as described by Alwine et al. (16) and diazophenylthioether paper was prepared by an unpublished procedure of B. Seeds (California Institute of Technology) and gave more reproducible results. Hybridization was performed similarly to Alwine et al (16). Paper was prehybridized at 44°C in 50% formamide, 5X SSC, 0.2% SDS, 0.02 M Na phosphate (pH 6.8) 0.002 M EDTA, 200 µg/ml sheared denatured calf thymus DNA and 5X Denhardt's solution (37) for 2-6 hr. SSC is 0.015 M sodium citrate and 0.15 M NaCl. Hybridization was for 24 hr in the above buffer except the concentration of calf-thymus DNA was 50 µg/ml and Denhardt's solution was 1X. Nick-translated subclones or isolated restriction fragments (17) were added to 25 ng/ml. Restriction fragments 6A, 6B, 6C, 6D, 7A and 7B (Fig. 1) were isolated from acrylamide gels (21). These fragments were well separated from each other and showed no evidence of cross-contamination in subsequent hybridizations.

Subcloning of λgt-DNA phages. λgtMr974 (clone 3) was obtained from N. Arnheim (State University of New York at Stony Brook); it contains rDNA

sequences complementary to part of the non-transcribed spacer, the 5' external transcribed spacer and the 5'-two-thirds of 18S rRNA (18). λgtMr100 was obtained from P. Leder (National Institute of Health) and contains rDNA sequences complementary to the 3' one-third of 18S rRNA, the internal transcribed spacer and most or all of 28S rRNA (19). Sections of these rDNA-phages were subcloned into pBR322 (No. 3,4,6 and 7 in Figure 1) to facilitate further mapping and analysis of rDNA. Sucblones 5A and 5B were subcloned from a pBR322-rDNA clone obtained from N. Arnheim (20).

S1 nuclease mapping. 28S rRNA was isolated from urea-agarose gels as described (15). Subclone 7 was digested with Ava I, treated with phosphatase, labeled with γ-[^{32}P]-ATP and polynucleotide kinase, and the Ava I fragments A and B were isolated from an acrylamide gel (21). These fragments were hybridized to 28S rRNA at 57°C in 80% formamide, 0.4 M NaCl, 0.02 M Pipes (pH 6.8) and 0.001 M EDTA. The hybrids were diluted 10 fold with 0.25 M NaCl, 0.03 M sodium acetate (pH 4.75), 0.001 M $ZnSO_4$ and 5% glycerol, and digested with the indicated amounts of S1 nuclease at 30°C (22).

RESULTS

The structure of the internal transcribed spacer. Cloned mouse rDNA complementary to the internal transcribed spacer DNA was mapped with restriction enzymes. The 3'-terminus of 18S, both termini of 5.8S and the 5'-terminus of 28S rRNA were located on this cloned rDNA. These experiments were undertaken to determine the previously unspecified location of 5.8S rRNA within the internal transcribed spacer and to identify restriction fragments of rDNA useful for examining the structure of pre-rRNAs by RNA gel transfer and blot hybridization.

The 3'-terminus of 18S rRNA was mapped to the Eco RI-Sal I restriction fragment 6 (see Fig. 1) based on the known conserved sequence of the 3' terminus of 18S rRNA (23,24). This sequence predicts a Sau 3A or Hinf 1 restriction enzyme site 6 nucleotides from the 3' end of 18S rDNA. Furthermore, based on the known size of 18S rRNA (2000 nucleotides) and the lengths of 18S rDNA in fragment 5A (550 nucleotides) (our unpublished results) and 5B (1150 nucleotides), fragment 6 should contain approximately 200-400 nucleotide of 18S rRNA sequence. Sau 3A and Hinf 1 restriction enzyme sites were mapped by partial digestion of the ^{32}P end labeled fragment 6 (Fig. 2B) (25). Only the Sau 3A site that is 260 bp from the beginning of fragment 6 is within this expected region of fragment 6. Thus, fragment 6 most probably contains about 260 bp of 18S rRNA sequence.

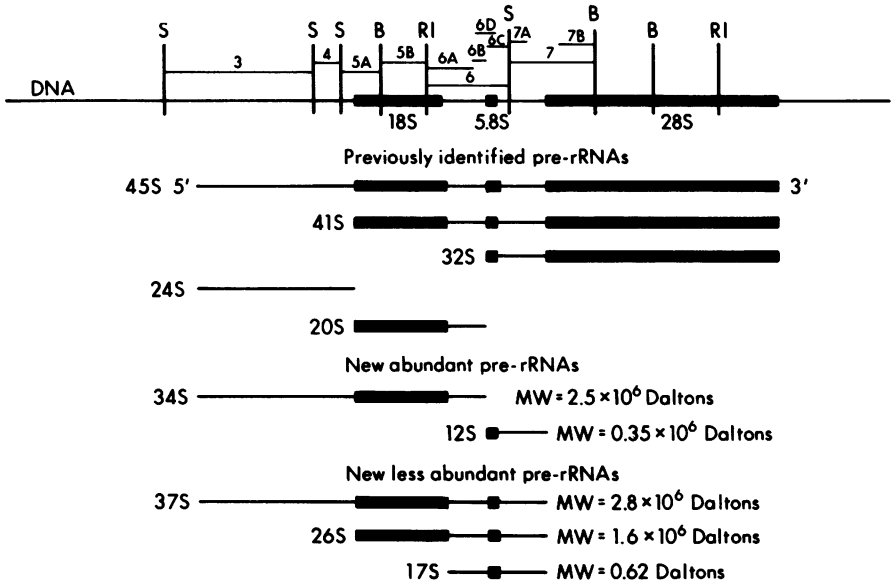


Figure 1. Restriction map (3,18,19,27,28,29,30) of mouse rDNA and the structure of the pre-rRNAs detected. S is Sal 1, B is Bam H1 and R1 is Eco R1. The location of restriction fragments 6A, 6B, 6C and 6D are shown in more detail in Fig. 2 and 7A and 7B in Fig. 3. The molecular weights of the new pre-rRNAs were determined from their migration on agarose gels using 28S and 18S as molecular weight standards. The molecular weights of 28S and 18S rRNA were assumed to be 1.74 x 10^6 and 0.68 x 10^6 Daltons respectively (7).

The 5' terminus of 28S rRNA was located by S1 nuclease protection experiments (22). Ava I and Sau 3A restriction enzyme sites were mapped on the Sal I-Bam H1 restriction fragment 7 by he partial digestion of end labeled fragment (25) (Fig. 3c). The Sau 3A restriction fragments of fragment 7 were separated by acrylamide gel electrophoresis and transferred to diazo-paper. Hybridization of ^{32}P labeled 28S rRNA to this DNA-paper indicated that the 5'-terminus of 28S rRNA was approximately 400-700 bp from the Sal I site (data not shown). Restriction fragment 7 was digested with Ava I and 5' end labeled with γ-[^{32}P]-ATP. The two Ava I restriction fragments localized to this region were isoated and hybridized to purified 28S rRNA. The hybrids were digested with S1 nuclease and separated by acrylamide gel electrophoresis under denaturing conditions. Figure 3 shows that 28S rRNA completely protected Ava I fragment B. Only 260 nucleotides of the Ava I fragment A were protected, indicating that the 5' end of 28S is about 260 bp from this Ava I site. This places the 5' terminus of 28S about 1160 bp from the Bam HI end of fragment

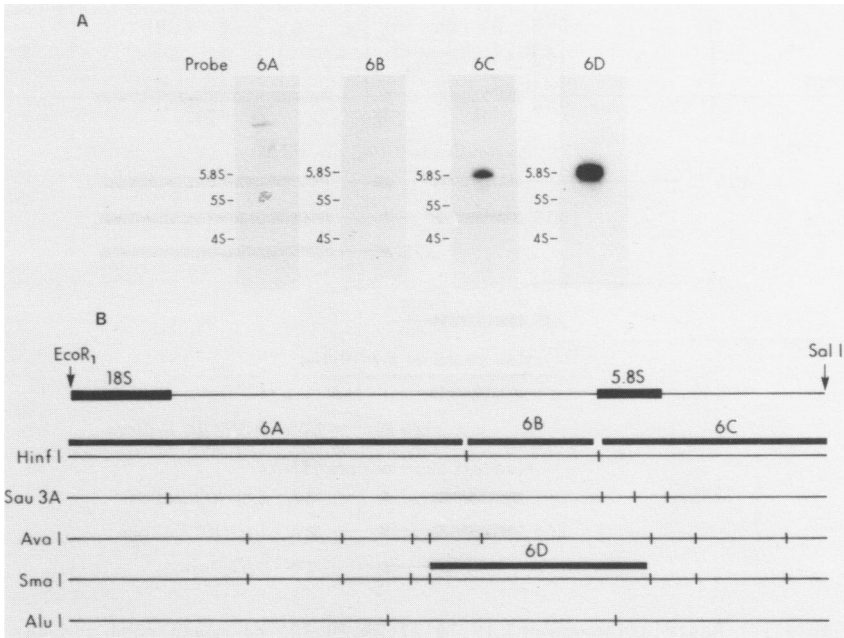


Figure 2. The location of the 18S and 5.8S rRNA sequences and the restriction enzyme map of fragment 6. A. Cytoplasmic rRNA was fractionated in a 9% acrylamide gel containing 7 M urea, transferred to dizao-paper and hybridized to the indicated labeled restriction fragments. B. The restriction enzyme map of fragment 6. The known 5.8S rRNA sequences predict the following restriction enzyme sites in 5.8S rDNA: Hinf 1 at 2, Sau 3A at 16 and 89, Alu 1 at 54 and Ava I and Sma 1 at 119 and 130 bp of the 5.8S rDNA sequence.

7. Since fragment 6 is 1850 bp long and contains 260 bp of 18S rRNA sequence, and fragment 7 is 1800 bp long and contains 1160 bp of 28S rRNA sequence, then the internal transcribed spacer must be about 2200 bp. This is consistent with previous experiments (7).

5.8S rRNA has previously been mapped to the internal transcribed spacer (39), but its location with respect to the 3' terminus of 18S rRNA and the 5' terminus of 28S rRNA is unknown. Hybridization of labeled 5.8S rRNA to fragments 6 and 7 indicated that fragment 6 contains the 5.8S rRNA sequence (data not shown). Fragment 6 was then mapped with several restriction enzymes (Fig. 2B) by partial digestions of the end-labeled fragment 6 (25). The 5.8S rDNA sequence was positioned within fragment 6 from its characteristic pattern of restriction enzyme sites. This pattern is derived from the known and generally conserved 5.8S rRNA sequence from a large number of species (26). Comparison

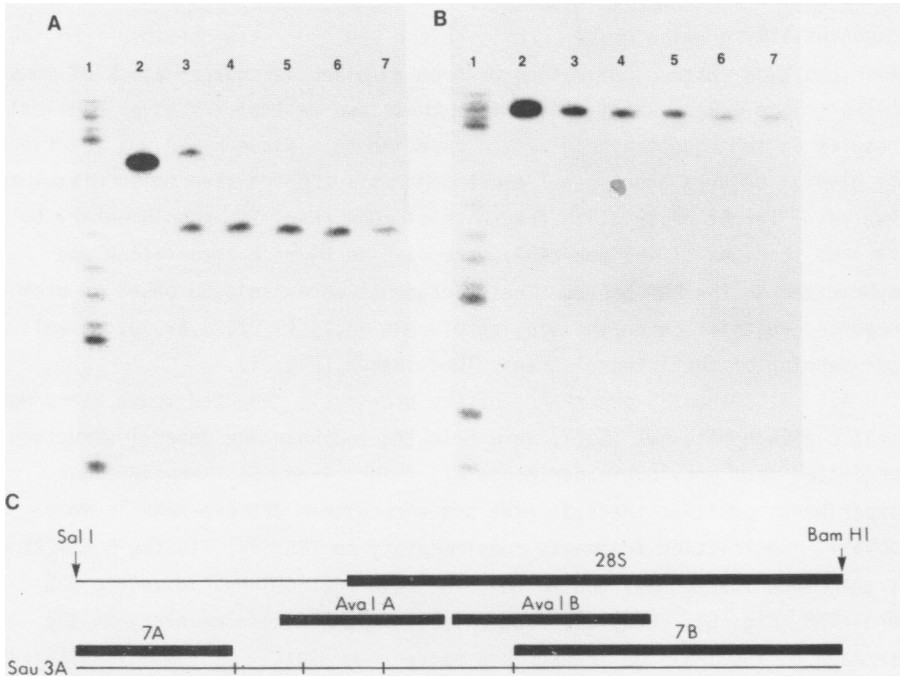


Figure 3. The location of the 28S rRNA sequence. A. 28S rRNA was hybridized with 5' end labeled Ava 1A fragment (see Material and Methods) and digested with (2) 0, (3) 1000, (4) 3000, (5) 7,000, (6) 18,000, and (7) 36,000 units of S1 nuclease per 30 λ of hybridization mix. (1) is pBR322 Hae III digest molecular weight markers. B. Identical to A except 28S rRNA was hybridized to fragment Ava 1B.

of the restriction enzyme map of fragment 6 to that predicted for 5.8S rRNA sequences suggests that 5.8S rDNA sequence begins 1330 nucleotides from the Eco R1 end of fragment 6 (Fig. 2). To verify this location, cytoplasmic RNA was separated by acrylamide-urea gel electrophoresis, transferred to diazo-paper and hybridized to the labeled Hinf I and Sma I restriction fragments (6A, 6B, 6C, and 6D) (Fig. 2). Only Hinf I fragment 6C and Sma I fragment 6D hybridized to 5.8S rRNA (Fig. 2A), and the only sequences these fragments share is the predicted 5.8S rDNA sequence. This places 5.8S rRNA in the middle of the internal transcribed spacer. The 18S-5.8S rRNA transcribed spacer and the 5.8S-28S rRNA transcribed spacer are each about 1100 bp long.

The detection of pre-rRNAs of the mouse L and HeLa cell cleavage pathways. The sequence content of mouse L cell pre-rRNAs was examined by RNA gel

transfer and blot hybridization (16). Nuclear RNA was isoalted from exponentially growing mouse L cells (clone 929) recently obtained from the American Type Culture Collection or from an older laboratory stock of mouse L cells (clone 929). RNA isolated from these two cell stocks gave identical results in subsequent hybridization experiments. Nuclear RNA was fractionated by glyoxal or urea agarose gel electrophoresis and transferred to diazo-paper. Twelve different restriction fragments of rDNA (Fig. 1), complementary to various sections of 45S pre-rRNA, were labeled by nick-translation and hybridized to the RNA-paper. These fragments were isolated based on previously reported restriction enzyme mapping of rDNA (3,18,19,27,28,29,30) as well as our mapping of the internal transcribed spacer (Fig. 1).

45S, 41S, and 32S pre-rRNAs, of the previously reported mouse L and HeLa cell cleavage pathways (5,7), were detected and have the general structure predicted from previous experiments (7) (Figs. 4 and 5). However, our experiments position the 5.8S rRNA sequence within 32S pre-rRNA in more detail. Restriction fragments complementary to 28S (7B), to the 5.8S-28S rRNA transcribed spacer (7A) and to the 5.8S rRNA (6C, 6D) hybridized to 32S pre-rRNA (Fig. 5). However, fragment 6B, which is complementary to the section of the 18S-5.8S transcribed spacer immediately proximal to 5.8S rRNA, did not hybridize to 32S pre-rRNA (Fig. 5). Thus, the 5' terminus of 32S

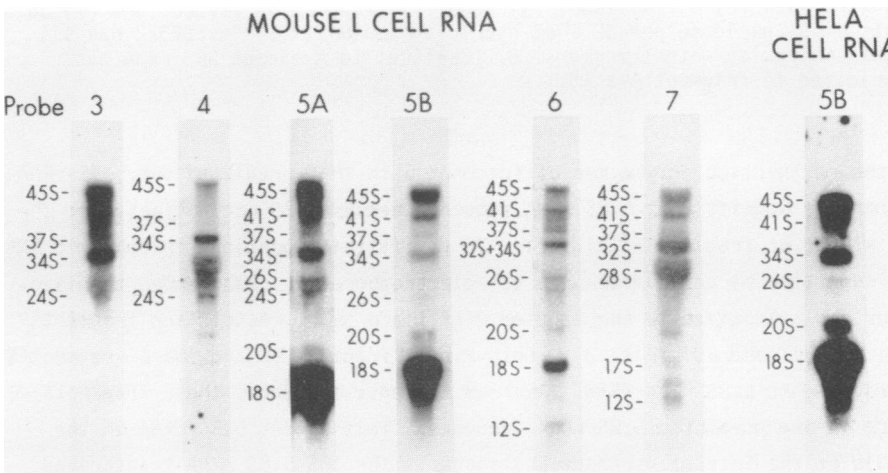


Figure 4. Mouse L or HeLa cell nuclear RNA was denatured with glyoxal, separated by agarose gel electrophoresis, transferred to diazo-paper and hybridized to the indicated labeled restriction fragments (Fig. 1).

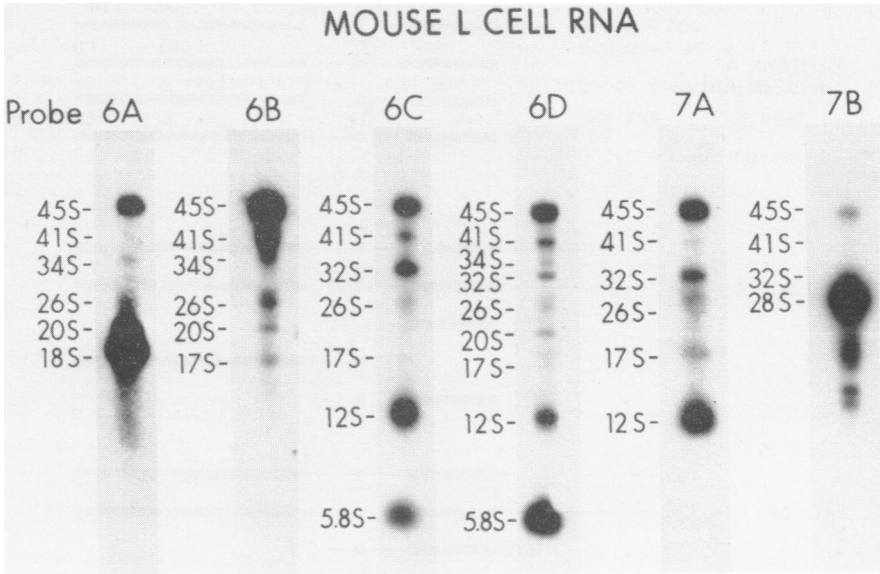


Figure 5. Mouse L cell nuclear RNA was separated by urea-agarose gel electrophoresis, transferred to diazo-paper and hybridized to the indicated labeled restriction fragments (Figs. 1, 2 and 3).

pre-rRNA must be very near the 5' terminus of 5.8S rRNA.

A 36S pre-rRNA complementary to the entire internal transcribed spacer and 28S rRNA is characteristic of the known mouse L cell cleavage pathway (7). No RNA species of this size and structure were detected in RNA separated by either glyoxal or urea agarose gel electrophoresis (Figs. 4 and 5). The absence of 36S pre-rRNA suggests that our mouse L cells do not use the previously documented cleavage pathway for mouse L cell pre-rRNA. However, 20S pre-rRNA, characteristic of the cleavage pathway used by HeLa cells (5,6), was readily detected in RNA separated by either gel system. Restriction fragments complementary to 18S rRNA (5A, 5B, 6, 6A) and to the 18S-5.8S transcribed spacer (6A, 6B) hybridized to 20S pre-rRNA (Figs. 4 and 5). This 20S pre-rRNA has not previously been detected in mouse L cells. Its presence suggests that under our culture conditions mouse L cells use the pathway first documented in HeLa cells (pathway A, Fig. 6).

The detection of new pre-rRNAs suggestive of new pre-rRNA cleavage pathways. An abundant 34S species hybridized to restriction fragments complementary to the 5'-external transcribed spacer (fragments 3, 4), to 18S

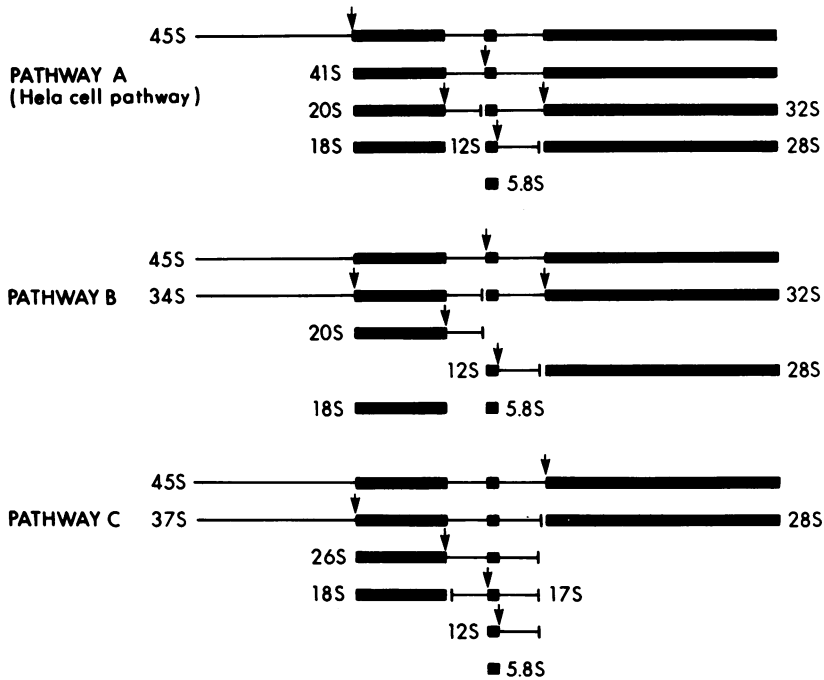


Figure 6. Pre-rRNA cleavage pathways.

rRNA (fragments 5A, 5B, 6) and to the 18S-5.8S transcribed spacer (fragments 6A, 6B) (Fig. 4). This species was poorly resolved from 32S pre-rRNA by glyoxal gel electrophoresis (Fig. 4). However, 34S and 32S pre-rRNA were well separated by urea agarose gel electrophoresis (Fig. 5). The presence of the 34S species suggests that some 45S molecules are first cleaved near the 5' terminus of 5.8S rRNA in L cells instead of near the 5'-terminus of 18S rRNA, as in the previously described mouse L and HeLa cell cleavage pathways (5,7). Pathway B (Fig. 6) is inferred from the presence of this 34S pre-rRNA. Other cleavages of this pathway are inferred from the presence of the appropriate intermediates.

Four other less abundant new pre-rRNAs were also detected. A 37S RNA was detected in nuclear RNA separated by glyoxal gel electrophoresis (Fig. 4) but not in nuclear RNA separated by urea agarose gel electrophoresis. Based on its molecular weight and its hybridization to restriction fragments 3,4,5A, 5B, 6 and 7, 37S pre-rRNA is comprised of the external transcribed spacer, 18S rRNA and the entire internal transcribed spacer. 37S pre-rRNA could arise

from cleavage of 45S near the 5' end of 28S rRNA. A 26S species hybridized to restriction fragments complementary to 18S rRNA (5A, 5B, 6, 6B), to the 18S-5.8S transcribed spacer (6B), to 5.8S (6C, 6D), and to the 5.8S-28S rRNA transcribed spacer (7A) (Figs. 4 and 5). This species could simply result from cleavage of 41S pre-rRNA molecules near the 5' terminus of 28S. A 17S RNA was detected in RNA separated by urea agarose gel electrophoresis (Fig. 4). Based on its hybridization to restriction fragments 6B, 6C, 6D and 7A, it is complementary to the internal transcribed spacer. However, its molecular weight is slightly less than that of the entire internal transcribed spacer (Fig. 1). This suggests that one of the cleavages producing 17S pre-rRNA occurs 100-300 nucleotides from the mature termini of 18S or 28S. Further, a 24S pre-rRNA was detected by hybridization to restriction fragment 6, but it was not abundant enough to determine its sequence content. The presence of 37S, 26S and 17S pre-rRNAs suggests that 45S is processed into mature pre-rRNAs by at least one other cleavage pathway (pathway C, Fig. 6).

Restriction fragments complementary to 28S rRNA hybridized to some rRNA species that are shorter than 28S. We believe these species are the result of limited cleavages of 28S rRNA occurring during RNA isolation and fractionation, since the amounts of these species vary from one RNA preparation to another. In contrast, the relative amounts of all the other rRNA species are very reproducible.

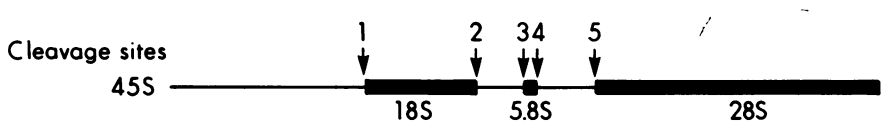
HeLa cells contain the 34S and 26S pre-rRNAs. Nuclear RNA isolated from exponentially growing HeLa cells was also examined by gel transfer and blot hybridization to determine whether HeLa cells also contain these pre-rRNAs. Minor pre-rRNAs have previously been detected in HeLa cells (5). Hybridization of a restriction fragment complementary to 18S rRNA (5B) to HeLa cell nuclear RNA detected the 34S and 26S rRNAs as well as the previously known 45S, 41S, and 20S pre-rRNAs (Fig. 4). The detection of 34S and 26S pre-rRNA in HeLa and mouse L cells suggests that alternate cleavage pathways for the production of mature rRNA may be a characteristic of most mammalian cells.

A new precursor to 5.8S rRNA. An abundant 12S RNA species hybridized to restriction fragments complementary to 5.8S rRNA (6C and D) and to the 5.8S-28S transcribed spacer (7A), but not to the fragment 6B which is complementary to the region immediately proximal to the 5' end of 5.8S rRNA (Fig. 5). This species is apparently formed by cleavages occurring near the 5' terminus of 5.8S rRNA and near the 5' terminus of 28S rRNA. Such a precursor to 5.8S rRNA has not been previously identified in mammalian cells,

although an analogous species has been identified in Drosophila (31).

The detection of transcribed spacer sequences unlinked to mature rRNA. Hybridization of RNA-paper to restriction fragments complementary to the external transcribed spacer detected the already known 24S external transcribed sequence (Fig. 4). Additional RNAs smaller than 24S were also detected by hybridization with restriction fragments 3 and 4. These species are localized to the nucleus (data not shown) and are presumably discrete breakdown products of the external transcribed spacer. In addition, a "broad diffuse band" of unknown significance migrates between 32S and 25S. In contrast, internal transcribed spacer sequences not linked to mature rRNA sequences were not detected. This indicates that after their cleavage from mature rRNA sequences these sequences are more rapidly degraded than are sequences from the external transcribed spacer.

The pre-rRNA cleavage sites used. Pre-rRNA cleavage sites are inferred from the structures of the detected pre-rRNAs (Fig. 1, Table 1). The presence of the new pre-rRNAs as well as the previously known pre-rRNAs indicates that



Pre-RNA	cleavage sites present	Cleavage sites used maximum	Cleavage sites used minimum
45S	1,2,3,4,5	1,3,5	1,3,5
41S	2,3,4,5	3,5	3
37S	1,2,3,4	1,3	1
34S	1,2	1	1
32S	4,5	5	5
26S	2,3,4	2,3	2
20S	2	2	2
17S	3,4	3	3
12S	4	4	4

Table 1. An alternate arangement for the minimum sites used is: sites 1 and 5 are used for 45S pre-rRNA and sites 1 and 3 are used for 37S pre-rRNA. All other pre-rRNAs have the same sites used in this alternate arrangement.

many pre-rRNAs can be cleaved at more than one site. However, not every site in each pre-rRNA is cleaved (Table 1). The maximum number of cleavage sites used is inferred from the presence or absence of the appropriate cleavage products (Table 1). In cases where the same pre-rRNAs could be formed by cleavage of different molecules at identical sites, it was assumed that all are possible. The minimum number is the least number of sites used to produce the detected intermediates, and is not a unique arrangement (see Table 1). This analysis suggests that the cleavage sites (1,3 and 5) at or near the 5' termini of 18S, 5.8 and 28S rRNA can be cleaved in 45S pre-rRNA and most probably in all pre-rRNAs. In contrast, the cleavage sites 2 and 4 at or near the 3' termini of 18S and 5.8S rRNA can be cleaved only in the immediate precursors to these mature rRNAs.

DISCUSSION

Pre-rRNA cleavage pathways. The presence of 45S, 41S, 32S and 20S pre-rRNAs in mouse L cells suggests that these cells use cleavage pathway A (Fig. 6), the pathway previously shown in HeLa cells (5,6,12). The detection of a new abundant 34S pre-rRNA as well as the new, less abundant 37S, 26S and 17S pre-rRNAs suggests the existence of at least 2 other cleavage pathways (Fig. 6, pathways A and C). Some ambiguity exists as to how many and what cleavage pathways are actually used, since the pre-rRNAs detected could also be part of other cleavage pathways. A minimum of 3 and a maximum of 7 pre-rRNA cleavage pathways can be inferred from the pre-rRNAs detected. The new abundant 12S precursor to 5.8S rRNA (Fig. 1) is an intermediate in all these cleavage pathways. These cleavage pathways differ only in the temporal order of cleavage at apparently identical sites.

The new pre-rRNAs were previously overlooked in mouse L cells probably because previous techniques could not distinguish them from other abundant pre-rRNAs. We believe these new pre-rRNAs are authentic processing intermediates because they are formed by cleavage at previously known sites and because they were detected in nuclear RNA isolated from exponentially growing cells. Several studies of the labeling kinetics of 45S pre-rRNA and 18S and 28S rRNAs indicate that exponentially growing cells do not degrade detectable amounts of 18S and 28S rRNA sequences during processing (11,32).

Previous studies have detected a 36S but not 20S pre-rRNA in mouse L cells, and a 20S but not 36S pre-rRNA in HeLa cells (5,6,7). Since these two pre-rRNAs are characteristic of different cleavage pathways, mouse L and HeLa cells were thought to use different cleavage pathways. Our experiments

detected 20S pre-rRNA but not 36S pre-rRNA in mouse L cells. This discrepancy is not due to HeLa cell contamination of our mouse L cells, since the latter were certified to be mouse L cells by the American Type Culture Collection. The discrepancy may reflect differences in cell culture conditions or RNA isolation procedures.

Other cells also process 45S pre-rRNA by more than one cleavage pathway. Previous studies have suggested that BHK cells (8), resting lymphocytes (9) and Drosophila (31) may use two pre-rRNA cleavage pathways, and rat liver (13) cells may use multiple cleavage pathways. We have detected 34S and 26S pre-rRNAs in HeLa cells. These may be identical to the minor species previously detected in HeLa cells (5). Their presence suggests that HeLa cells use the pre-rRNA cleavage pathway B (Fig. 6) in addition to the previously known HeLa cell pathway (pathway A; Fig. 6).

Preferred cleavage pathways. RNA gel transfer and blot hybridization experiments measure the steady state amounts of pre-rRNAs and therefore do not determine what fraction of 45S pre-rRNA is processed into mature rRNAs by the different pathways. However, some pre-rRNA cleavage pathways are probably preferred. A careful analysis of pre-rRNA labeling kinetics will be required to determine this. It is also likely that different cells have different preferred pre-rRNA cleavage pathways, because the relative concentrations of pre-rRNAs differ (5,6,7,8,9,10).

In both the previously known mouse L and HeLa cell cleavage pathways the initial cleavage occurs at cleavage site 1 (Table 1) followed by cleavages at more distal sites. This finding led to the idea that cleavages at proximal 45S or 41S pre-rRNA cleavage sites alter the conformation of pre-rRNA to expose the previously buried more distal cleavage sites (2). Our results are consistent with this notion with respect to cleavage sites 2 and 4 (Table 1). However, our results suggest that sites 1,3, and 5 are always exposed because they can be cleaved in any molecule (Table 1). This finding suggests that pre-rRNA conformation changes are not required to expose these cleavage sites. Thus, preferred cleavage pathways simply reflect the relative rate constants for cleavage of the exposed sites. These relative rates could be altered by variables like base sequence, methylation, ionic environment or bound proteins to produce different preferred cleavage pathways in different cells.

Pre-rRNA cleavage sites. Cleavage sites in mouse L cells were shown to be near the 5' and 3' termini of 18S rRNA, near the middle of the internal transcribed spacer and near the 5' terminus of 28S rRNA (7). Our results

confirm these findings. Further, our experiments show that the 5.8S rRNA sequence is located at nearly the exact middle of the internal transcribed spacer, and that the cleavage site within the internal transcribed spacer is at or near the 5' terminus of 5.8S rRNA (Table 1). Thus, the cleavage sites detected thus far are all at or near the termini of mature rRNA sequences. We do not detect any abundant pre-rRNAs whose structures suggest the presence of additional cleavage sites within 45S pre-rRNA spacer sequences. However, the RNA species of 26-34S that are complementary to the external transcribed spacer, and the new 17S pre-rRNA that is complementary to the internal transcribed spacer could conceivably result from cleavages within transcribed spacer sequences. Of course, our gel transfer and blot hybridization experiments are not sensitive enough to determine whether cleavages at these sites truly produce the mature termini of 18S, 5.8S and 28S rRNA. Some authors have suggested that further trimming is necessary to produce the mature termini of 18S, 5.8S and 28S RNAs (33,34,35) as in bacteria (36). Determination of the 5' and 3' terminal sequences of pre-rRNAs and mature rRNAs is required to test this possibility.

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