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Characterization of mouse 45S ribosomal RNA subspecies suggests that the first processing cleavage occurs 600 ± 100 nucleotides from the 5′ end and the second 500 ± 100 nucleotides from the 3′ end of a 13.9 kb precursor

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ABSTRACT

Mouse fibroblasts labeled 1-9 h with ^{3}H-uridine contained radioactive 45S rRNA subspecies of 13.9, 13.3, and 12.8 kb, as determined by hybrid-selection with rDNA plasmids and by electrophoresis in agarose-formaldehyde. The 13.9 kb subspecies contained 5' and 3' terminal rDNA sequences known from the work of Grummt and colleagues to be at or near the ends of the primary transcript. The 13.3 kb subspecies contained the 3' terminal sequence but lacked the 5' terminal sequence. The 12.8 kb subspecies lacked both terminal sequences. Washed nuclei produced one discrete species of 13.9 kb. The results suggested that synthesis of the primary transcript terminated 500±100 nucleotides beyond the 3' end of 28S rRNA, that the first processing cleavage occurred 600±100 nucleotides from the origin of synthesis, and the second cleavage occurred near the 3' end of 28S rRNA. Changes in relative radioactivities among the subspecies after serum stimulation or after treatment with low concentrations of cycloheximide suggesting that processing was not perfectly coupled with synthesis and that cycloheximide inhibited one cleavage more than others.

INTRODUCTION

The precursor of mammalian 28S and 18S ribosomal RNA (rRNA) is the well known 45S rRNA which is synthesized and cleaved repeatedly in the nucleolus (1). The 45S rRNA has often been assumed to be the primary transcript but recent evidence has suggested that most or all 45S rRNA in mouse cells has been cleaved at least once after transcription. Miller and Sollner-Webb (2) showed that most 45S rRNA was cleaved at 650 nucleotides from the origin, as confirmed by Grummt (3) and Mishima et al (4). Kominami et al (5) suggested that mouse rRNA synthesis extended 30 nuceotides beyond the 3' end of 28S RNA; however, Grummt et al (6) recently found that mouse rRNA synthesis *in vitro* using truncated plasmid templates did not terminate before 339 nucleotides beyond the 3' end of 28S RNA. No rRNA is known to be conserved beyond the 3' end of 28S RNA, hence an early processing cleavage could occur near the 3' end as well as near the 5' end of the primary transcript. It is not yet known, however, where the primary transcript terminates or if it ever exists as a whole molecule.

Here I report three distinct subspecies of 45S rRNA in mouse cells, confirming an earlier observation of Tiollais et al (7). In the earlier work (7) the largest of the three was preferentially labeled in short pulses, 5 minutes of ^{3}H-uridine, suggesting that the largest subspecies was a precursor to the smaller two. The present work extends the earlier findings by using hybrid-selection with rDNA plasmids and continuation of rRNA synthesis in isolated nuclei. I found evidence that the largest subspecies contained sequences missing in the smaller subspecies, in support of the earlier suggestion (7) that the two smaller subspecies were derived from the largest. The results presented here also confirmed that most or all mouse 45S rRNA terminated hundreds of nucleotides beyond the 3' end of 28S rRNA, in agreement with the recent results mentioned above (6), and furthermore, suggested a specific site of termination, 500±100 nucleotides beyond the 3' end of 28S rRNA. The data also give the order of the first two processing cleavages. Variations in relative amounts of the three 45S subspecies were observed under different culture conditions and are discussed.

MATERIALS AND METHODS

Cell culture

The SV40-transformed Balb/c mouse line SVT2 (8) was seeded in monolayer culture at 200,000 cells per sq cm in Dulbecco-Eagle medium plus 5% calf serum and was cultured 18-24 h. The untransformed mouse line Balb 3T3 (9) was seeded at 10,000 cells per sq cm in the same medium and was cultured 3 to 8 days. By 3 days 3T3 cells formed a quiescent confluent sheet with fewer that 0.5% of the cells in S phase. Quiescent cultures could be stimulated to reenter the cell cycle by stimulation with 20% calf serum. DNA synthesis began in over 90% of the cells by 12 to 20 h after stimulation (10).

Preparation of ^{3}H-labeled nucleic acids

Cultures of 8 sq cm or 20 sq cm were labeled with 10 to 20 µCi of ^{3}H-uridine for periods of 1 to 9 hours. The labeled compound was either (5-^{3}H)-uridine at 18 Ci/mmol or (5,6-^{3}H)-uridine at 40 Ci/mmol. In one study, cycloheximide was added at 1, 3, and 10 µM during the last 30 min of labeling.

Degradation of RNA during extraction was minimized by rapid lysis of labeled cells on the culture dish, as follows: Cultures were chilled within 2 sec by pouring off the warm growth medium and pouring on ice-cold phosphate buffered physiological saline (PBS). The cultures were rinsed twice at 0-4° C with PBS by pouring, 5 sec per rinse, and then lysed on the dish at 20° C with 1 ml or 2 ml of 150 mM NaCl, 50 mM Tris-HCl pH 8.1, 25 mM EDTA, 0.5% SDS, and 100 µg/ml self-digested pronase (11). The lysed cells were scraped into a tube, incubated 30 min at 45° C, and extracted once with phenol-chloroform. The aqueous phase of the extract was precipitated with 2.5 volumes of 95% ethanol. The precipitate was washed with 70% ethanol, dried under vacuum, and dissolved in an R-loop buffer (12) which contained 400 mM NaCl, 100 mM PIPES pH 6.8, 10 mM EDTA, and 70% (v/v) formamide. The solution contained all nucleic acids of the cell at about 1 mg/ml; the specific radioactivity of RNA was estimated at 100,000 cpm/µg.

Plasmids containing mouse rDNA

Three mouse rDNA plasmids were obtained from Dr. I. Grummt. The first was a plasmid known be homologous to all 45S molecules, a second was known to be homologous to the first few nucleotides at the 5' end of the primary transcript, and a third was known to be homologous to a short region at or near the 3' end of the primary transcript. The first plasmid was pMrSalB (13) which contained approximately 2500 nucleotides homologous to 45S rRNA in a region near the 5' end of the primary transcript, entirely within the nonconserved external transcribed spacer. The second, the 5' terminal clone pMrSP (14), contained 461 nucleotides of mouse DNA which overlapped the site of initiation and was homologous to the first 292 nucleotides of the primary transcript. The third, the 3' terminal clone pUCMrSB (6), contained 282 nucleotides of mouse DNA corresponding to nucleotides +58 to +339, beyond the 3' end of the 28S rRNA. As mentioned above, the 3' terminus of the primary transcript has not been well defined.

Preparation of DNA-nitrocellulose filters

Plasmid DNA was isolated (15) and bound (12) to nitrocellose as described, except for the following modifications. DNA was dissolved in 10 x SSC at 2 micrograms per ml and applied at 0.5 ml per well to a 96-well BRL "Hybridot" apparatus with a sheet of nitrocellulose in place. Vacuum was applied to draw the solution through the nitrocellulose. The nitrocellulose was baked and washed as described (12) and then circles of DNA-nitrocellulose, 3 mm in diameter, were punched from the sheet. Each 3 mm circle contained about 1 µg of plasmid DNA according to a fluorometric measurement (10).

DNA-RNA hybrid selection

One circle of DNA-nitrocellulose was incubated with 5 µl of RNA solution, prepared as described above, for 3 h at 52-54° C. The circle was washed (12), and selected RNA was eluted by heating at 90° C for 10 min in 10 µl of elution buffer which contained 91% (v/v) formamide, 30 mM sodium-PIPES pH 6.8, and 2 mM EDTA.

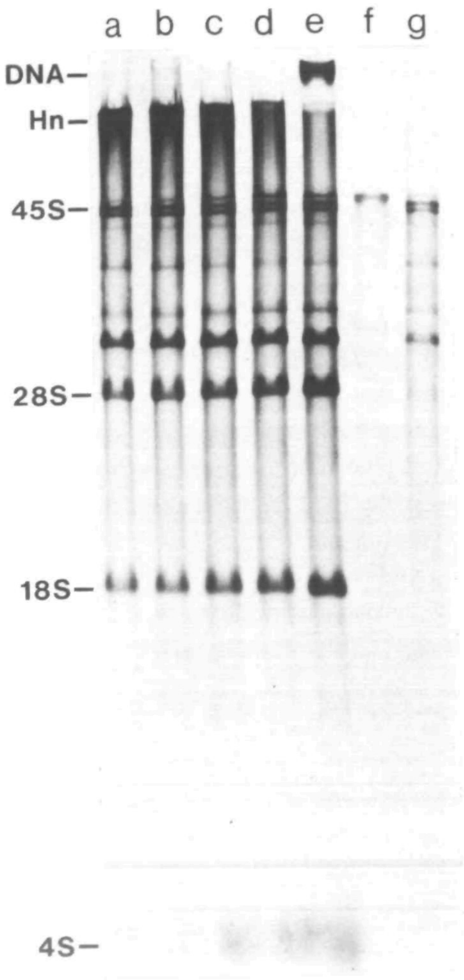

Figure 1: 45S RNA labeled in quiescent 3T3 cells, in stimulated 3T3 cells, and comparison with RNA labeled in isolated nuclei. 3T3 cells were labeled 2 h with (5,6-^{3}H)uridine, and extracted nucleic acids were electrophoresed for 650 volt hours in 0.7% agarose, 2 M formaldehyde. The amounts of radioactivity applied to lanes a-e were adjusted to give about the same radioactivities of the smaller two 45S subspecies. Lane f contained radioactive RNA synthesized by isolated SVT2 nuclei incubated with alpha-amanitin 20 min, to compare with sizes of 45S rRNA synthesized in cells. (See also Fig. 2.)
Lane a Confluent 3T3 cells, 8 days after confluence, unstimulated.
Lane b Confluent 3T3 cells, 3 days after confluence, unstimulated.
Lane c As in b, but stimulated 2 h with 20% serum.

Lane d As in b, but stimulated 5 h.

Lane e As in b, but stimulated 20 h. Note DNA labeled by (5,6-^{3}H)uridine in this lane only. DNA synthesis began 12-15 h after stimulation.

Lane f Radioactivity from synthesis by isolated SVT2 nuclei, labeled 20 min *in vitro*. DNase was used here but not in lanes a-e.

Lane g Radioactivity from nuclei isolated from SVT2 cells which were labeled 1 hour *in vivo* before nuclear isolation. DNase was used here; note that the DNA band was missing.

Electrophoresis of RNA

RNA in elution buffer was heated to 90° C, cooled to 55° C, supplemented with 37% (w/w) formaldehyde to 2 M, and incubated 15 min at 55° C. The solution was applied to gel of 0.7% agarose in 2 M formaldehyde (12), of dimensions 14 x 19 x 0.4 cm. Electrophoresis was for 650 or 1000 volt-hours at room temperature. Resolution of the 45S subspecies was improved somewhat by waiting 2 to 3 h after applying the RNA samples before turning on the power. After electrophoresis, the gel was fixed in acid, dehydrated in methanol, impregnated with 2,5-diphenyloxazole (PPO), dried, and fluorographed with X-ray film (12).

Preparation of end-labeled single-stranded DNA used as size standards

Bacteriophage lambda DNA was digested (separately) with Bam HI and with Bgl II. The recessed 3' ends of the double-stranded fragments were labeled with [alpha-^{32}P]dGTP and the Klenow fragment of E. coli DNA polymerase (16). The end-labeled fragments were denatured in 91% formamide and reacted with formaldehyde as described above. The use of formaldehyde is known to be fully denaturing for both RNA and DNA (17).

Isolation of nuclei and cell-free RNA synthesis

Monolayer cultures of 54 sq cm were washed twice with cold PBS as in the isolation of labeled RNA above and were then lysed on the culture dish at 0-4° C with a non-ionic detergent in 5 ml of a lysis-washing solution which contained 30 mM Tris-HCl pH 7.9, 80 mM KCl, 7 mM magnesium acetate, 7 mM 2-mercaptoethanol, 10 nM EDTA, 10% (v/v) glycerol, and 0.5% (v/v) Nonidet P-40. After 3 min the lysis-washing solution was decanted. Nuclei and components of the cytoskeleton remained attached to the culture dish although soluble components of the cells were removed (18, 19). The nuclei were washed once more on the dish with 5 ml of the same lysis-washing solution, which was also decanted. The tenuously attached nuclei and cytoskeletons were then suspended in 3 ml of fresh lysis-washing solution by gentle pipetting and were then sedimented (2000 x g, 3 min, 2° C), thus washing the nuclei altogether three times. The pellet was resuspended at 40,000 nuclei/µl in an incubation buffer which was the lysis-washing solution plus 1 mM ATP, 0.4 mM GTP and CTP, 50 µM ^{3}H-UTP (10 Ci per mmol), and 300 µg/ml alpha-amanitin. The suspension was divided into samples of 5 and 30 µl and incubated at 30° C for times up to 60 min. The samples of 5 µl were used to measure incorporation into acid-insoluble material, normalized to nuclear DNA (10). Nucleic acids were extracted from the samples of 30 µl, as described above, with the addition of a digestion with RNase-free DNase I (20). Conditions of DNase digestion were 50 µg per ml DNA and 1 Kunitz unit per ml of iodoacetate-treated DNase-I in 0.2 M sodium acetate pH 6.9, 10 mM magnesium sulfate, for 30 min at 2-4° C. Digestion with 5 Kunitz units per ml with otherwise the same conditions did not degrade HeLa 45S rRNA in a separate test (21).

RESULTS

Observation of 45S RNA subspecies

Cultures labeled 1 or 2 h with ^{3}H-uridine contained three radioactive RNA species in the 45S or 12-14 kb size region (Figs. 1-4). The three subspecies were observed in cultures labeled 1h, 3 h, 4 h, and 9 h, with approximately the same relative radioactivities among the three subspecies (not shown).

The sizes of the subspecies were estimated at 13.9, 13.3 and 12.8 kb, with a latitude of ±100 nucleotides by using a much larger version of Fig. 4. The references used for determining the sizes were denatured restriction fragments of bacteriophage lambda DNA, not RNA. Within the same gel lanes, however, the sizes of mouse 32S and 28S were determined by the same methods to be 6.2 and 5.05 kb, respectively. These values are close to published values (1), so the sizes of the 45S subspecies were not corrected.

The finding of multiple subspecies of 45S RNA confirms an earlier observation of Tiollais et al (7) who found three subspecies in labeled mouse L cells using sliced tube gels of polyacrylamide. The sizes were estimated in sedimentation coefficients to be 47S, 46S, and 45S. Tiollais et al also observed two or more 45S subspecies, even more closely spaced, in human HeLa cells (7).

In other studies, not on rRNA explicitly, I incidentally confirmed the the presence of two 45S RNA subspecies in HeLa cells (22), and found multiple species in Syrian hamster cells (23), monkey cells and chicken cells (unpublished observations). Of the five animal species examined, mouse had the most pronounced separations between individual 45S RNA subspecies.

Identification of the 45S subspecies as rRNA by DNA-RNA hybrid selection

The rDNA plasmid pMrSalB, homologous to about 2500 nucleotides of transcribed rRNA near the origin of synthesis (13), selected the three 45S subspecies

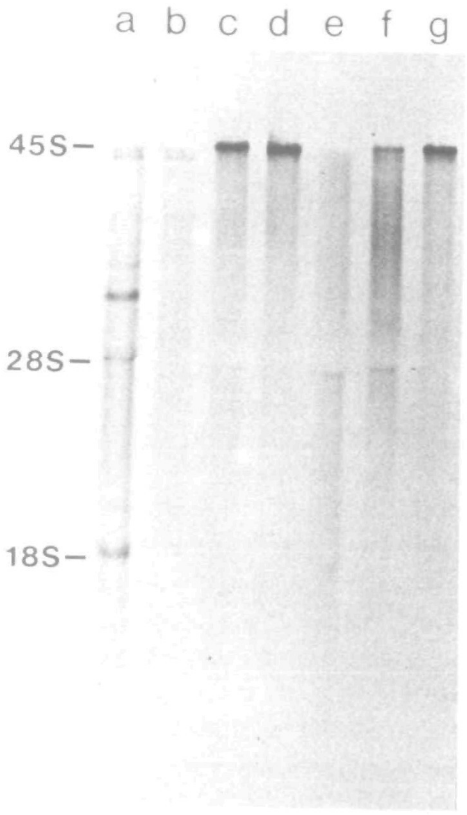

Figure 2: Further studies of sizes of labeled rRNA produced by washed mouse nuclei. Crude isolated nuclei from 3T3 and SVT2 cells were incubated at 30° C with ^{3}H-UTP and alpha-amanitin. Nucleic acids were extracted, treated with DNase, and electrophoresed as in Fig. 1. The band of radioactivity near 28S in lanes e-g was probably an artifact resulting from overloading the lanes with cold 28S rRNA from the nuclei.

Lane a SVT2 RNA labeled *in vivo* 1 h with ^{3}H-uridine for comparison.
Lane b 45S RNA from lane a, fractionated by sucrose gradient.
Lane c Isolated nuclei labeled 20 min, quiescent 3T3 cells.
Lane d Isolated nuclei labeled 20 min, stimulated (20 h) 3T3 cells.
Lane e Isolated nuclei labeled 1 min, SVT2 cells.
Lane f Isolated nuclei labeled 5 min, SVT2 cells.
Lane g Isolated nuclei labeled 20 min, SVT2 cells.

discussed above plus 2 or 3 minor subspecies near 11-12 kb and one near 6 kb (Fig. 3, lane d). The rDNA plasmid pMrSB, homologous to the first 292 nucleotides of transcribed rRNA (14), selected only the 13.9 kb subspecies (Fig. 3, lane e). The rDNA plasmid pUCMrSB (6), homologous to 282 nucleotides of non-conserved rRNA near the 3' end of the primary transcript, selected the 13.9 and 13.3 kb subspecies but no other discrete species (Fig. 3, lane f). As discussed below, one interpretation of the results is that mouse rRNA terminated beyond the 3' end of 28S rRNA and that a cleavage near the 5' end of mouse 45S rRNA preceded a cleavage near the 3' end of the 28S rRNA.

Comparison of the 45S subspecies with rRNA synthesized *in vitro*

Isolated nuclei are able to elongate nascent transcripts, but initiation and processing are nearly eliminated in washed nuclei (24). The use of high concentrations of alpha-amanitin with isolated nuclei confines synthesis to rRNA (25, 26). It has been known for several years that isolated nuclei can produce rRNA of about 45S in size (27, 28), suggesting that termination or processing might be accurate *in vitro*. This possibility was explored further by making a comparison of sizes of rRNA synthesized in isolated nuclei with sizes of subspecies labeled *in vivo*. Results are shown in Figs. 1 and 2.

Labeling *in vitro* for one min produced labeled molecules in a continuum of sizes, with no obvious discrete species, from smaller than 2 kb to larger than 12 kb (Fig. 2, lane e). Five min of labeling produced larger molecules with an upper limit near 14 kb (Fig. 2, lane f). Incorporation stopped increasing by 10 to 15 min at 0.2 to 1.5 p mole UTP incorporated per microgram of nuclear DNA. The amount of incorporation depending on the growth state of the cells, as has been observed before (18). The labeled products produced in 20 min of labeling, after incorporation had reached a plateau, were a single discrete species at 13.9 kb plus some smaller species of many sizes (Fig. 2, lanes c, d, and g; and Fig. 1, lane g). The same result was obtained with incubations of 40 and 60 min (not shown). The size of the rRNA produced in isolated nuclei did not depend on the growth state of the cells (Fig. 2, lanes c, d, and g). The size of the major discrete product produced *in vitro* was not distiguishable from the 13.9 kb species labeled *in vivo*. As above, the size analysis was electrophoresis of whole molecules in 0.7% agarose-formaldehyde gels.

Variations in relative radioactivities among subspecies

The relative radioactivities between the subspecies were found to change in mouse 3T3 cells stimulated to reenter the cell cycle from a quiescent state. The distributions of radioactivity among subspecies in quiescent 3T3 cells (Fig. 1, lanes a and b), and in rapidly dividing SVT2 cells (Fig. 2, lane a and Fig. 3, lane c) were similar; the two smaller subspecies were more radioactive than the largest (13.9 kb) by a factor of 2 to 4. The estimates of relative radioactivities were made by comparing different film exposures of the same gel. Between 5 and 20 h

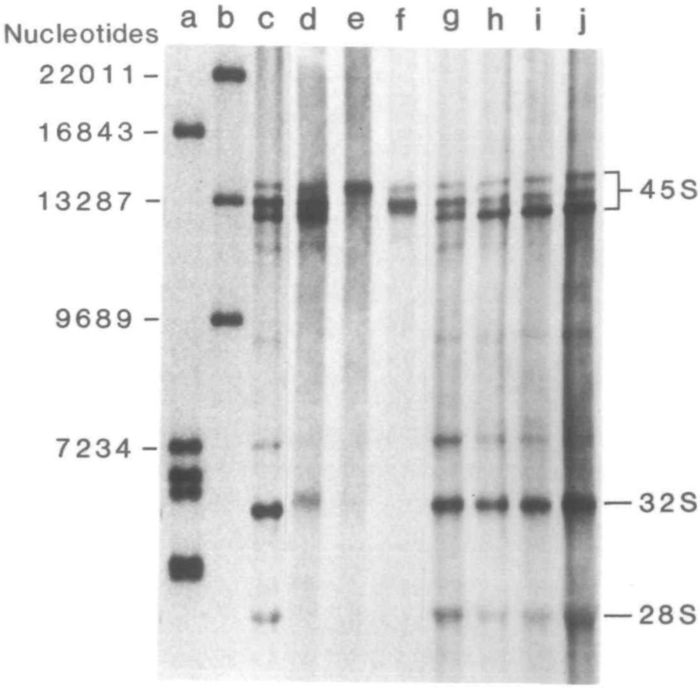

Figure 3: Comparison of nucleic acid sizes, hybrid selection of 45S RNAs, and effect of cycloheximide on relative radioactivities of 45S RNAs. The figure is a composite of fluorographs of three agarose-formaldehyde gels (a-c, d-f, and g-j) each run for 1000 volt-hours. The sizes of restriction fragments of bacteriophage lambda DNA are shown on the left.

Lane a Lambda DNA cleaved with Bam HI, ^{32}P end-labeled, single stranded.
Lane b Lambda DNA cleaved with Bgl II, ^{32}P end-labeled, single-stranded.
Lane c SVT2 RNA labeled 1 h with ^{3}H-uridine.
Lane d ^{3}H RNA as in (c) hybrid-selected with pMrSalB.
Lane e ^{3}H RNA as in (c) hybrid-selected with pMrSP.
Lane f ^{3}H as in (c) hybrid-selected with pUCMrSB.
Lane g SVT2 RNA labeled 1 h with ^{3}H uridine, no cycloheximide.
Lane h ^{3}H RNA as in (g), 1 μM cycloheximide added during last 30 min.
Lane i ^{3}H RNA as in (g), 3 μM cycloheximide added during last 30 min.
Lane j ^{3}H RNA as in (g), 10 μM cycloheximide added during last 30 min.

after serum stimulation, however, the 13.9 kb subspecies became about as radioactive as the other two, (Fig. 1, lanes c, d, and e). The time of labeling with ^{3}H-uridine (1, 2, or 4 h; not shown) did not affect the relative radioactivities among 45S subspecies in stimulated 3T3 cells. The amount of 45S RNA synthesized in stationary contact-inhibited 3T3 cells was probably much greater than that synthesized in stationary mouse ascites cells (3), for unknown reasons possibly related to differences between the cell lines or culture conditions.

The pattern of relative radioactivies also changed after exposing the cells to low concentrations of cycloheximide, 1, 3, and 10 µM, during the last 30 min of labeling (Fig. 3, lanes g-j). These low levels of cycloheximide do not halt protein synthesis but slow ribosome translocation enough (compared to the rate of initiation of protein synthesis) to recruit most of the free ribosomes into polysomes (29, 30). Under these conditions of partial inhibition of protein synthesis, the 12.8 kb subspecies became 2 to 4 times as radioactive as the other two which were of equal radioactivity (Fig. 3, lanes h, i, and j). The effect of cyloheximide was the same on cells in all of the growth states examined in this report. There were no significant differences in effect between the three concentrations of cycloheximide, 1, 3, and 10 micromolar (Fig. 3; h, i, and j).

DISCUSSION

Hybrid selection with rDNA plasmids showed that the three 45S RNA subspecies were all rRNA, since all three were selected by pMrSalB (Fig. 3, lane d). The hybrid selection also showed that the three subspecies shared non-conserved rRNA sequences near the origin. It should be mentioned that it is possible that more subspecies existed than the three observed.

Of the three 45S subspecies observed, only the 13.9 kb subspecies contained some or all of the more restricted sequence in pMrSP, 292 nucleotides of rRNA at the extreme 5' end of the primary transcript (Fig. 3, lane e). The 5' end had been defined previously using cell-free initiation and runoff transcription on fragments of cloned plasmid rDNA as templates (2, 3, 4). Since only one of the three 45S subspecies contained the sequence in pMrSP at the 5' end, it is probable that the other two were generated by processing of the 13.9 kb subspecies, as suggested by Miller and Sollner-Webb who found that most 45S rRNA lacked 650 nucleotides at the 5' end (2). Another hypothetical explanation of the origin of subspecies, not yet excluded, is the existence of hypothetical alternate site(s) of initiation *in vivo*, which could not be used *in vitro*, for unknown reasons.

The plasmid pUCMrSB (6), which contained a short nonconserved sequence near the 3' end of the primary transcript, selected the 13.9 and 13.3 kb subspecies but not the 12.8 kb subspecies (Fig. 3, lane f). This observation suggests that cleavage at the 3' end, or perhaps an alternate site of termination, distinguished the 13.9 and 13.3 kb subspecies from the 12.8 kb subspecies. The 13.9 kb subspecies appeared to contain both the 5' and the 3' terminal sequences since it was selected by both the 5' and 3' terminal plasmids (Fig. 3, lanes e and f).

The plasmid pUCMrSB (6) selected the 13.9 and 13.3 kb subspecies but not the 12.8 kb subspecies (Fig. 3, lane f). More than half of the 45S rRNA was selected yet the only mouse DNA the plasmid contained was 58-339 nucleotides beyond the 3' end of the 28S molecule. By contrast, previous work using mapping by S1 nuclease determined a terminus only 30 nuceotides beyond the 3' end of 28S (5). The reason for the discrepancy between these results and those obtained with S1 nuclease mapping (5) is not known; however, the results presented here are in agreement with the studies of cell-free synthesis (6) which showed no termination signal *in vitro* before 339 nucleotides beyond the 3' end of 28S rRNA.

The 500 nucleotide difference in size between the 13.3 and 12.8 kb subspecies could give an approximate distance between the site of termination and the end of 28S if it is assumed that all rRNA terminates at one site. In principle, however, it is still possible that the 13.3 and 12.8 kb subspecies were generated by different sites of termination *in vivo* which could not be reproduced *in vitro* using particular plasmid templates or incubation conditions.

Another conceivable cause of heterogeneity in the size of 45S rRNA is heterogeneity in nonconserved spacer regions, from transcription of different rDNA templates in the large array of reiterated rDNA in mouse. A small amount of heterogeneity in spacer DNA has been found in yeast (31), *Xenopus* (32), and rat (33). The possibility of template heterogeneity could not be tested in previous studies using initiation of synthesis *in vitro* or S1 nuclease studies (2-6) since both approaches used cloned plasmid templates which could not represent all possible templates *in vivo*.

The possibilities of alternate sites of initiation and termination plus heterogeneity in template DNA as being causes of the different 45S subspecies were addressed by measuring sizes of 45S rRNA produced in isolated nuclei. Since the vast majority of rRNA synthesis in nuclei is expected to have initiated *in vivo*, any alternate sites of initiation should have been present in the products of synthesis of the isolated nuclei. The same is likely to be true for possible alternate template sizes or alternate sites of termination. If heterogeneity existed in initiation, termination, template lengths, or processing *in vitro*, we would have found an uninterpretable collection of different sizes produced *in vitro*. The spectrum of observed sizes was simple, however; only one discrete species was found which was of the same mobility as the largest of the three subspecies observed *in vivo*. Of course, the similarities in mobility in no way

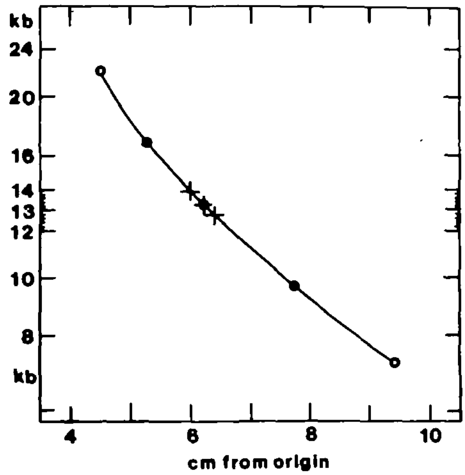

Figure 4: Calibration of sizes of 45S RNAs. ^{3}H-RNA as in Fig. 3 g was run with ^{32}P-labeled single-stranded lambda DNA fragments (in Fig. 3 a and b) in adjacent lanes of an agarose-formaldehyde gel. The mobilities of DNA fragments (o) are compared with three 45S RNAs (+) on a semi-log plot. The single-stranded lambda Bam HI B fragment (13.3 kb) was of the same mobility as the middle-sized 45S RNA. The data were not corrected for the difference between DNA and RNA. From a much larger version of this plot, the sizes of the three 45S RNAs were determined to be 13.9, 13.3 and 12.8 kb, ± 0.1 kb.

proved identity of the 13.9 kb rRNAs labeled by the two methods, but the observation did not confirm a prediction of heterogeneous sizes from heterogeneous start or stop sites and/or heterogeneous templates. The simplest interpretation, though not an exclusive one, is that no heterogeneity in spacers or initiation sites existed, and that synthesis terminated at the same site *in vivo* and *in vitro*. From the differences in sizes of the subspecies (Fig. 4), the site of termination *in vivo* is predicted to be 500 ± 100 nucleotides beyond the 3' end of the 28S rRNA. More cell-free synthesis, sequencing, and S1 mapping will be required to test the prediction.

The smear of smaller molecules labeled in isolated nuclei might be explained by premature termination *in vitro*. The extent of synthesis in nuclei was considerable, several thousand nucleotides, so perhaps some premature termination is not surprising.

It is still possible that the 13.9 kb subspecies was cleaved from a longer precursor, but the present work serves to limit the discussion of that possibility. If the 13.9 kb RNA was cleaved from a larger molecule, then that particular cleavage must have been essentially complete *in vitro*, in washed nuclei, where further cleavages were not detected.

The variations in relative radioactivities between the subspecies could have originated from different relative amounts of the subspecies and/or different relative specific radioactivities. A difference in specific radioactivity might have ocurred if synthesis of the subspecies used nucleoside triphosphate precursors of different radioactivities. Since the subspecies appeared to be processed from the same primary transcript, a difference in specific radioactivity could be explained instead by a changing nucleoside triphosphate pool. There are precedents for a change in the triphosphate pools after stimulation (34), but that explanation seems unlikely in this instance because the lifetime of all 45S rRNA is only a few minutes (1), and the lifetimes of subspecies must have been even shorter. The change in specific activity of an intracellular triphosphate, ATP, has been characterized in our 3T3 cells using the same culture conditions used here (30), but the rate of change was relatively slow, measured in hours not minutes, at the time of labeling, 1-9 hours after stimulation. From these considerations, it seems safe to conclude that different radioactivities among the subspecies represented different steady-state amounts of the subspecies. The different relative amounts, between different cultures, would then have resulted from differences in rates of processing.

If we assume differences in processing, there was a distinct difference between the processing which produced the 45S subspecies and later processing which produced smaller rRNAs. In Fig. 1, processing to rRNA smaller than 32S became apparently faster in stimulated 3T3 cells than in quiescent cells, since the smaller species became more radioactive, relative to larger species. (Compare in Fig. 1 lane a with lane e.) This effect has been found repeatedly, in several cell systems (35). Among the 45S subspecies, however, the opposite was found after stimulation; processing appeared to become slower after stimulation since the 13.9 kb subspecies became relatively more radioactive than the two smaller 45S subspecies. (Compare in Fig. 1 lane a with lane d.) This observation suggests that the rate of rRNA synthesis increased faster than did the rate of processing in recently stimulated 3T3 cells.

The effect of cycloheximide, an accumulation of the 12.8 kb subspecies, relative to the other two (Fig. 3; h-j), appears to have been a specific inhibition of rRNA processing after the production of 12.8 kb RNA. An accumulation of another intermediate in processing at about 8 kb (Fig. 3, lanes h-j) was also noted in low cycloheximide. Two details of this cycloheximide effect are worth noting. First, the effect occurred within 30 min. If rRNA processing required ongoing protein synthesis and 1 µM cyclheximide inhibited that synthesis, there must have been a tiny pool of a specific protein required for particular cleavages since 1 µM cycloheximide does not halt protein synthesis (29, 30). Second, the effect was a partial inhibition of processing since some labeled smaller rRNA was found, yet the inhibition did not become more pronounced with higher concentrations of cycloheximide. (In Fig. 3 it was found that 1, 3, and 10 micromolar cycloheximide produced the same effect.) This observation was especially puzzling since we found in previous work that the low levels of cycloheximide did not stop protein synthesis (30) but were increasingly effective at slowing synthesis in this range of concentrations. If, as these considerations suggest, the effect did not depend on inhibition of protein synthesis, then perhaps cycloheximide acted more directly on processing. Since cycloheximide inhibits protein synthesis by binding to peptidyl transferase of the 60S ribosomal subunit (36), then perhaps it can also inhibit rRNA processing by binding to a site with the same affinity in the nascent ribosome in the nucleolus. The prediction might be tested using cell-free processing.

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REFERENCES

1. Perry, R.P. (1976) Annu. Rev. Biochem. **45**, 605-629.
2. Miller, K.G. and Sollner-Webb, B. (1981) Cell **27**,165-174.
3. Grummt, I. (1981) Nucl. Acids Res. **9**, 6093-6102.
4. Mishima, Y., Yamamoto, O., Kominami, R., and Muramatsu, M. (1981) Nucl. Acids Res. **9**, 6773-6785.
5. Kominami, R., Mishima, Y., Urano, Y., Sakai, M., and Muramatsu, M. (1982) Nucl. Acids Res. **10**, 1963-1979.
6. Grummt, I., Sorbaz, C., Hofmann, A., and Roth, E. (1985) Nucl. Acids Res. **13**, 2293-2304.
7. Tiollais, P., Galibert, F., and Boiron, M. (1971) Proc. Natl Acad. Sci. USA **68**, 1117-1120.

8. Aaronson, S.A. and Todaro, G.J. (1968) Science **162**, 1024-1026.
9. Aaronson, S.A. and Todaro, G.J. (1968) J. Cell. Physiol. **72**, 141-148.
10. Foster, D.N. and Gurney, T. (1976) J. Biol. Chem. **251**, 7893-7898.
11. Kavenoff, R. and Zimm, B.H. (1973) Chromosoma **41**, 1-27.
12. Gurney, T., Sorenson, D.K., Gurney, E.G., and Wills, N.M. (1982) Anal. Biochem. **125**, 80-90.
13. Grummt, I. and Gross, H.J. (1980) Molec. Gen. Genet. **177**, 223-229.
14. Grummt, I. (1982) Proc. Natl Acad. Sci., USA **79**, 6908-6911.
15. Ish-Horowicz, D. and Burke, J.F. (1981) Nucl. Acids Res. **9**, 2989-2998.
16. Drouin, J. (1980) J. Mol. Biol. **140**, 15-25.
17. Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker. H. (1977) Biochemistry **16**, 4743-4751.
18. Tsai, M. and Green, H. (1973) Nature New Biol. **243**, 168-170.
19. Herman, R.C., Williams, J.G., and Penman, S. (1976) Cell **7**, 429-439.
20. Zimmerman, S.B. and Sandeen, G. (1966) Anal. Biochem. **14**, 269-277.
21. Gurney, T. and Gurney, E.G. (1984) In *Methods in Molecular Biology*, J.M. Walker, ed., Vol II, pp 13-20, Humana Press, Clifton, N.J.
22. Gurney, T. and Collard, M.W. (1984) Anal. Biochem. **139**, 25-34.
23. Herz, C., Stavnezer, E., Krug, R.M., and Gurney, T. (1981) Cell **26**, 391-400.
24. Mory, Y.Y. and Gefter, M.L. (1977) Nucl. Acids Res. **4**, 1739-1757.
25. Roeder, R.G. and Rutter, W.J. (1970) Proc. Natl Acad. Sci. USA **67**, 675-682.
26. Zylber, E.A. and Penman, S. (1971) Proc. Natl Acad. Sci. USA **68**, 2861- 2868.
27. Grummt, I. (1975) Eur. J. Biochem. **57**, 159-167.
28. Udvardy, A. and Seifart, K.H. (1976) Eur. J. Biochem. **62**, 353-363.
29. Stanners, C.P. and Becker, H. (1970) J. Cell. Physiol. **77**, 31-41.
30. Bandman, E. and Gurney, T. (1975) Exp. Cell Res. **90**, 159-168.
31. Bayev, A.A., Georgiev, O.I., Hadjiolov, A.A., Nikolaev, N., Skryabin, K.G., and Zakhareev, V.M. (1981) Nucl. Acids Res. **9**, 789-799.
32. Stewart, M.A., Hall, M.C., and Maden, B.E.H. (1983) Nucl. Acids Res. **11**, 629-646.
33. Georgiev, O.I., Nosikov, Braga, E.A., and Hadjiolov, A.A. (1984) Biochem. International **8**, 225-229.
34. Foster, D.O. and Pardee, A.B. (1969) J. Biol. Chem. **244**, 2675-2681.
35. Hadjiolov, A.A. (1980) Subcell. Biochem. **7**, 1-80.
36. Leder, P. (1973) Advan. Protein Chem. **27**, 213-242.