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Molecular cloning and *in vitro* transcription of rat 4.5S RNA_H genes

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ABSTRACT

4.5S RNA_H (4.5S RNA associated with poly A containing RNA) has extensive homology to major interspersed repeat B1 in rodent genomes. We developed a new cloning technique for screening genomic library that eliminates the signal produced by repeated sequences or pseudogenes and applied it to cloning of 4.5S RNA_H genes. Six phage clones (2, 3, 6, 9, 10 and 15) which hybridize with 4.5S RNA_H were isolated from a rat gene library by this method. The restriction fragments containing the 4.5S RNA_H locus were subcloned into plasmids and sequenced. Clones 2, 3, 9 and 15 contained one to five base substitutions in the coding region for 4.5S RNA_H and were probably pseudogenes. In clone 2, the 4.5S RNA_H locus was linked directly with the identifier sequence. Clone 6 contained three copies of the 4.5S RNA_H gene (6a, b and c) which were clustered in the same direction within 455 base pairs. 6b was linked directly with 6c and ubiquitous repetitive DNA sequences B2 were inserted immediately after 6a and 6c. These three sequences as well as the sequence in clone 10 were colinear with rat 4.5S RNA_H. In an *in vitro* transcription system, only clone 10 gave intact 4.5S RNA_H.

INTRODUCTION

4.5S RNA_H, one of the small nuclear RNAs (snRNAs), has been isolated from rodent cells, Moloney murine leukemia virus and Friend spleen focus forming virus (1-4). This RNA is specifically associated with viral genomic RNAs (1,3) and with poly(A)-containing RNAs of mouse, rat and hamster cells (2-4). However, it has not been found in human, monkey, cat, mink, rabbit or chicken cells (4). 4.5S RNA_H is also associated with protein and this ribonucleoprotein complex is precipitated by anti-La antibody from serum of patients with systemic lupus erythematosus (5).

The total nucleotide sequences of 4.5S RNA_H from mouse and hamster cells have been determined (6,7) and found to have extensive homology to interspersed repeated sequences in rodent genomes, the so called B1 family or type 1 Alu-equivalent sequences (7-9). Although several functions have been suggested from circumstantial evidence, the exact functions of 4.5S RNA_H are not yet known. This paper reports the cloning, sequencing and *in*

vitro transcription of rat 4.5S RNA_H genes.

MATERIALS AND METHODS

Preparation of 4.5S RNA_H.

Nuclei were prepared from the liver of 6-week-old male Wistar Rats as described (10). Nuclear RNA was extracted from the nuclei by the hot phenol-SDS method (11) and was fractionated by gel filtration on Sephacryl-S200 (Pharmacia). 4.5S RNA_H was eluted in a high molecular weight RNA fraction, not in the 4.5S RNA fraction. Thus probably almost all the 4.5S RNA_H was associated with high molecular weight RNA. The high molecular weight RNA fraction was heat denatured and separated by two dimensional polyacrylamide gel (2-D gel) electrophoresis by a modification (3) of the method described previously (12, 13). The spots of 4.5S RNA_H were detected under ultraviolet light and eluted from appropriate pieces of the gel. The purified 4.5S RNA_H was labeled with [5'-^{32}P]pCp (Amersham) at the 3'-end (14), purified by 2-D gel electrophoresis and used as a probe in genomic cloning or sequencing.

Sequence analysis of rat 4.5S RNA_H.

Uniformly ^{32}P-labeled 4.5S RNA_H from normal rat kidney (NRK) cells was purified by 2-D gel electrophoresis as described previously (4). The sequences of oligonucleotide fragments obtained by complete digestion with RNase T1 (Sankyo) or RNase A (Sigma) were determined as described previously (6). The total sequence of the RNA was determined by the chemical sequencing method (15) using 3'-end-labeled RNA.

Screening of phage clones.

A gene library in lambda phage Charon 4A, which was constructed with partial Eco RI digests of liver DNA from a Sprague-Dawley rat, was provided by H. Esumi (16). About 3×10^5 phages were screened with post-labeled 4.5S RNA_H (17). Hybridizations were carried out in 50% formamide, 5x SSC (1x SSC is 0.15M NaCl plus 0.015M sodium citrate), 5x Denhardt's solution (18), 0.2% SDS and 10% dextran sulfate sodium salt (19). After hybridization for 18 hours at 42°C, the filters were washed with two changes of 2x SSC containing 0.2% SDS for 20 min each time at 50°C and then with 0.1x SSC containing 0.2% SDS at 50°C for an hour. The filters were autoradiographed for several hours and then washed briefly with 2x SSC to remove SDS. They were then immersed in TKE buffer [10mM Tris-HCl(pH7.3), 0.33M KCl, 1mM EDTA(20)] containing 0.5 or 2 µg/ml of RNase A. After incubation at room temperature for 15 minutes, RNase A was inactivated with iodoacetate (20)

and the filters were washed in 0.1x SSC containing 0.2% SDS for an hour at 50°C and autoradiographed for periods of 16 hours to several days.

Southern blot analysis.

DNA from Sprague-Dawley rat liver, phage clones or plasmids was digested with a restriction enzyme (Takara Shuzo) and the digests were subjected to electrophoresis in agarose gel. Blotting of DNA fragments from the gel was as described by Southern (21). The blotted filters were hybridized with 3'-end-labeled 4.5S RNA_H probe. The procedures for hybridization, washing and RNase A treatment were as described in the previous section.

Subcloning and sequencing of 4.5S RNA_H genes.

DNAs from purified phage clones were digested with Eco RI (clones 3 and 10), Hind III (clone 9) or Eco RI/Hind III (clones 2, 6 and 15), and ligated to the corresponding restriction sites of pKH47 (21, obtained from K. Hayashi). Ligated DNAs were used to transform E. coli K12 strain HB101 (23) and ampicillin-resistant transformants were selected by colony hybridization (24) with ^{32}P-labeled 4.5S RNA_H as a probe. Plasmid DNAs were digested with various restriction enzymes and restriction endonuclease maps were obtained. The region encoding 4.5S RNA_H was sequenced by the method of Maxam and Gilbert (25).

In vitro transcription and characterization of the transcripts.

A soluble whole cell extract for in vitro transcription (26) was prepared from Friend erythroleukemia cells T3 K-1 (27). In a standard reaction in 50 µl volume (26), 1 pmole of plasmid DNA was incubated with 20 µl of cell extract and 10 µCi of [α-^{32}P]GTP (Amersham) for an hour at 30°C. Transcripts extracted with phenol were subjected to electrophoresis on 8% polyacrylamide gel (0.6 mm thick x 40 cm long) in 7M urea and 1x TBE (25). After autoradiography, each band was eluted, digested with either RNase T1 or RNase A and fingerprinted (28).

RESULTS

Isolation of recombinant phage clones carrying the 4.5S RNA_H sequence.

4.5S RNA_H has high homology (more than 80%) with the rodent repetitive sequence B1 or type 1 Alu equivalent sequence (6-9) which is repeated more than 10^5 times throughout the genome. Therefore, almost every recombinant phage containing a 15kbp rat DNA fragment will hybridize to the 4.5S RNA_H probe. In fact, ^{32}P-labeled 4.5S RNA_H hybridized extensively to a

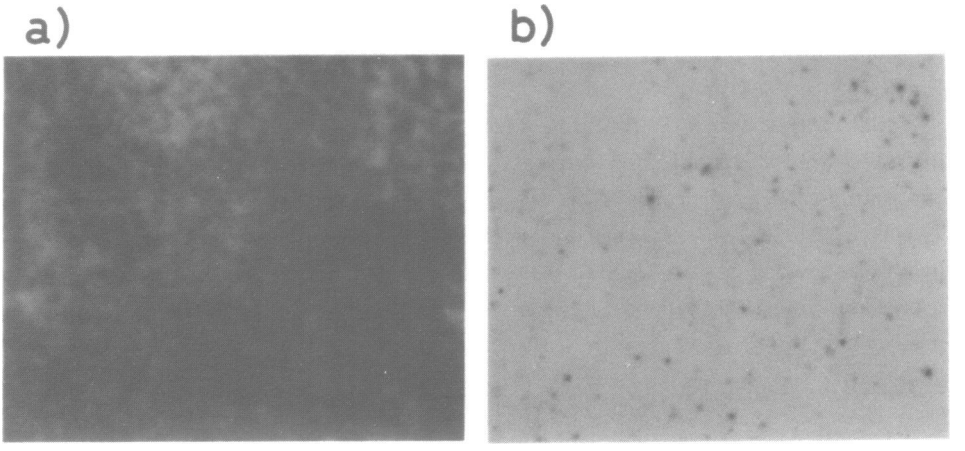

Figure 1. Screening of the rat genomic library. The autoradiograms shown are of nitrocellulose membrane filters hybridized with 3'-^{32}P-labeled 4.5S RNA_H (a) and after treatment with RNase A (b).

nitrocellulose filter to which the bacteriophage plaques had been transferred from the culture plate (Fig. 1a).

On the other hand, 4.5S RNA_H has oligo U at the 3'-terminus and this is not present in the corresponding position of the consensus sequence of

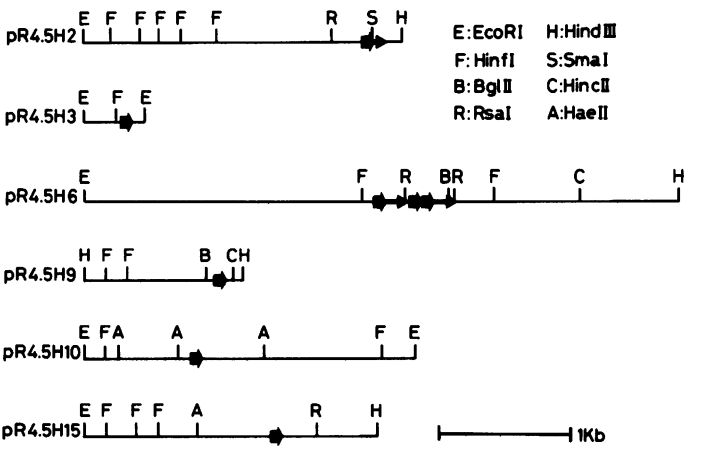

Figure 2. Restriction maps of the inserts of the plasmid clones containing the 4.5S RNA_H gene or pseudogene. Thick arrows represent 4.5S RNA_H related sequences and thin arrows indicate the ID sequence (pR4.5H2) or B2 sequences (pR4.5H6). There were many other Hinf I sites in pR4.5H6 but only the longest Hinf I fragment is shown.

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RNA  GCCGGTTGTGGTGGCGCAC[G/A]CCGGTAGGATTTGCTGAAGGAGGCAGAGGCAGGAGGATCACGAGTTCGAGGCCAGCCTGGGCTACACATTT(TTT)
 2   ..................A.....................................G........C.............TTT
 3   .......C..........GG.....................................TG...................A.........TT
6a   ..................A.....................................
6b   ..................G.....................................T
6c   ..................G.....................................
 9   ..T...........T....G.....................................TT
10   ..................G.....................................TTTT
15   ..............T....A.....................................TTTTT
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Figure 3. Nucleotide sequences of rat 4.5S RNA_H and the corresponding regions of six clones. In the RNA sequence the letter T has been used in place of a U for easier comparison with the DNA sequences. A dot at any position indicates the same nucleotide as in the 4.5S RNA_H sequence and a letter indicates a different nucleotide from that in the RNA sequence.

the repetitive sequence (6-9). Therefore, if the hybridized filter is treated with RNase A, the radioactivity of 4.5S RNA_H hybridized to the repetitive sequence should be eliminated from the filter and only 4.5S RNA_H genes should be selected. Fig. 1b shows an autoradiogram of an RNase A treated hybridized filter. About $3x10^5$ phage plaques were screened and about 1500 positive clones were obtained by this method.

Thirty-two phage clones giving intense signals were plaque-purified and phage DNAs were isolated. Each DNA was digested with Eco RI and characterized by Southern blot analysis (21). Sixteen different hybridizing patterns were obtained (data not shown).

Subcloning and sequencing.

Six clones (clones 2, 3, 6, 9, 10 and 15) giving different hybridizing patterns were selected and digested with Eco RI, Hind III, Bam HI or combinations of these enzymes. The shortest fragment containing the 4.5S RNA_H sequence from each phage DNA was subcloned into pKH 47 and a restriction map was constructed (Fig. 2). The maps were all different, indicating that the fragments were from different loci in the genome.

The nucleotide sequences of the regions homologous to 4.5S RNA_H and the flanking sequences were determined. Fig. 3 shows the sequences of rat 4.5S RNA_H and the corresponding regions of the six genomic clones. 4.5S RNA_H of rat cells gave the same sequence as that of hamster cells (7) except for microheterogeneity at position 20 from the 5'-terminus (70% of the RNA contained G and 30% contained A). The corresponding sequences of clones 2, 3, 9 and 15 contained 2, 5, 2 and 1 base substitutions, respectively. The sequence of clone 10 was colinear with the RNA. Clone 6 contained three corresponding sequences (6a, b and c) oriented in the same

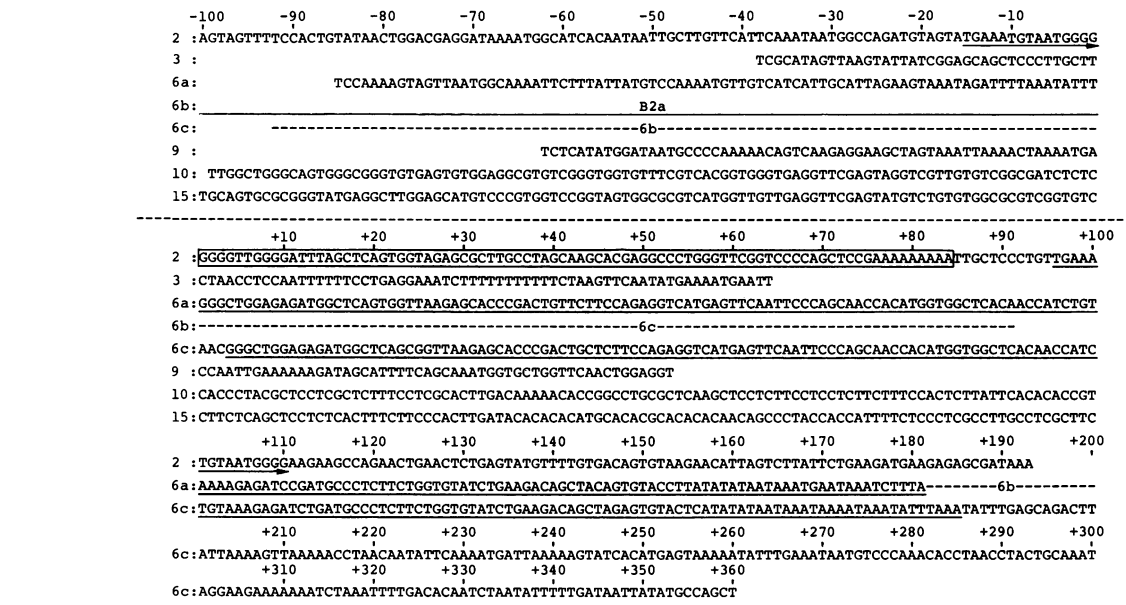

Figure 4. Nucleotide sequences of the flanking regions of eight 4.5S RNA_H related sequences. Upper sequences are 5'-flanking regions, while lower sequences are 3'-flanking regions. Arrows indicate direct repeats, boxed sequence shows the ID sequence (clone 2) and underlines represent B2 sequences (clone 6).

direction within 455 base pairs; 6a was 181 nucleotides away from 6b and 6b was directly linked with 6c. Although the three sequences were colinear with the RNA, 6a and 6c contained only three Ts at their 3'-termini.

Fig. 4 shows the flanking sequences of the 6 clones. In clone 2, the 4.5S RNA_H sequence was directly linked with an identifier (ID) sequence (29). A 15 nucleotide-long sequence, TGAAATGTAATGGGG, immediately adjacent to the 5'-end of the 4.5S RNA_H gene was repeated downstream of the ID sequence. In clone 6, the sequence between 6a and 6b was almost identical to the sequence downstream of 6c and these sequences were quite homologous to another rodent repetitive sequence B2, or the type 2 Alu equivalent sequence (30,31) and were named B2a and B2b. A palindromic octanucleotide AAATATTT immediately adjacent to the 5'-end of 6a was directly repeated immediately adjacent to the 3'-end of the second B2 sequence (B2b). Therefore, the structure of this region is summarized as direct repeat-6a-B2a-6b-6c-B2b-direct repeat. About 60 nucleotides of the 5' and 3' flanking sequences of clones 10 and 15 resembled each other (about 70%

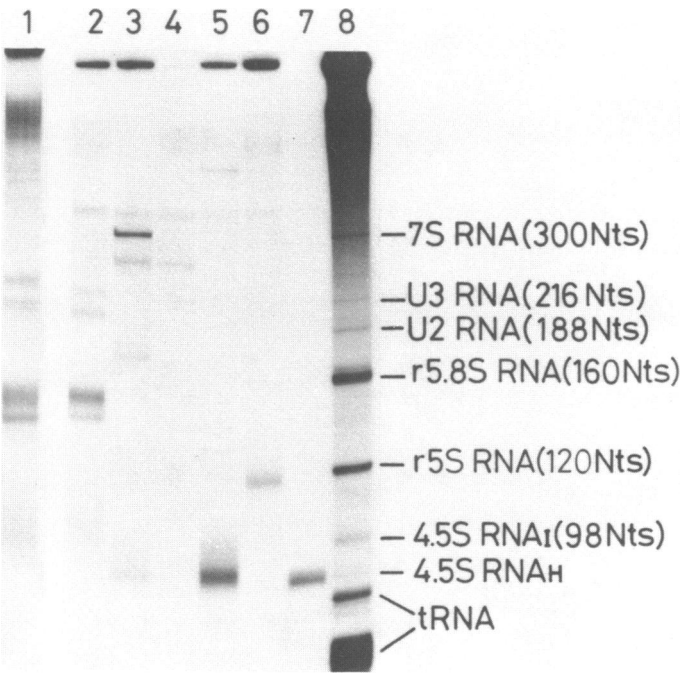

Figure 5. Analysis of the RNA products from a cell-free transcription reaction with plasmids containing the 4.5S RNA_H gene. Transcription products were separated on 8% polyacrylamide gel. Lane 1, Hae II digested pR4.5H2; lane 2, pR4.5H2; lane 3, pR4.5H6; lane 4, pR4.5H9; lane 5, pR4.5H10; lane 6, pR4.5H15; lane 7, uniformly labeled 4.5S RNA_H from NRK cells; lane 8, uniformly labeled total RNA from NRK cells.

homology). The flanking sequences of clones 3 and 9 did not have remarkable characters.

In vitro transcription of plasmids containing 4.5S RNA_H genes.

Since 4.5S RNA_H contains 5'-triphosphates, 3'-oligo U and intragenic promoter sequences (6,7,32,33), this RNA has been presumed to be a transcript of RNA polymerase III. To ascertain whether 4.5S RNA_H can be transcribed *in vitro* by RNA polymerase III, we incubated plasmid subclones of 4.5S RNA_H genes in a soluble mouse whole-cell extract and analyzed the transcripts by polyacrylamide gel electrophoresis (Fig. 5). Since this transcription is insensitive to 1 ug/ml of α-amanitine, but sensitive to 200 ug/ml of α-amanitine (data not shown), all transcripts in this figure must have been synthesized by RNA polymerase III. Each RNA band was eluted, digested with either RNase T1 or RNase A and analyzed by fingerprinting.

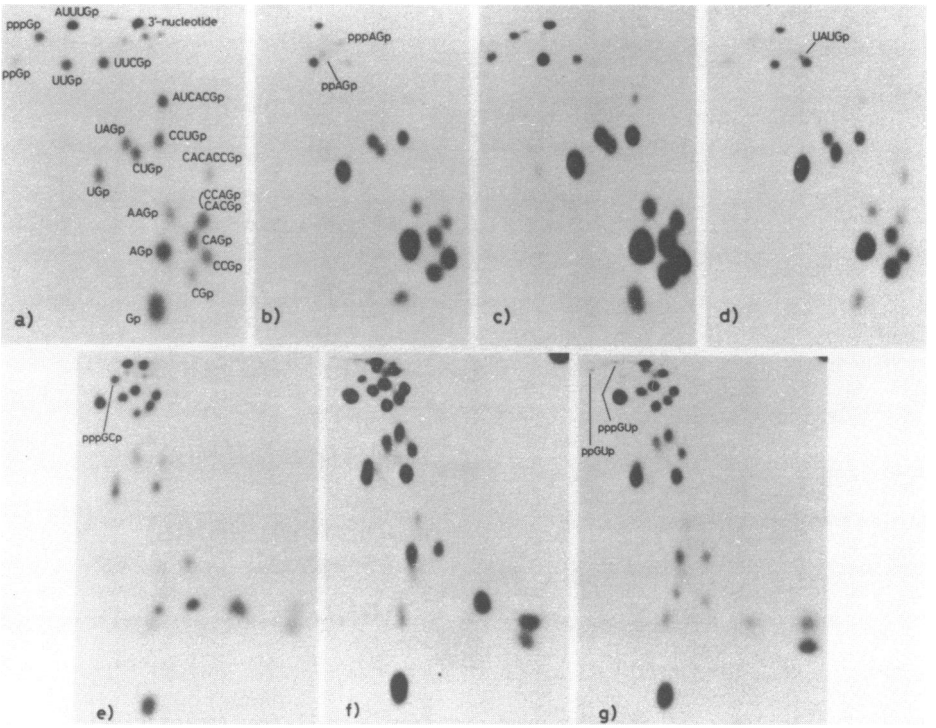

Figure 6. Fingerprints of RNase T1 (a-d) and RNase A (e-g) digests of uniformly labeled 4.5S RNA_H from NRK cells (a and e), 4.5S RNA from pR4.5H6 (b), 4.5S RNA from pR4.5H10 (c and f) and 5S RNA from pR4.5H15 (d and g). Electrophoresis in the first dimension, from right to left, was carried out on cellulose acetate in pyridine acetate buffer (pH 3.5)-7M urea-2.5mM EDTA. Electrophoresis in the second dimension, from top to bottom, was carried out on DEAE-cellulose in 7% formic acid.

Subclone 2 (pR4.5H2) gave 5 bands (about 230, 205, 145, 135 and 100 nucleotides). The RNase T1 fingerprints of the four longer RNAs were similar, but were not related to that of 4.5S RNA_H or to the presumed transcript of the ID sequence. The unrelatedness of these RNAs to the ID sequence was confirmed by an experiment using Hae II digested pR4.5H2 (Fig.5, lane 1) since the ID sequence contains an Hae II site between two intragenic promoter sequences (see Fig. 4). The transcripts from digested plasmid subclones were the same as those from the intact one. These longer RNAs must be transcribed from an other part in this subclone. A transcript of 100 nucleotides long gave the RNase T1 fingerprint which was expected from the sequence of the 4.5S RNA_H gene of clone 2 except for an additional

oligonucleotide (2A, 2U)Gp. Therefore, this gene must be transcribed from nine nucleotides upstream of the normal initiation site.

Subclone 6 (pR4.5H6) gave 4 major in vitro transcripts (about 320, 270, 180 and 90 nucleotides). The RNase T1 fingerprint of the 90 nucleotide RNA species was the same as that of the major species of 4.5S RNA_H except that the 5'-terminal nucleotide pppGp was replaced by pppA-Gp (Fig. 6b). Since in three 4.5S RNA_H genes in this subclone, only 6b was flanked by A at the 5'-terminus, this RNA must be transcribed from 6b. A transcript of 180 nucleotides gave an RNase T1 fingerprint corresponding to the B2 family. The B2 family has been shown to be a template for in vitro transcription by RNA polymerase III (34). An RNase T1 fingerprint of a 270 nucleotide-long transcript contained oligonucleotides derived from the B2 family and 4.5S RNA_H containing CACACCG instead of CACGCCG. Therefore this 4.5S RNA_H sequence must be transcribed from 6a (see Fig. 3) and the 270 nucleotide RNA species must be fused RNA derived from 6a-B2a. Since the longest transcript gave the RNase T1 fingerprint that was expected from the sequence of the B2 family and 3'-flanking sequence of B2b, this RNA must be the read through product of B2b. These results showed that in this transcription system the TTT sequence (the 3'-end of 6a) was not sufficient for transcriptional termination by RNA polymerase III, whereas TTTT (the 3'-end of 6b) was sufficient. In the case of the B2 family, TATTT (the 3'-end of B2b) was not sufficient for termination, whereas TCTTT (the 3'-end of B2a) was effective. The latter sequence is analogous to the termination signal TTCTTT of the Xenopus laevis $tRNA^{Leu}$ gene (33).

Although subclone 9 (pR4.5H9) was a very poor template, a faint band at the position of 4.5S RNA_H gave the RNase T1 fingerprint expected from the gene structure.

Subclone 10 (pR4.5H10) was the best template. A major transcript was found at the position of 4.5S RNA_H and an RNase T1 or an RNase A fingerprint of this RNA was identical to that of the major species of 4.5S RNA_H (Fig. 6c and f).

Subclone 15 (pR4.5H15) gave a major band of about 115 nucleotides long and a smaller amount (about one tenth as much) of a band of 4.5S RNA. The RNase T1 fingerprint of the 4.5S RNA band was identical to that of the minor 4.5S RNA_H, whereas the RNase T1 or RNase A fingerprint of the major band of RNA was slightly different from that of 4.5S RNA_H (Fig. 6d and g). In the RNase T1 fingerprint, a new spot, U-A-U-Gp, was observed and the intensities of oligonucleotides (2U,C)Gp, (U,C)Gp, U-Gp and C-Gp were

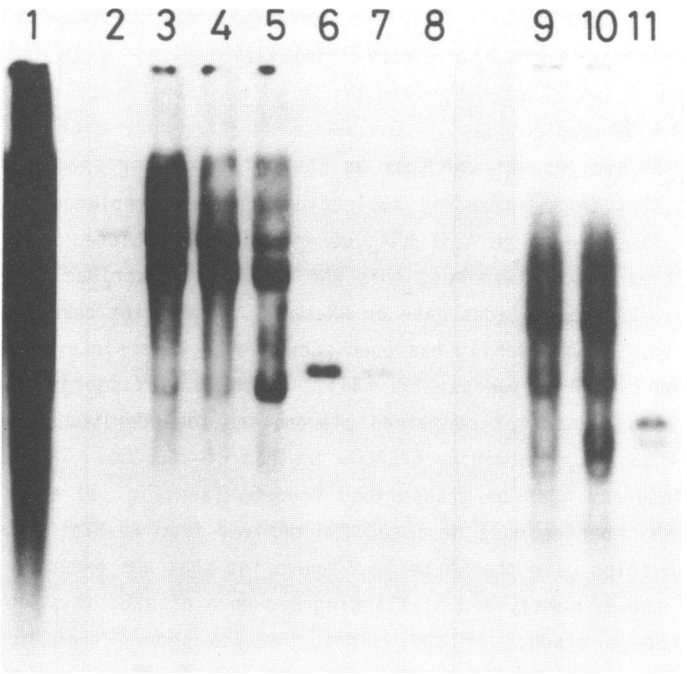

Figure 7. Whole genome Southern blots probed with 3'-^{32}P-labeled 4.5S RNA_H. Samples of 5 ug of Sprague-Dawley rat liver DNA digested with Eco RI (lanes 1, 3, 4 and 5) or Eco RI/Hind III (lanes 9 and 10) was electrophoresed in 0.8% agarose gels and transferred to nitrocellulose sheets. In lane 3, an Eco RI digest of 200 copy equivalents of phage clone 15 was coelectrophoresed, and in lane 5, an Eco RI digest of 1000 copy equivalents of pR4.5H10 was coelectrophoresed as internal markers. In lane 10 an Eco RI/Hind III digest of 300 copy equivalents of phage clone 15 and 600 copy equivalents of pR4.5H10 was coelectrophoresed. Lanes 2, 6, 7, 8 and 11 are gene copy number control lanes that contain 200 copy equivalents of an Eco RI digest of phage clone 15 (lane 2), 1000 (lane 6), 300 (lane 7) or 100 (lane 8) copy equivalents of an Eco RI digest of pR4.5H10 or a mixture of an Eco RI/Hind III digest of phage clone 15 (300 copy equivalents) and pR4.5H10 (600 copy equivalents) (lane 11). Lane 1 is a filter before RNase A treatment and lanes 2-11 are filters after RNase A treatment.

increased. In the RNase A fingerprint, the 5'-terminal oligonucleotide pppG-Cp was replaced by pppG-Up. From these observations and the size of the major band of RNA, we concluded that the transcriptional initiation site must be 26 nucleotides upstream from the usual initiation site.

Sequence in subclones 10 and 15 repeated hundreds of times in rat DNA.

For analysis of the copy number and the genomic distribution of the 4.5S RNA_H gene, post-labeled 4.5S RNA_H was hybridized to restriction enzyme

digests of rat liver DNA (Fig. 7). Although the 4.5S RNA_H probe hybridized extensively to the Eco RI digest of rat DNA (lane 1), most of the radioactivity was eliminated by RNase A treatment and several discrete bands appeared (lane 4). Each band corresponded to 100 to 1000 copies per haploid genome judging from its intensity. One of these bands coincided with a band (5.5 kbp) containing the 4.5S RNA_H gene in the Eco RI digest of phage clone 15 (lanes 2 and 3) and another band (2.3 kbp) coincided with that of the Eco RI digest of pR4.5H10 (lanes 5 and 6). Lanes 9-11 show Eco RI and Hind III digests of DNAs. Again several bands were observed after RNase A treatment and two of them coincided with the digestion products of clone 15 (2.1 kbp) and pR4.5H10 (2.3 kbp). The intensities of these bands suggest that there may be about 200 copies of the sequences corresponding to clones 10 and 15 per rat haploid genome. A similar experiment was done with restriction enzyme digests of clones 2, 3, 6 and 9, but no such bands were obtained (data not shown).

DISCUSSION

We developed a new method to clone genomes for RNA with a homologous sequence to the repetitive sequence. The essential features of this method were use of terminally labeled RNA as a hybridization probe and treatment of hybrids with RNase for elimination of repetitive sequences in hybrids. By this method we obtained many genomic clones for 4.5S RNA_H from a rat gene library. The same method was applied in molecular cloning of genes for rat 4.5S RNA_I (accompanying manuscript) and for human endogenous retroviruses (manuscript in preparation) with satisfactory results.

By this method we obtained about 1500 RNase A resistant clones from $3x10^5$ recombinant phage clones. Since the average length of the insert in recombinant phage is 15 kbp and the rat haploid genome is $3x10^6$ kbp, the copy number of 4.5S RNA_H related sequences was estimated to be 1000 per haploid. However since some clones contain multiple copies, this number should be an under-estimated. By Southern blot analysis of restriction enzyme digested rat DNA (Fig. 7), the copy number was roughly estimated to be 3000. At any rate there are some thousands of copies of 4.5S RNA_H related sequences in the rat haploid genome as a subfamily of B1 repetitive sequences.

We determined eight 4.5S RNA_H related DNA sequences from six genomic clones. Four (6a, 6b, 6c and 10) of them were colinear with 4.5S RNA_H. However 6a, 6b and 6c might be pseudogenes, since subclone 6 did not give a

correct transcript for 4.5S RNA_H in an *in vitro* transcription system. On the other hand, the sequence in subclone 10 was transcribed very efficiently and gave correct 4.5S RNA_H. Therefore, we believe that the sequence homologous to 4.5S RNA_H in subclone 10 is a *bona fide* gene for 4.5S RNA_H.

The other four sequences (2, 3, 9 and 15) contained one to five base substitutions in 4.5S RNA_H related sequences and might be pseudogenes. Subclone 15 gave a transcript that was 26 nucleotides longer than 4.5S RNA_H in an *in vitro* transcription system. This phenomenon might be caused by the appearance of a new 5'-internal promoter sequence GTGGCGCGTCGG (residues -16 to -5), which fits the consensus block A sequence GTGGYNNRGTGG (32) much better than the original block A sequence GTGGCGCACGCC (residues 11 to 22). Since the 5'- and 3'- flanking sequences of 4.5S RNA_H genes from clones 10 and 15 resembled each other, these sequences might be derived from a common ancestral sequence. There are about 200 copies of the sequences of subclone 15 and subclone 10 in the rat haploid genome (Fig. 7). Therefore, some of these sequences may be *bona fide* genes.

The 4.5S RNA_H sequences in clones 2 and 6 are linked with other repetitive sequences. In clone 2, the ID sequence (29) is directly linked to the 4.5S RNA_H gene. ID sequences have been found in introns of brain-specific genes of the rat genome (35) and also about 170 bp downstream of the rat U1 and U2 snRNA genes in reverse orientation (36,37). In the latter cases, ID sequences, like the transposable element, were flanked by 7 or 15 bp direct repeats (36,37). The 4.5S RNA_H gene-ID sequence in clone 2 was also flanked by a 15 bp direct repeat as if these sequences formed one unit. Although the cloned ID sequence is reported to be transcribed by RNA polymerase III *in vitro* (38), the ID sequence in clone 2 was not transcribed. Since the promoter sequences of the two ID sequences were identical, other conditions, probably some specific sequence in the 5'-flanking sequence, might be necessary for transcription. In clone 6, three 4.5S RNA_H genes and two B2 sequences were arranged in the order 6a-B2a-6b-6c-B2b. Generally the B2 family has the common structure, direct repeat-B2 sequence-oligo dA stretch-direct repeat (30,31). B2a contains only one dA after the B2 sequence and a very long direct repeat (6a and 6b), whereas B2b contains three dAs after the B2 sequence but no remarkable direct repeat. On the other hand, this cluster was flanked by a palindromic octanucleotide AAATATTT. It has been suggested that short

interspersed sequences have a tendency to insert into each others A-rich tails, and this has generated composits which are themselves propagated as single retroposons (39). Although 4.5S RNA_H genes do not have A-rich tails, clusters in clones 2 and 6 may be fused by the same or similar mechanism.

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