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4.5S RNA Is Encoded by Hundreds of Tandemly Linked Genes, Has a Short Half-Life, and Is Hydrogen Bonded In Vivo to Poly(A)-Terminated RNAs in the Cytoplasm of Cultured Mouse Cells

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4.5S RNA is a group of RNAs 90 to 94 nucleotides long (length polymorphism due to a varying number of UMP residues at the 3′ end) that form hydrogen bonds with poly(A)-terminated RNAs isolated from mouse, hamster, or rat cells (W. R. Jelinek and L. Leinwand, Cell 15:205–214, 1978; F. Harada, N. Kato, and H.-O. Hoshino, Nucleic Acids Res. 7:909–917, 1979). We have cloned a gene that encodes the 4.5S RNA. It is repeated 850 (σ = 54) times per haploid mouse genome and 690 (σ = 59) times per haploid rat genome. Most, if not all, of the repeats in both species are arrayed in tandem. The repeat unit is 4,245 base pairs long in mouse DNA (the complete base sequence of one repeat unit is presented) and approximately 5,300 base pairs in rat DNA. This accounts for approximately 3 × 10^6 base pairs of genomic DNA in each species, or 0.1% of the genome. Cultured murine erythroleukemia cells contain 13,000 molecules per cell of the 4.5S RNA, which can be labeled to equilibrium in 90 min by [^{3}H]uridine added to the culture medium. The 4.5S RNA, therefore, has a short half-life. The 4.5S RNA can be cross-linked in vivo by 4′-aminomethyl-4,5′,8-trimethylpsoralen to murine erythroleukemia cell poly(A)-terminated cytoplasmic RNA contained in ribonucleoprotein particles.

The 4.5S RNA is a low-molecular-weight RNA that was isolated because it forms hydrogen bonds with poly(A)-terminated RNAs from a variety of rodent species (12, 16). Its base sequence is similar to that of the type I *Alu*-like dispersed repeat family (also known as the "B1" sequence) of rodents (11, 13), but it has a region of base sequence not shared with the rodent *Alu*-like sequence and, therefore, is not transcribed from conventional members of that dispersed repeat sequence family.

The function of the 4.5S RNA is unknown, but the following observations deserve consideration. The 4.5S RNA forms hydrogen bonds with purified poly(A)-terminated RNAs, suggesting a function in association with mRNA. Other properties are also consistent with such a function. Two examples have been reported in which changes in the amount of 4.5S RNA per cell accompany changes in mRNA populations. Upon induction of myotube formation in cultured rat myoblasts 4.5S RNA synthesis ceases, coincident with a change in the mRNA population from that characteristic of the growing myoblasts to that of differentiated myotubes (21). Likewise, the amount of 4.5S RNA was observed to decrease during meiotic maturation of mouse oocytes, a time of transition from stored maternal mRNAs to newly synthesized embryonic RNAs (10, 19). Casual observation suggested a short half-life and a relatively low copy number per cell of 4.5S RNA in cultured Chinese hamster ovary cells (W. R. Jelinek, unpublished data), two properties expected of molecules used once or only a few times during their lifetime and of molecules whose abundance in cells could be quickly changed. A short half-life of approximately 30 min has been documented in cultured rat myoblasts (21). To extend these observations, we investigated further the properties of the 4.5S RNA and its genes.

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MATERIALS AND METHODS

Cell growth and labeling. Murine erythroleukemia (MEL) cells were maintained in suspension at a concentration of 5 × 10^5 to 1 × 10^6 cells/ml in Iscove modified Dulbecco medium (15) supplemented with 15% fetal bovine serum. For labeling with [^{3}H]uridine, cells were concentrated to 5 × 10^6 cells per ml and incubated in growth medium supplemented with 100 μCi of [5-^{3}H]uridine (26.5 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml.

Cross-linking of RNA in vivo and photoreversal of the cross-linking. Cross-linking by treatment of cells with 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT; HRI Associates, Inc., Emeryville, Calif.) was achieved as described by Richards et al. (26). Briefly, MEL cells were concentrated by centrifugation and suspended at approximately 10^8 cells per ml in 0.15 M NaCl–0.01 M Tris hydrochloride (pH 7.4) in a Corning T150 tissue culture flask. AMT at a concentration of 1 mg/ml was added to give a final concentration of 20 μg/ml, and the flask was placed on a Chromato-Vue Transilluminator, TL-33 (peak emission at 365 nm; Ultraviolet Products, Inc., San Gabriel, Calif.), in a 4°C cold room. A glass plate was placed between the flask and the illuminator to screen out any stray shortwave UV light. The cells were illuminated for 5 min, after which more AMT was added to give a final concentration of 40 μg/ml, and the cells were again illuminated for 5 min. This process was repeated 10 times for a total of 50 min of illumination and a final concentration of 200 μg of AMT per ml. The cross-linking of RNA molecules was photoreversed by suspending the purified RNA in ETS buffer (0.01 M Tris hydrochloride, pH 7.4, 0.01 M EDTA, 0.2% sodium dodecyl sulfate [SDS]) in a 1.5-ml Eppendorf tube and illuminating the tube for 30 min with short-wavelength UV light from a Minerallight lamp, model R-52 (peak emmission at 254 nm; Ultraviolet Products, Inc.), positioned 3 cm above the tube while it rested on a bed of ice, essentially as described by Calvet and Pederson (6).

Cell-fractionation and RNA purification. MEL cells were

harvested by centrifugation and washed twice in phosphate-buffered saline (160 g of NaCl, 4 g of KCl, 23.6 g of Na_2HPO_4, and 4 g of KH_2PO_4 per liter) followed by a wash in ice-cold iso-hi pH buffer (0.01 M Tris hydrochloride, pH 8.2, 0.14 M NaCl, 0.0015 M Mg_2). The cells were suspended in iso-hi pH buffer, and Nonidet P-40 was added to a final concentration of 0.5%. The cells were homogenized with two strokes of a "B" Dounce homogenizer and the homogenate was centrifuged at 2,000 rpm in the JS4.5 rotor of a Beckman J6 centrifuge. The supernatant was withdrawn as the cytoplasmic fraction. The nuclear pellet was washed once by centrifugation in iso-hi pH buffer containing 0.5% Nonidet P-40, and the wash was added to the first supernatant. The nuclei was lysed by treatment with DNase in high-salt buffer (0.5 M NaCl, 0.01 M Tris hydrochloride, pH 7.4, 0.002 M $CaCl_2$, 0.05 M $MgCl_2$). DNase (electrophoretically pure) was obtained from Worthington Diagnostics (Freehold, N.J.) and treated with iodoacetate according to Zimmerman and Sandeen (31). Nuclear RNA was extracted at 65°C by the SDS/phenol method of Soeiro and Darnell (29). If RNA was to be isolated directly from the cytoplasmic fractions they were added to an equal volume of an equal mixture of phenol and chloroform, shaken at 37°C, and centrifuged to separate the phases. The aqueous phase was removed and repeatedly re-extracted until the interphase was clear. RNA was precipitated by adding 2.5 volumes of 95% ethanol. If the cytoplasmic fraction was to be subfractionated further by centrifugation, it was made 0.01 M in EDTA and 0.5% each in Brij-58 and sodium deoxycholate and layered on 10 to 30% linear sucrose gradients made in 0.01 M Tris hydrochloride (pH 7.4)–0.01 M NaCl–0.01 M EDTA. Fractions from the gradients were collected, made 0.2% in SDS, and RNA precipitated by adding 2.5 volumes of 95% ethanol. The precipitated RNA was suspended and extracted with phenol as described above.

Labeling of RNA and DNA in vitro. DNAs to be used as hybridization probes were labeled in vitro by the oligo-labeling method of Feinberg and Vogelstein (9). Synthetic deoxyoligonucleotides were end labeled in vitro with [^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) and T4 phage polynucleotide kinase. RNA was labeled in vitro by first cloning the appropriate DNA sequences into plasmid SP64 or SP65, which contain the phage SP6 transcription promotor (25). The plasmid DNAs were cleaved once with either *Bam*HI or *Hin*dIII and used as template for the SP6 RNA polymerase in the presence of the [^{32}P]UTP (6,000 Ci/mmol; New England Nuclear). The specific activity of the synthesized RNA was controlled by diluting the labeled UTP with its unlabeled counterpart. Conditions for use of the SP6 RNA polymerase were as specified by the supplier (Promega Biotec, Madison, Wis.).

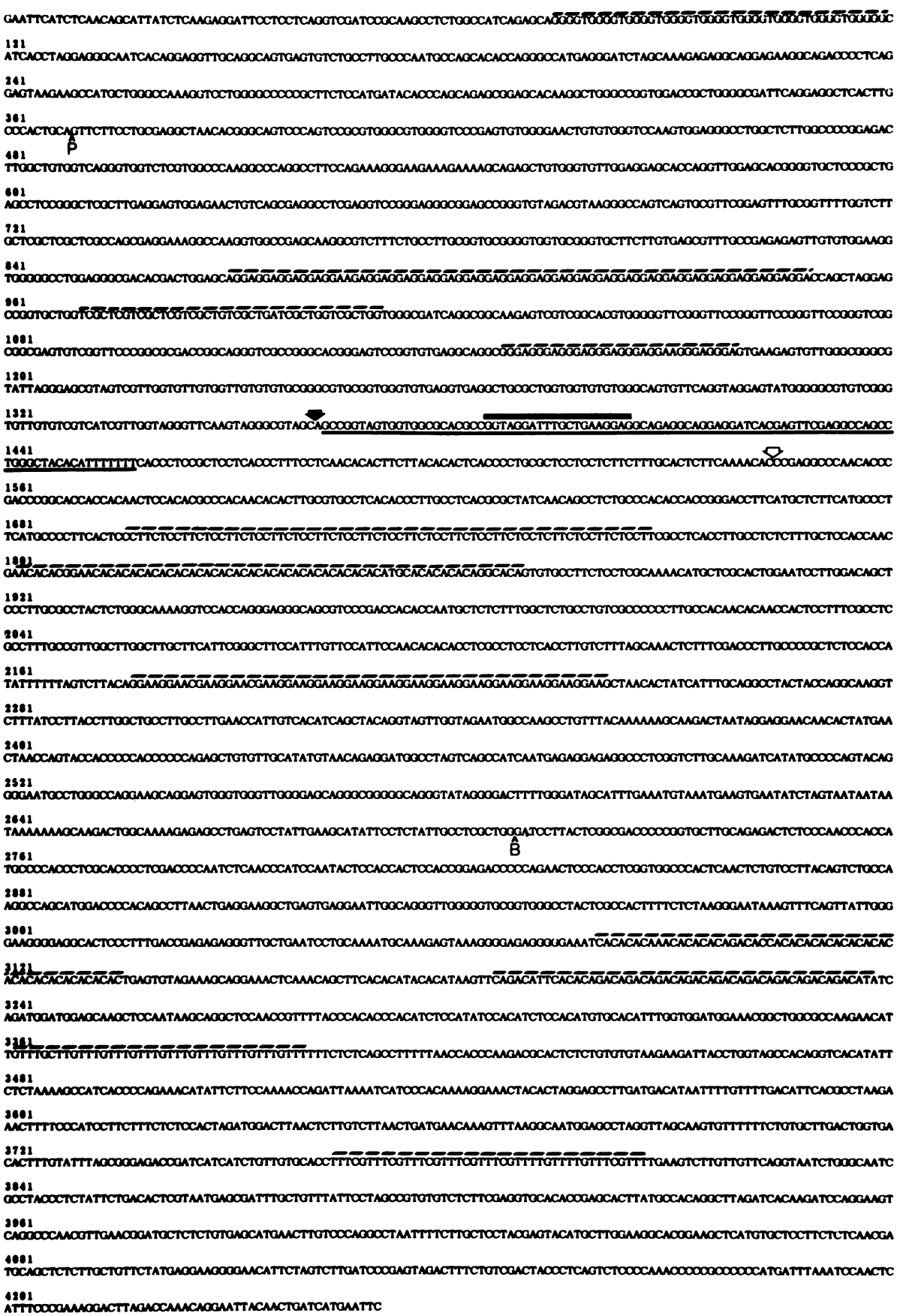
Molecular hybridization. Different hybridization conditions were used depending on the purpose of each particular experiment. For hybridization to DNA fragments separated by agarose gel electrophoresis, the DNA was transferred from the gel to nitrocellulose paper according to Southern (30). Hybridization occurred at 65°C in 0.05 M sodium phosphate (pH 6.5)–0.8 M NaCl–0.001 M EDTA–0.1% SDS–2.5× Denhardt solution–250 μg of denatured herring sperm DNA per ml. Following hybridization the nitrocellulose was washed extensively in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 68°C. For hybridization to RNA that was size fractionated by electrophoresis in polyacrylamide gels, the conditions of electrophoresis and transfer to aminobenzylmethyl (ABM) paper were those suggested by the supplier of the paper (Bio-Rad Laboratories, Richmond, Calif.). Hybridization was carried out at 45°C for 18 h in 2× SSC–0.01 M EDTA–0.1% SDS–0.02 M sodium phosphate (pH 7.5)–10% dextran sulfate–50% formamide–1× Denhardt solution, after which the paper was washed with 0.1× SSC at 72°C. Hybridization to "slot blots" was performed as follows. The nitrocellulose filter was first prehybridized at 55°C for 4 h in 0.05 M sodium phosphate (pH 6.5)–0.8 M NaCl–0.001 M EDTA–0.1% SDS containing 2.5× Denhardt solution and 250 μg of denatured herring sperm DNA per ml. A total of 6.25×10^5 cpm of ^{32}P-end-labeled synthetic oligonucleotide per ml used as a probe to isolate the cloned 4.5S RNA gene (see text) was added, and hybridization was allowed to proceed for 48 h. The filter was washed in 6× SSC containing 0.1% SDS at 45°C and exposed to X-ray film. Samples of in vivo labeled cellular RNA were each hybridized to five nitrocellulose filter disks containing 100 μg of plasmid DNA containing the 4.5S RNA gene. Hybridization occurred in 0.01 M *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0)–0.3 M NaCl–0.01 M EDTA–0.2% SDS at 68°C for 18 h. Following hybridization the filters were washed twice with 2× SSC at 68°C and then treated with 5 U of RNase T_1 and 5 μg of RNase A per ml at 37°C for 30 min. The RNase was eliminated by a 60-min treatment with 5 mg of 30-min predigested nonspecific protease per ml from *Streptococcus griseus*, and the filters were washed again in 2× SSC at 68°C for 15 min. Hybridization of in vitro-labeled 4.5S anti-sense RNA to unlabeled cellular RNA was performed in 30 μl of 80% formamide–0.4 M NaCl–0.04 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)–0.001 M EDTA, containing 4.6×10^{11} molecules (5.2×10^5 cpm) of 4.5S anti-sense RNA and low-molecular-weight cytoplasmic RNA. The hybridization reactions were heated to 90°C for 5 min and then incubated at 45°C for 12 h, after which they were diluted 10-fold with 0.3 M NaCl–0.01 M Tris hydrochloride (pH 7.5)–0.005 M EDTA containing 40 μg of RNase A and 2 μg of RNase T_1 per ml and incubated at 30°C for 1 h. SDS and proteinase K were added to final concentrations of 0.6% and 150 μg/ml, respectively, and incubation was continued at 37°C for 15 min followed by phenol extraction and ethanol precipitation.

Isolation and poly(A)-terminated RNA. RNA samples from which poly(A)-terminated molecules were to be purified were dissolved in ETS buffer, made 0.4 M in NaCl, and applied to a column of AGPOLY(U) Type 6 (Pharmacia Diagnostics, Piscataway, N.J.). The column was washed in ETS buffer and then in ETS buffer containing 15% formamide, and finally the bound RNA was eluted from the column with 70% formamide in ETS buffer.

Immobilization of DNA on nitrocellulose. DNA to be loaded on nitrocellulose disks was diluted to 100 μg/ml in 0.1× SSC and 0.1 volume of 1 M NaOH was added, resulting in a DNA solution with a pH of 13. The solution was kept at room temperature for 15 min, after which 10 ml of 2 M NaCl was added for each initial 0.2 ml, resulting in a solution of pH 11.6. This DNA was applied to nitrocellulose filter disks under suction, and the filters were subsequently washed copiously with 6× SSC and baked at 80°C. DNA to be loaded on nitrocellulose by means of a slot blot apparatus was denatured in NaOH and subsequently neutralized with ammonium acetate as recommended by the manufacturer of the apparatus (Schleicher & Schuell, Inc., Keene, N.H.).

Densitometry of X-ray film. X-ray films were scanned with a laser densitometer. The areas of peaks were determined by computer-assisted integration.

DNA sequence determination. The method of Maxam and Gilbert (23) was used to determine DNA base sequences.



RESULTS

4.5S RNA genes are arranged in a tandem repeating array(s) with a unit of repeat 47 to 60 times longer than the gene itself.

(i) Cloning and base sequence determination of a mouse 4.5S RNA gene. Mouse DNA was cleaved with *Eco*RI and size fractionated by electrophoresis in an agarose gel, and the appropriately sized DNA (T. P. Toomey, Ph.D. dissertation, Rockefeller University, New York, N.Y., 1981) was cloned in Charon 16A lambda phage (3). The resulting phage plaques were screened by hybridization with an end-labeled synthetic oligonucleotide that is perfectly matched to a region of the 4.5S RNA sequence but mismatched with respect to the rodent *Alu*-type sequence, with which the 4.5S RNA shares base sequence similarity (17). The base sequence of the *Eco*RI-generated DNA fragment insert in the selected recombinant phage was determined, and the sequence of the DNA strand of the same sense as the 4.5S RNA is given in Fig. 1. This DNA fragment is a monomer unit of a tandem repeating array. The sequence contains an unusually high number of short tandem repeats (indicated by dashed overlining), some of which are composed exclusively (or almost so) of either purines or pyrimidines asymmetrically distributed to one DNA strand. Others contain alternating purines and pyrimidines. The biological significance of these short tandem repeats is unknown.

The region of the sequence between the arrows in Fig. 1, which contains the coding region for the 4.5S RNA, was subcloned into plasmids containing phage SP6 promotor (24). A *Hin*dIII linker was ligated at the position indicated by the filled arrow and a *Bam*HI linker was ligated at the position indicated by the unfilled arrow. The resulting DNA fragment was cloned into plasmid SP64 (pSP64-4.5S), from which 4.5S sense RNA could be transcribed from the SP6 promoter, and into plasmid SP65 (pSP65-4.5S), from which 4.5S anti-sense RNA could be transcribed. For some experiments the *Hin*dIII- and *Bam*HI-linked fragment was excised, labeled in vitro, and used as a hybridization probe. It is referred to below as the 4.5S RNA gene DNA.

(ii) Tandem repeats of the 4.5S RNA gene in mouse and rat DNA. Mouse genomic DNA was cleaved with *Hin*dIII, which does not cleave within the *Eco*RI fragment whose sequence is given in Fig. 1, *Bam*HI, *Eco*RI, or *Pst*I, each of which has a single cleavage site (Fig. 1). The cleaved DNA was separated in an agarose gel, blotted to nitrocellulose, and hybridized with radiolabeled 4.5S RNA gene DNA. The results are shown in Fig. 2, lanes A to D. The *Hin*dIII-cleaved DNA (lane A) showed a background throughout, due to cross-hybridization with the type 1 *Alu*-like sequence (the film shown was intentionally overexposed to reveal possible minor bands). In addition, a band of approximately 40 kilobases (kb) was seen. The more important observation is that the other three enzymes each yielded a single major band of approximately 4.2 kb. The expected size was 4.245 kb (Fig. 1). If the film was overexposed even more than the one shown in Fig. 2, some minor bands became visible below the 4.245-kb band (not shown). Likewise, rat liver DNA was digested to completion with *Bam*HI (lane J), *Eco*RI (lane K), or *Hin*dIII (lane L) and analyzed. Both *Bam*HI and *Hin*dIII gave a minor band of approximately 5.3 kb and *Eco*RI gave two major bands, one of 5.3 kb and the other of approximately 2.2 kb. Other minor bands are also visible. These data are most easily explained if the majority of the mouse and rat 4.5S RNA genes are organized in tandem repeating arrays with unit lengths of 4.245 and approximately 5.3 kb, respectively. The most common 4.5S RNA genes in rat DNA are polymorphic with respect to *Eco*RI cleavage sites. There may be one *Hin*dIII cleavage site for approximately each 10 repeat units in mouse DNA.

Tandem repeating arrays of DNA sequence can be revealed by partial digestion with a restriction enzyme that cleaves once per repeat unit. Accordingly, mouse DNA was cleaved with *Bam*HI, and five aliquots were removed at various times during the digestion and analyzed for 4.5S RNA sequences as described above. Figure 2, lanes E to I, shows the results. Lane E was loaded with DNA digested for the shortest length of time and showed bands (indicated by arrowheads) at positions expected for the monomer repeat unit (4.245 kb), the dimer, the timer, and the tetramer. With increasing time of digestion (lanes F, G, H, and I) the abundance of the multimers decreased as the abundance of the monomer increased until finally, when digestion was complete (lane I), only the monomer band was visible. Likewise, rat liver DNA was digested with *Hin*dIII and analyzed. Figure 2, lanes M to P, shows the results. DNA digested for the shortest length of time (lane M) showed bands (indicated by arrowheads) at positions expected of the monomer, dimer, and trimer. As digestion proceeded the amount of the multimers decreased and the amount of monomer increased. We conclude from these data that there are multiple copies of the 4.5S RNA gene in both mouse and rat DNA and that for the most part they are arranged in tandem repeating arrays with repeat lengths approximately 47 and 60 times longer than is necessary to encode the 4.5S RNA.

(iii) There are 850 copies of the 4.5S RNA gene per haploid MEL cell genome and 690 copies per haploid rat genome. The method of Kafatos et al. (18) were used to determine the number of copies of the 4.5S RNA gene in MEL cell and rat liver DNAs. Three 10-μg samples of genomic DNA from MEL cells and two 10-μg samples of genomic DNA from rat liver were applied to a nitrocellulose filter by means of a slot blot apparatus. Ten 10-μg samples of HeLa cell DNA, which does not contain the 4.5S RNA gene, were mixed with 10 different amounts of a plasmid DNA containing the cloned 4.5S RNA gene and applied to the nitrocellulose filter in 10 different slots to serve as standards. The HeLa cell DNA was used so that all slots would be loaded with the same quantity of DNA to obviate any potential artifact of hybridization due to variable quantities of DNA per slot. The amount of plasmid DNA added was calculated so that the total amount of 4.5S RNA gene DNA added per slot would be that mass expected in 10 μg of genomic DNA if there

FIG. 1. Base sequence of a monomer unit of the mouse genomic tandem repeat containing the 4.5S RNA gene. The base sequence of an *Eco*RI mouse genomic DNA fragment containing a 4.5S RNA gene is shown. This is a monomer unit of a tandem repeating array. The region of base sequence corresponding to the 4.5S RNA is indicated by solid underlining. The sequence corresponding to a synthesized 20-nucleotide-long oligonucleotide used as a probe to identify this cloned DNA fragment is indicated by the thick solid overlining. The longer regions of tandem repeats of short di- or oligonucleotides are indicated by broken overlining. The downward arrows demarcate a region of DNA that was subcloned into plasmids containing the phage SP6 promotor as described in the text. The positions of the unique *Pst*I and *Bam*HI cleavage sites are indicated, respectively, by "P" and "B."

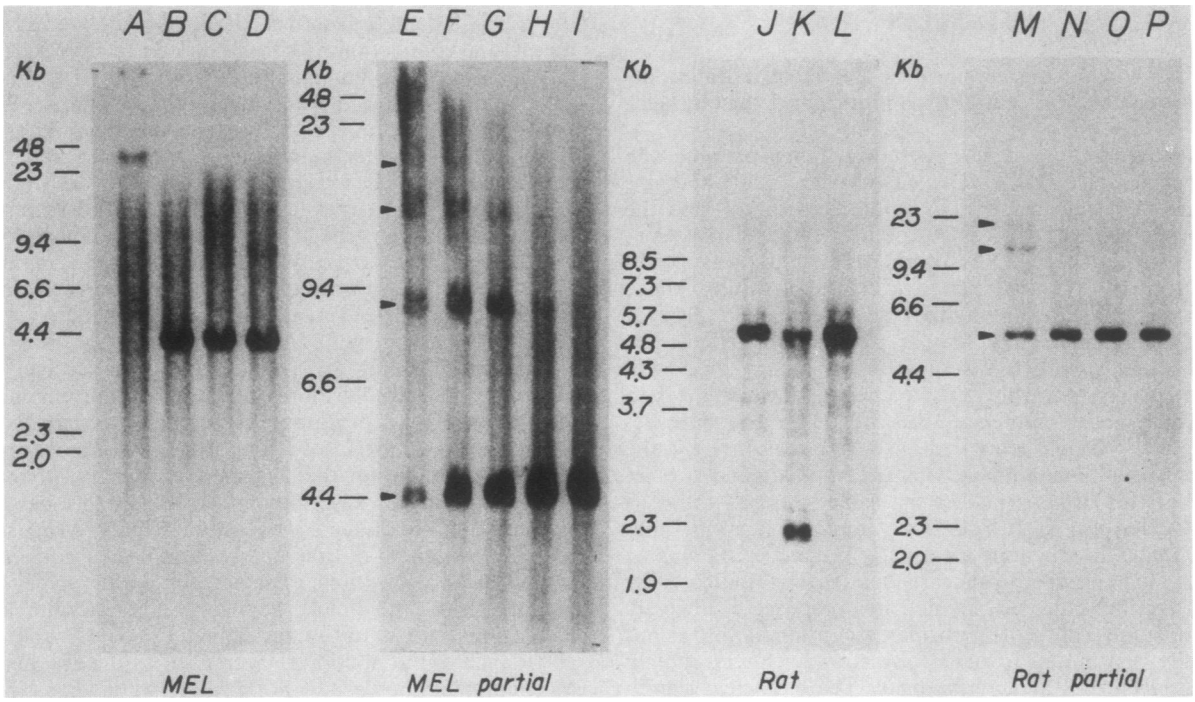


FIG. 2. Organization of 4.5S RNA genes in mouse and rat genomic DNA. (Lanes A to D) Complete digestion of MEL cell DNA. A 2-μg amount of MEL cell DNA was digested to completion with *Hin*dIII (lane A), *Bam*HI (lane B), *Eco*RI (lane C), or *Pst*I (lane D) and subjected to electrophoresis in a 0.8% agarose gel. Following electrophoresis the DNA was transferred from the gel to a sheet of nitrocellulose and hybridized with in vitro labeled 4.5S RNA gene DNA. (Lanes E to I) Partial digestion of MEL cell DNA. MEL cell DNA was digested with *Bam*HI, and samples were removed from the digestion reaction at various times, heated to 65°C, subjected to electrophoresis in a 0.6% agarose gel, transferred to nitrocellulose, and hybridized with in vitro labeled 4.5S RNA gene DNA. Lane E contained DNA digested for the shortest length of time, lane I contained DNA digested for the longest length of time, and lanes F, G, and H contained DNA digested for intermediate lengths of time. Each lane of the gel was loaded with 2 μg of DNA. The arrowheads point to the positions of observable bands of sizes equal to the 4.5S gene monomer, dimer, trimer, and tetramer. (Lanes J to L) Complete digestion of rat DNA. A 2-μg portion of rat liver DNA was digested to completion with *Bam*HI (lane J), *Eco*RI (lane K), or *Hin*dIII (lane L) and analyzed as described for lanes A to D. (Lanes M to P) Partial digestion of rat DNA. Rat liver DNA was digested with *Hin*dIII, and samples were removed from the digestion reaction at various times and analyzed as described for lanes E to I. Lane M contained DNA digested for the shortest length of time, lane P contained DNA digested for the longest length of time, and lanes N and O contained DNA digested for intermediate lengths of time. Each lane of the gel was loaded with 2 μg of DNA. The arrowheads point to the positions of observable bands of sizes equal to the major 4.5S gene monomer, dimer, and trimer. The positions to which markers DNAs are known sizes (in kilobases) migrated in each gel are indicated at the left of each set of gel lanes. Conditions of hybridization are given in Materials and Methods.

were, respectively, 0, 100, 200, 300, 400, 500, 600, 800, 900, or 1,000 copies of the gene per haploid genome. The filter was hybridized with the ^{32}P-end-labeled synthetic oligonucleotide whose sequence is indicated in Fig. 1 and exposed to X-ray film. The film was scanned with a laser densitometer, and the areas under the peaks were integrated. Figure 3 presents the results. Panel a shows a photograph of the X-ray film of the three slots loaded with the MEL cell DNA and the two slots loaded with the rat liver DNA. The densitometric tracing of the film is shown in panel c, with the integrated peak areas given below each peak. Similarly, a photograph of the slots loaded with the standards is shown in panel b, and the corresponding tracings and peak areas are shown in panel d. A direct comparison of the peak areas for the slots loaded with the mouse and rat genomic DNA with those for the slots loaded with the standards allows the determination of the number of copies of the 4.5S RNA gene per haploid genome's worth of DNA. The peak areas for the standards were subjected to a linear regression analysis. When the average peak area for the mouse genomic DNA was compared with this analysis, a value of 850 (σ = 54) copies per haploid genome was calculated. Likewise, a similar comparison for the rat DNA indicated 690 (σ = 59) copies per haploid genome.

The number of 4.5S RNA molecules utilized per cell may exceed the transcriptional capacity of one gene per haploid genome. The similar organization of the 4.5S RNA genes in mouse and rat DNAs in tandem repeats that contain the gene and its flanking sequences suggested that this arrangement is an evolutionarily conserved, functional structure. Similar arrangements have been observed for genes whose products are required in amounts incapable of being supplied by only one copy of the gene per haploid DNA equivalent, e.g., genes for rRNAs (4) and genes that become amplified as a result of environmental conditions requiring larger quantities of the gene's product than can be supplied by a single haploid copy of the gene for the continual life of the cells in such conditions (for review, see reference 27). To determine whether one copy of the 4.5S RNA gene per haploid DNA equivalent is sufficient to supply the required amount of 4.5S RNA per cell, experiments were performed to measure the number of molecules of 4.5S RNA synthesized per cell per unit time and then to calculate the number of genes required to maintain this rate. Two measurements were required, the half-life and the number of molecules of 4.5S RNA per cell.

(i) 4.5S RNA has a short nuclear and cytoplasmic half-life. The half-life of 4.5S RNA was estimated by determining the time required for it to become fully labeled with [^{3}H]uridine

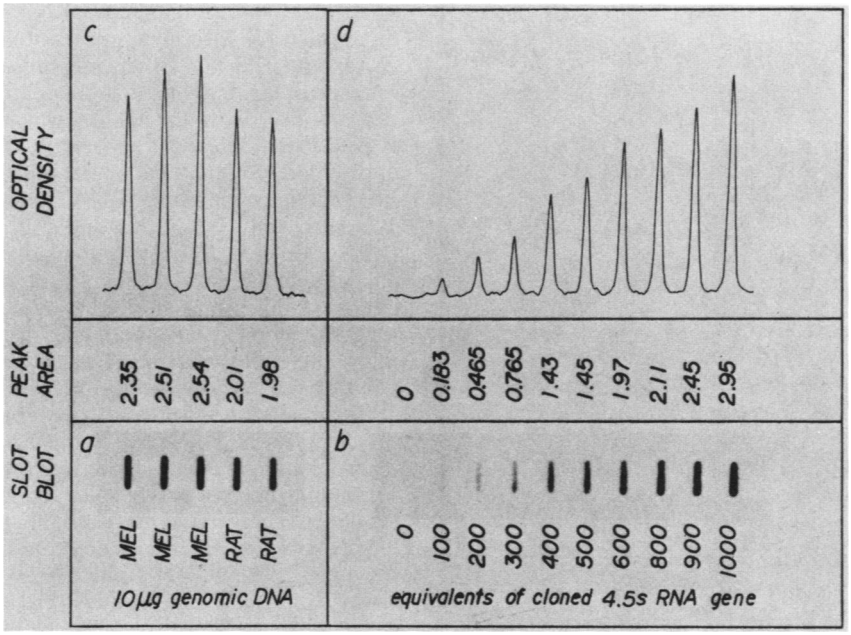


FIG. 3. Determination of the number of 4.5S RNA genes per haploid genome. (a) Three samples of 10 μg each of MEL cell genomic DNA were denatured in 0.8 M NaOH, neutralized by adding an equal volume of 2 M ammonium acetate, and applied to a nitrocellulose filter by means of a Minifold II slot blot apparatus (Schleicher & Schuell). Likewise, two samples of 10 μg each of rat liver genomic DNA were similarly applied. (b) Ten samples of 10 μg each of HeLa cell DNA were mixed with different amounts of a plasmid DNA containing the cloned 4.5S RNA gene and applied to a nitrocellulose filter as indicated in (a). The amounts of the cloned 4.5S RNA gene added were calculated to be equivalent to 0, 100, 200, 300, 400, 500, 600, 800, and 1,000 copies of the gene (as indicated) per haploid genome, which was assumed to be 3.2×10^9 base pairs. The filter was hybridized with the end-labeled synthetic oligonucleotide indicated in Fig. 1 and exposed to X-ray film. The film was scanned with a laser densitometer, and the areas under the peaks were integrated. (c) Densitometer tracing of the X-ray film for slots in (a). (d) Densitometer tracing of the X-ray film for slots in (b). The integrated areas under the peaks shown in (c) and (d) are given.

supplied in the cell growth medium. [^{3}H]uridine was added to a culture of MEL cells; aliquots of cells were withdrawn from the culture at 6, 15, 30, 45, 60, 90, and 120 min after the addition of label and fractionated into nuclear and cytoplasmic fractions, and RNA was isolated from each. The amount of labeled 4.5S RNA was determined by hybridizing the total labeled nuclear or cytoplasmic RNA to excess quantities of the cloned 4.5S RNA gene DNA affixed to nitrocellulose filters. Incorporation of tritium into nuclear 4.5S RNA reached a maximum after approximately 45 min (Fig. 4). During the first 40 min of the labeling period the amount of [^{3}H]uridine in cytoplasmic 4.5S RNA lagged behind that in the nucleus, but then surpassed the nuclear amount and reached a plateau at approximately 90 min.

(ii) 1.3×10^4 molecules of 4.5S RNA per cell. The number of molecules of 4.5S RNA in the cytoplasm of MEL cells was measured by using radioactive 4.5S anti-sense RNA of known specific activity synthesized in vitro from the pSP65-4.5S plasmid as "driver" to hybridize unlabeled RNA isolated from the cytoplasms of known numbers of MEL cells. Total cytoplasmic RNA was isolated from 10^9 MEL cells, denatured, and size fractionated by sucrose gradient sedimentation. The RNA that sedimented at less than approximately 8S was collected and used for hybridization as described below. Nine hybridization reactions were assembled, each containing 4.6×10^{11} molecules of ^{32}P-labeled 4.5S anti-sense RNA at a specific activity of 1.13×10^{-6} cpm per molecule, for a total of 5.2×10^5 cpm per hybridization reaction. The low-molecular-weight cytoplasmic RNA from 0, 7.8×10^4, 1.56×10^5, 3.125×10^5, 6.25×10^5, 1.25×10^6, 2.5×10^6, 5×10^6, or 10^7 MEL cells was added to the various hybridization reactions and hybridization was allowed to

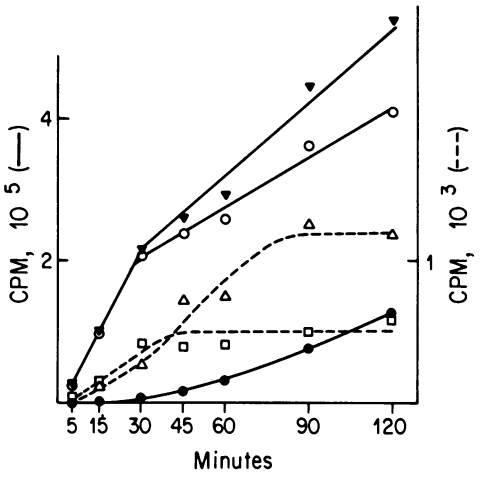


FIG. 4. Time course of 4.5S RNA synthesis in nucleus and cytoplasm. [^{3}H]uridine (26.5 Ci/mmol; New England Nuclear) was added to a final concentration of 100 μCi/ml to a culture of MEL cells, and at the indicated times aliquots of cells were withdrawn and fractionated into nuclei and cytoplasm, and RNA was isolated from each fraction as described in Materials and Methods. Symbols: ●, incorporation into 1% of total cytoplasmic RNA; ○, incorporation into 1% of total nuclear RNA; ▼, sum of the counts per minute incorporated into 1% of nucler and cytoplasmic RNA; □, filter-bound counts of nuclear RNA; △, filter-bound counts of cytoplasmic RNA. One-half of the RNA from each sample was hybridized to nitrocellulose filters bearing plasmid DNA containing the cloned 4.5S RNA gene DNA. Following hybridization the filters were processed as described in Materials and Methods, and filter-bound counts per minute were determined by scintillation spectroscopy.

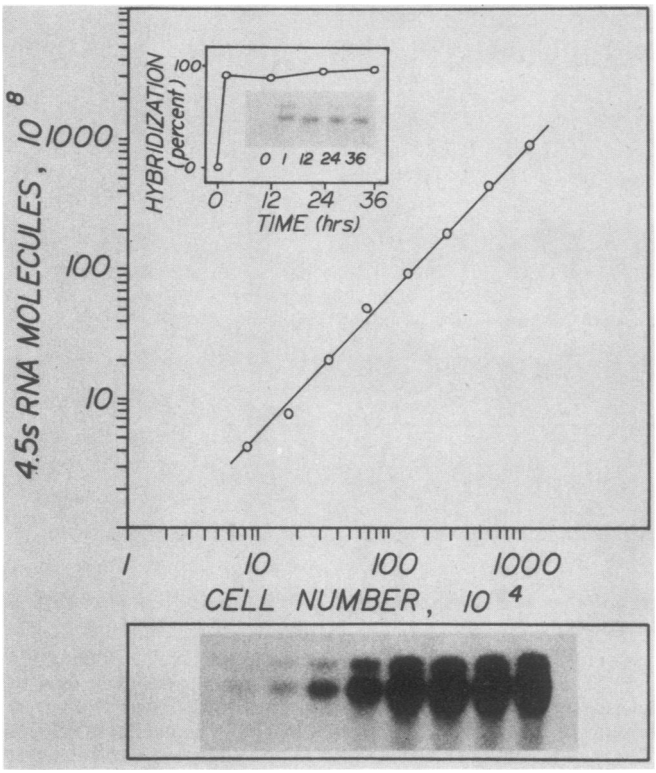


FIG. 5. Determination of the number of 4.5S RNA molecules per MEL cell in the cytoplasm. Radiolabeled 4.5S anti-sense RNA was synthesized in vitro from SP65-4.5S plasmid DNA, using a mixture of [^{32}P]UTP and unlabeled UTP to give a specific activity of 1.13×10^{-6} cpm per molecule. The RNA was purified by preparative electrophoresis in a polyacrylamide gel, and 4.6×10^{11} molecules (5.2×10^5 cpm) were used in separate reactions to drive the hybridization of low-molecular-weight cytoplasmic RNA from 0, 7.8×10^4, 1.56×10^5, 3.125×10^5, 6.25×10^5, 1.25×10^6, 2.5×10^6, 5×10^6, or 10^7 MEL cells as described in Materials and Methods. The hybrids were digested with RNase and subjected to electrophoresis in a 10% polyacrylamide–8 M urea gel. The gel was exposed to X-ray film, the film was scanned with a laser densitometer, and the areas under the peaks were integrated. The areas were converted to numbers of molecules by comparison to the areas obtained from scanning gel lanes loaded with known numbers of molecules of the radiolabeled, unhybridized, undigested 4.5S anti-sense RNA used as hybridization "driver" that were loaded on the gel as calibration standards for the densitometry. A correction factor of 19/43 was applied because there were 43 [^{32}P]UMP residues per molecule of the 4.5S anti-sense driver RNA but only 19 residues of [^{32}P]UMP per molecule of the RNase-resistant, hybridized, 4.5S anti-sense RNA. The graph shows the number of molecules of 4.5S RNA as a function of the number of cell's worth of low-molecular-weight RNA input to the hybridization. A photograph of the X-ray film is positioned below the graph with the appropriate lanes aligned with the abscissa. (Inset) Time course of hybridization. A hybridization reaction was assembled as described above with low-molecular-weight RNA from the cytoplasm of 5×10^5 MEL cells. At the indicated times aliquots were removed and processed as described above. The percent maximal hybridization is plotted as a function of time of the hybridization reaction. The inset to the inset is a photograph of the radioautograph that was scanned to obtain the plot. The time of hybridization is indicated below each lane of the gel.

proceed for 12 h, after which the hybrids were treated with RNase and assayed by polyacrylamide gel electrophoresis. Figure 5 shows a plot of the number of cells worth of RNA input to the hybridization (abscissa) versus the number of molecules of 4.5S anti-sense RNA protected from RNase digestion (ordinate). As expected, this is a linear relationship consistent with the driver anti-sense RNA being in excess for each of the amounts of input cellular RNA. A photograph of the X-ray film of the gel that was traced to obtain the data is positioned below the plot so the gel lanes align with the appropriate designations on the abscissa. For the hybridization reactions containing RNA from 2.5×10^6, 5×10^6, and 10^7 cells, only 0.5, 0.25, and 0.125, respectively, of the RNase-resistant RNA was loaded on the gel, so that the capacity of the X-ray film would not be exceeded under exposure times that allowed imaging of the lanes from the reactions with lower amounts of input cell RNA. Accordingly, the values obtained from tracing the film were multiplied by the appropriate factor before being plotted.

It can be seen that two closely spaced bands appeared in the X-ray film. The sizes of the RNA in both bands were determined by subjecting them to electrophoresis in a polyacrylamide gel that was also loaded with five Maxam-Gilbert sequence determination reactions performed on an end-labeled DNA fragment of known base sequence. The size of the upper band was determined to be 106 nucleotides and the lower band was 89 nucleotides. The size of the labeled input 4.5S anti-sense RNA was 195 nucleotides (Fig. 1). RNase T_1 fingerprints of the RNA in the two bands were identical (data not shown). Therefore, the sum of the RNA in both bands was used in the plot of Fig. 5. The number of molecules of 4.5S RNA per cell clytoplasm can be calculated by dividing the value of the ordinate for each point on the curve by its corresponding value on the abscissa. Accordingly, there are 9.2×10^3 molecules per cell of 4.5S RNA in MEL cell cytoplasm. Since the cytoplasm contains approximately 70% of the cellular 4.5S RNA and the nucleus contains the other 30% (Fig. 4), the total number of molecules of 4.5S RNA per cell is 1.3×10^4.

The measurements given above depend on the labeled driver RNA being in excess so that the hybridizations were driven to completion. Two variables of the hybridization can be independently adjusted to attempt to force the reaction to completion. Either the concentration of the reactants or the time duration of the hybridization reaction can be varied. The time course of hybridization was monitored by assembling a hybridization reaction containing the same amount of labeled driver 4.5S anti-sense RNA used in the experiment of Fig. 5 and low-molecular-weight cytoplasmic RNA (the same RNA preparations used in the experiment of Fig. 5) from 5.2×10^5 cells and withdrawing aliquots for analysis after various times of hybridization. The inset of Fig. 5 shows that after 1 h of hybridization essentially the maximum amount of the labeled driver 4.5S anti-sense RNA was converted to RNase resistance. Therefore, the hybridizations of the previous experiment that proceeded for 12 h must have gone to completion.

The number of molecules of 4.5S RNA synthesized per unit time can be calculated from the measurements described above. The time at which equilibrium of labeling of the 4.5S RNA occurred represents the time required to replace all unlabeled 4.5S RNA molecules present in the cells by newly synthesized, labeled molecules, i.e., the time required to completely turn over the cellular pool of 4.5S RNA molecules. This was approximately 90 min (Fig. 4). The number of molecules of 4.5S RNA per cell is 1.3×10^4 (Fig. 5). Therefore, 1.3×10^4 molecules are synthesized per cell per 90 min, or 2.4 molecules per s per cell.

The 4.5S RNA is synthesized by RNA polymerase III (21). The in vivo elongation rate of RNA polymerase III is

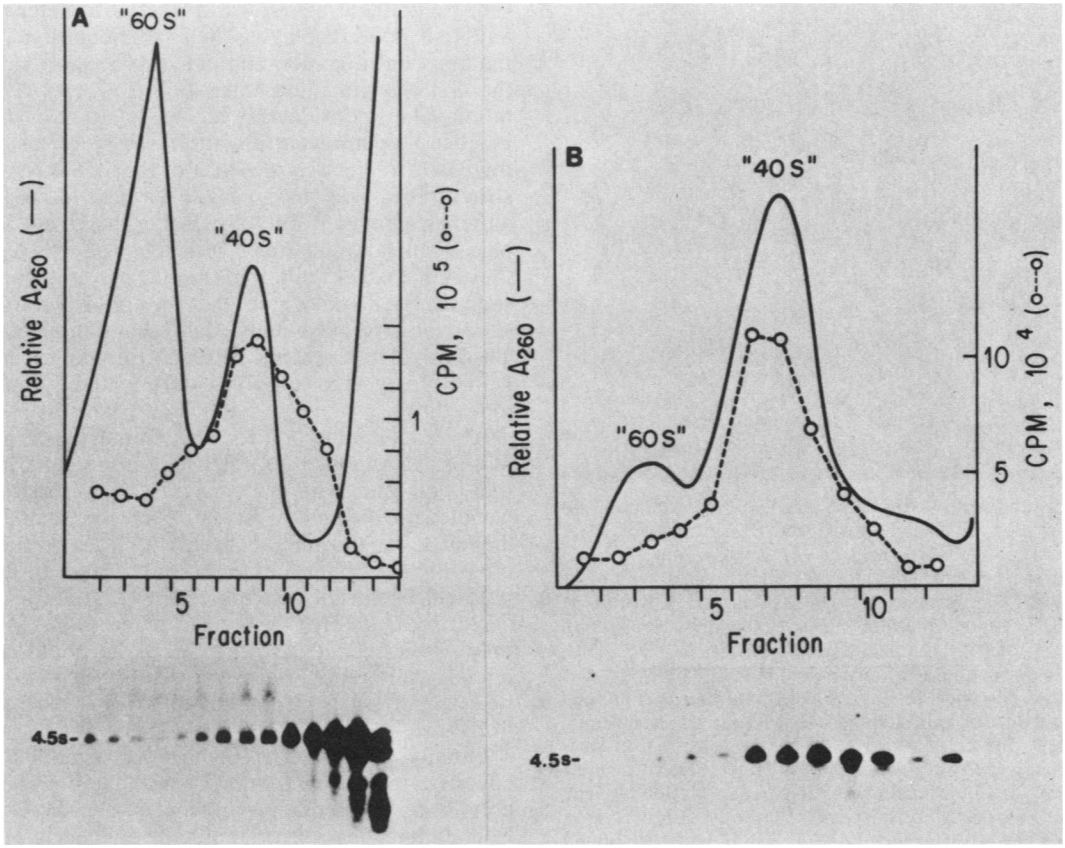


FIG. 6. Sedimentation analysis of 4.5S RNA from non-phenol-extracted cytoplasmic fraction of MEL cells. (A) The crude cytoplasm from 6×10^8 MEL cells (see Materials and Methods) was sedimented in two sucrose gradients. Fractions were collected from each gradient through a flow cuvette continuously monitored for A_{260}, and RNA was isolated from each fraction of one of the two gradients by phenol extraction. An aliquot of each fraction was subjected to electrophoresis in a 15% polyacrylamide–8 M urea gel, electrotransferred to ABM paper, and hybridized with in vitro labeled 4.5S RNA gene DNA as described in Materials and Methods. A second aliquot from each gradient fraction was denatured in formamide-formaldehyde and spotted onto nitrocellulose disks, which were subsequently hybridized with ^{32}P-end-labeled oligo(dT) to detect poly(A). (Upper) Solid line, relative A_{260}. Broken line, Counts per minute of oligo(dT) hybridized. The positions to which the "60S" and "40S" ribosomal subunits sedimented are indicated. (Lower) Radioautograph of the ABM paper hybridized with 4.5S RNA gene DNA. Each lane of the gene is aligned below its corresponding fraction from the sucrose gradient. (B) Immediately after collection, fractions 6 to 10 from the sucrose gradient centrifuged in parallel with the one analyzed in (A) were pooled, diluted, and layered on another 15 to 30% sucrose gradient, and analyzed for 4.5S RNA and poly(A) as described above. (Upper) Solid line, Relative A_{260}. Broken line, Counts per minute of oligo(dT) hybridized. The positions to which "60S" and "40S" ribosomal subunits sedimented are indicated. (Lower) Radioautograph of the ABM paper hybridized with the 4.5S RNA gene. Each gel lane is aligned with the sucrose gradient fraction from which the RNA with which it was loaded was isolated.

unknown, but if it is assumed to have an elongation rate equal to that measured for RNA polymerase II, which is approximately 50 to 100 nucleotides per s (28), it would take between 1 and 2 s to synthesize one molecule of 4.5S RNA from one gene. In a diploid cell containing one copy of the 4.5S RNA gene per haploid DNA equivalent, this would be between 1 and 2 molecules synthesized per s, a rate slightly slower than that required to meet the rate of 2.4 molecules per s calculated from the data given above. Therefore, it appears that one copy of the gene per haploid DNA equivalent may just meet the required synthesis rate. However, these considerations have not taken into account the possibility that initiation might be a rate-limiting step in transcription. If it is, and it is reasonable to assume that it is, then more than one copy of the gene would be required. The 850 (σ = 54) gene copies measured for MEL cell DNA seems excessive. However, the synthetic rates might be greater during the ontogeny of a complete organism than it is in cultured MEL cells, and a larger number of genes might be required.

4.5S RNA is associated with poly(A)-terminated RNAs in vivo. The function of the 4.5S RNA is unknown, but the strongest evidence currently available suggests that it may function in concert with poly(A)-terminated RNA molecules because it has been isolated hydrogen bonded to them. However, all demonstrations of hydrogen bonding of 4.5S RNA with poly(A)-terminated RNA have been made with cellular RNA after it was purified by phenol extraction, a process known to favor hydrogen bond formation between nucleic acids of complementary base sequence (20). We therefore sought evidence of such base pairing in vivo.

(i) Sedimentation analysis of 4.5S RNA from crude cytoplasmic extracts of MEL cells. If 4.5S RNA were hydrogen bonded to poly(A)-terminated RNA in vivo, it should sediment with poly(A) in ribonucleoprotein particles that have sedimentation values substantially larger than that of the 4.5S RNA itself. To test this, the crude cytoplasmic fraction was prepared from MEL cells and sedimented in two sucrose gradients as described in Fig. 6. Following centrifugation, the gradients were fractionated, and RNA was isolated

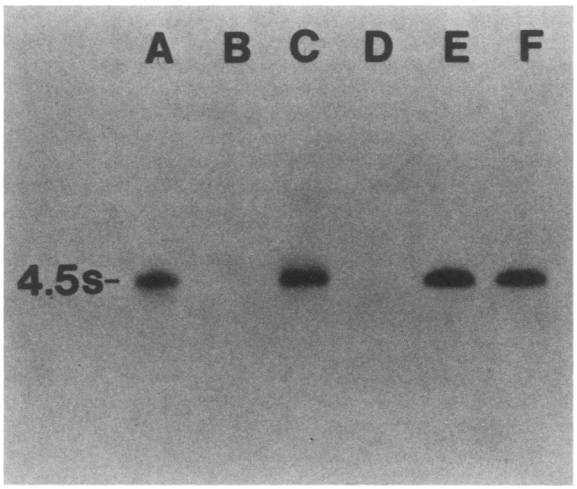


FIG. 7. Cross-linking in vivo of 4.5S RNA to MEL cell poly(A)-terminated cytoplasmic RNA. (Lanes A and B) Cytoplasmic RNA was isolated from 6×10^8 MEL cells as described in Materials and Methods and divided in half. One half was denatured by heating at 65°C for 5 min in 50% formamide–0.01 M Tris (pH 7.4)–0.01 M EDTA–0.2% SDS, diluted 100-fold in poly(U)-agarose application buffer (0.01 M Tris, pH 7.4, 0.2 M NaCl, 0.01 M EDTA, 0.2% SDS), and subjected to chromatography on a column of poly(U)-agarose as described in Materials and Methods. The second half was applied directly to a column of poly(U)-agarose without an intervening denaturation step. The column-bound RNA was subjected to electrophoresis in a 15% polyacrylamide–8 M urea gel, electrotransferred to ABM paper, and hybridized with radiolabeled 4.5S RNA gene DNA as described in Materials and Methods. Shown is a radioautograph of the ABM paper. (Lane A) Poly(U)-agarose-bound RNA not denatured before poly(U)-agarose chromatography. (Lane B) Poly(U)-agarose-bound RNA denatured as described above before poly(U)-agarose chromatography. (Lanes C and D) 10^9 MEL cells were treated with AMT and long-wavelength UV light to cross-link nucleic acids as described in Materials and Methods. Cytoplasmic RNA was isolated by phenol extraction, subjected to the denaturation regimen described above, and applied to a column of poly(U)-agarose. The column-bound RNA was divided in half. One half was treated with short-wavelength UV light, to effect a photoreversal of the cross-linking. The cross-linking in the other half was not photoreversed. The RNAs were subjected to electrophoresis in a 15% polyacrylamide–8 M urea gel, electrotransferred to diazobenzyloxymethyl paper, and hybridized with radiolabeled 4.5S RNA gene DNA. (Lane C) Poly(U)-agarose-bound RNA in which the cross-linking was photoreversed before electrophoresis. (Lane D) Poly(U)-agarose-bound RNA in which the cross-linking was not photoreversed before electrophoresis. (Lanes E and F) 2% of the flowthrough fraction from the poly(U)-agarose column was divided in half. On half was treated with short-wavelength UV light to photoreverse the cross-linking. The cross-linking was not photoreversed in the other half. Both halves were subjected to electrophoresis in the 15% polyacrylamide–8 M urea gel, electrotransferred to ABM paper, and hybridized with the radiolabeled 4.5S RNA gene DNA. (Lane E) Poly(U)-agarose flowthrough RNA in which the cross-linking was photoreversed before electrophoresis. (Lane F) Poly(U)-agarose flowthrough RNA in which the cross-linking was not photoreversed before electrophoresis. Conditions for hybridization and washing of paper are given in Materials and Methods.

from each fraction of one gradient by phenol extraction and assayed for poly(A) and 4.5S RNA (Fig. 6A). Fractions of the parallel sucrose gradient in the region where the poly(A)-terminated RNA was expected, and subsequently demonstrated, to sediment in ribonucleoprotein particles were pooled, immediately diluted, and centrifuged in a second sucrose gradient which was analyzed to determine whether any 4.5S RNA displaying high sedimentation values during the first centrifugation continued to do so (Fig. 6B). During the first centrifugation some 4.5S RNA displayed sedimentation values considerably higher than that of the 4.5S RNA itself and, upon recentrifugation, sedimented similarly. That there was a selective presence of the 4.5S RNA in the larger structures is indicated in Fig. 6A where it can be seen that, although a number of RNAs smaller than the 4.5S RNA have base sequence homology with the 4.5S RNA gene probe, they remained at the top of the sucrose gradient. These data suggest, but do not prove, that the 4.5S RNA was part of the same ribonucleoprotein particles that contained the mRNA. Therefore, experiments were performed to cross-link the 4.5S RNA in vivo to poly(A)-terminated cytoplasmic RNA molecules.

(ii) Cross-linking of 4.5S RNA to poly(A)-terminated cytoplasmic RNA in vivo. Psoralens are furocoumarin derivatives that photoreact with nucleic acids when irridated with long-wavelength UV light to give products that include mono- and diadducts to pyrimidine bases (25). Diaddition results in cross-linking by the formation of covalent bridges between base-paired nucleic acid molecules (1, 2, 7, 8). The psoralen derivative AMT (14) was used to cross-link 4.5S RNA to poly(A)-terminated RNAs in vivo. Its photo-cross-links to nucleic acids can be reversed by exposure to short-wavelength UV light, thereby allowing subsequent analyses of the cross-linked RNA species.

Initially, a routine procedure was established that was effective in removing non-cross-linked 4.5S RNA from poly(A)-terminated RNA molecules. Cytoplasmic RNA was isolated by phenol extraction and divided in half. One half was heated at 65°C in 50% formamide for 5 min, diluted 100-fold in buffer for application to the poly(U)-agarose column, and applied to the column. The second half was dissolved directly in poly(U)-agarose application buffer, without any intervening treatment that might disrupt hydrogen bonds, and applied to and eluted from the column. The bound RNAs were subjected to electrophoresis in a 15% polyacrylamide–8 M urea gel, electroblotted to ABM paper, and hybridized with radiolabelled 4.5S RNA gene DNA. If the RNA was not melted before application to the poly(U)-agarose column, the 4.5S RNA was present in the column-bound fraction (Fig. 7, lane A) as previously described (11, 16). If the RNA was melted before application to the column, the 4.5S RNA could not be detected in the bound fraction (Fig. 7, lane B). The melting procedure was effective.

Next, the cross-linking of 4.5S RNA to poly(A)-terminated RNA in vivo was demonstrated. If the 4.5S RNA and poly(A)-terminated RNA molecules are hydrogen bonded in vivo, the AMT should cross-link them and their association should become resistant to procedures that disrupt hydrogen bonds. MEL cells were treated with AMT while they were illuminated with long-wave UV light. Following the AMT-UV light treatment, cytoplasmic RNA was isolated by phenol extraction, subjected to the 50% formamide-65°C melting treatment described above, diluted 100-fold in poly(U)-agarose loading buffer, and applied to a column of poly(U)-agarose. The column-bound RNA was divided in half and one half was exposed to short-wavelength UV light to reverse the photo-cross-linking. The other half was kept in the dark. Each half was assayed for the 4.5S RNA as described above. If the column-bound RNA was treated to reverse the photo-cross-linking, the 4.5S RNA was detected (Fig. 7, lane C). If the photo-cross-linking was not reversed, no 4.5S RNA could be detected (Fig. 7, lane

D), presumably because it was cross-linked to high-molecular-weight poly(A)-terminated RNAs that were too large to enter the small-porosity polyacrylamide gel used for the detection. These results imply that some cytoplasmic 4.5S RNA is associated closely enough with poly(A)-terminated molecules in vivo, most likely by hydrogen bonding, to become cross-linked to them. Two aliquots of the RNA in the flowthrough fraction of the poly(U)-agarose column were examined for cross-linking. One aliquot was treated with short-wavelength UV light to reverse the photo-cross-linking; the other was kept in the dark. Both were assayed for the 4.5S RNA. There was no apparent difference between the samples (Fig. 7, lanes E and F). Apparently the majority of the 4.5S RNA not hydrogen bonded with poly(A)terminated molecules is also not associated closely enough with other RNA molecules to become cross-linked to them.

(iii) Cross-linking in vivo of 4.5S RNA to poly(A)-terminated cytoplsmic RNA in ribonucleoprotein particles. MEL cells were treated with AMT and long-wavelength UV light, and the crude cytoplasm was sedimented in a sucrose gradient. Figure 8A shows the optical density profile of the gradient. The material that sedimented at approximately 40S was collected ("pool A," Fig. 8A), diluted, and immediately layered on a second sucrose gradient. The material that sedimented at the top of the gradient ("pool B," Fig. 8A) was also collected and RNA was isolated from it by phenol extraction. Figure 8B shows the optical density profile of the second sucrose gradient, which was fractionated into pool I, pool II, and pool III as indicated, and poly(A)-terminated RNA was isolated from each pool by phenol extraction followed by heating at 65°C in 50% formamide and chromatography on a column of poly(U)-agarose as described above. An aliquot of the column-bound RNA from each pool was treated with short-wavelength UV light to photoreverse the cross-linking, subjected to electrophoresis in a polyacrylamide gel, electrotransferred to ABM paper, and hybridized with radiolabeled 4.5S RNA gene DNA. An aliquot of the RNA in pool B from the first sucrose gradient was also loaded on the gel. The results are shown in Fig. 8C. The 4.5S RNA was present in each of the pooled fractions from the second sucrose gradient. Therefore, the reason that some of the cytoplasmic 4.5S RNA displayed high sedimentation values in the preceding sedimentation analysis (Fig. 6) was because it was hydrogen bonded to poly(A)-terminated RNAs present in ribonucleoprotein particles. The sedimentation analysis of non-cross-linked, cytoplasmic 4.5S RNA described above (Fig. 6) provides a control for the current experiment by demonstrating that the cross-linking step did not artifically create the interactions between 4.5S RNA and poly(A)-terminated RNAs observed here. Some RNAs were present at the top of the first sucrose gradient (pool B) that migrated in the polyacrylamide gel faster than the 4.5S RNA and hybridized with the 4.5S RNA gene (Fig. 8). As in the sedimentation analysis of the non-cross-linked cytoplasmic 4.5S RNA (Fig. 6), they were not present in ribonucleoprotein particles with high sedimentation values, as was the authentic 4.5S RNA. This fact confirms the specificity of the interaction between 4.5S RNA and poly(A)-terminated RNAs and strengthens the argument that the cross-linking step did not artificially create RNA-RNA interactions that did not already exist in the cells at the time of the cross-linking.

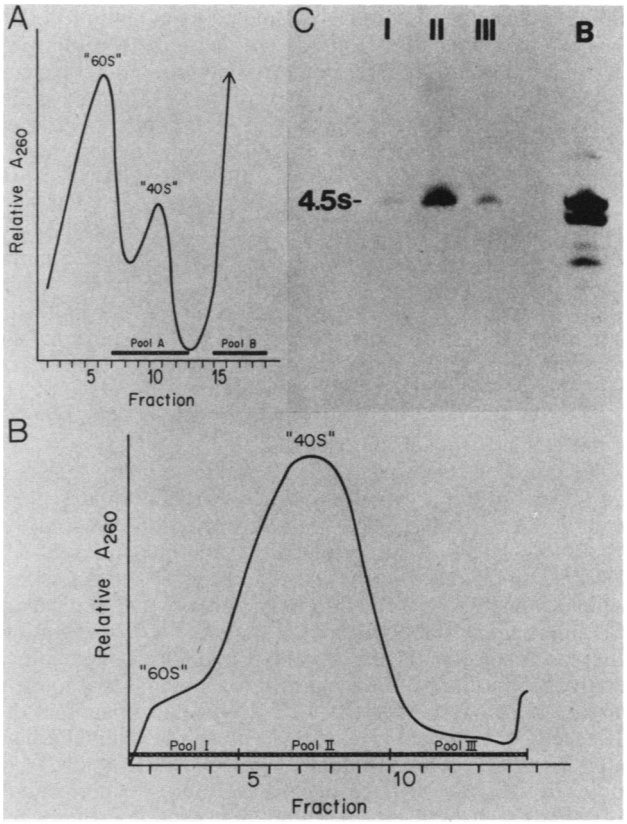


FIG. 8. Cross-linking in vivo of 4.5S RNA to cytoplasmic poly(A)-terminated RNA in ribonucleoprotein particles. A total of 10^9 MEL cells were treated with AMT and long-wavelength UV light as described in Materials and Methods to cross-link nucleic acids. Following the cross-linking regimen the cells were disrupted with Nonidet P-40, and the cytoplasmic fraction was subjected to centrifugation in sucrose gradients as described in the legend to Fig. 6. The gradients were fractionated by pumping from the bottom through a flow cuvette continuously monitored at 260 nm. (A) Relative optical density and indication of two regions of the gradient that were pooled for subsequent use, "pool A" and "pool B." RNA was isolated from pool B by phenol extraction and used as a marker for polyacrylamide gel electrophoresis described in (C). Pool A was diluted and layered on a second sucrose gradient as described in the legend to Fig. 6. (B) Optical density tracing of the second sucrose gradient and indication of three regions of the gradient that were pooled, "pool I," "pool II," and "pool III," for RNA extraction. RNA was isolated from each pool by phenol extraction and heated to 65°C in 50% formamide, and poly(A)-terminated RNA was selected by chromatography on a column of poly(U)-agarose as described in Materials and Methods. The column-bound RNA was exposed to short-wavelength UV light to photoreverse the cross-linking, and one-fourth of the RNA was subjected to electrophoresis in a 13% polyacrylamide–8 M urea gel, electrotransferred to ABM paper, and hybridized with radiolabeled 4.5S RNA gene DNA. (C) Radioautogram of the hybridized ABM paper. Lanes I, II, and III were loaded, respectively, with the poly(U)-agarose-bound RNA from pools I, II, and III of the sucrose gradient indicated in (B). Lane B was loaded with RNA from pool B of the sucrose gradient indicated in (A).

DISCUSSION

This work elucidates a number of characteristics of the 4.5S RNA and its genes. The genes for the 4.5S RNA, for the most part, are organized in a tandem repeating array(s) in both mice and rats. Casual observation suggested that this arrangement is also true of the 4.5S RNA genes in Chinese hamster DNA, but Chinese hamster DNA has not been investigated in sufficient detail to confirm this idea. The number of genes is approximately the same and they are

similarly organized in the DNA isolated from cultured MEL cells and in that isolated from rat liver immediately following sacrifice of the rat. Therefore, the repetition and organization of these genes are not aberrations of cultured cells. Most, if not all, of the 4.5S RNA genes in both species are present in DNA restriction fragments of uniform length generated by different restriction endonucleases that cleave once per unit repeat, and we have demonstrated that at least some of these are tandemly repeated. We do not know whether all of the unit-length DNA fragments that contain the 4.5S RNA gene are tandemly linked. Preliminary mapping data for the 4.5S RNA genes in mouse DNA indicates that most, if not all, are located on chromosome 6. We therefore think it likely that they are all tandemly linked at a single locus. The 850 (σ = 54) copies of the unit repeat (4,245 base pairs) in mouse DNA would occupy approximately 2% of the DNA of this chromosome.

The question arises as to why there are so many genes for the 4.5S RNA but only a relatively low number of molecules of the RNA per cell (13,000). For a comparison, we consider U1 RNA. There are approximately 10^6 molecules of U1 RNA per cell (5) and approximately 30 copies of the gene per haploid human genome (24). The major, obvious physiological difference in the two RNAs is that the U1 RNA is stable whereas the 4.5S RNA is unstable and turns over with a relatively short half-life. One line of reasoning is that to meet the high turnover rate of the 4.5S RNA and to maintain the observed cellular pool size, it must be synthesized at a high rate. To achieve this, rodents have evolved hundreds of copies of the gene. With respect to this idea, it is germane to note that the structure of the tandem repeating array of the 4.5S RNA genes is similar to those of other genes that have become stably "amplified" in the DNA of cultured cells placed under selection for increased production of particular gene products (27). The reason for the high turnover rate of 4.5S RNA is unknown, but we expect that its turnover is closely related to its function. Perhaps while performing its function it is destroyed. Each molecule might therefore be used only once before it is degraded.

The function of the 4.5S RNA remains unknown. That it does perform a function is suggested by its strong base sequence conservation, its relatively constant gene copy number, and its conserved gene organization among different rodent species. Although the 4.5S RNA shares much of its base sequence with the rodent type 1 *Alu*-like sequence family, it is unlike that family in that its genes are clustered and generally not dispersed throughout the genome as are the *Alu*-like sequences. It therefore cannot be classified as a "retroposon" as can the *Alu*-like sequences. The initial description of the 4.5S RNA demonstrated its base pairing with poly(A)-terminated RNAs that had been isolated from cells by phenol extraction (12, 16). This led to the suggestion that 4.5S RNA might function in concert with mRNA molecules. The demonstration here of the cross-linking of 4.5S RNA to poly(A)-terminated RNAs by AMT in live cells strengthens that suggestion.

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