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Isolation and characterization of genomic mouse DNA clones containing sequences homologous to tRNAs and 5S rRNA

James C.Hu +, Babette D.Cote, Elsebet Lund and James E.Dahlberg

Department of Physiological Chemistry, University of Wisconsin-Madison, Madison, WI 53706, USA

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ABSTRACT

We have cloned and characterized three fragments of Balb/c mouse DNA which hybridize to mouse cell tRNAs. Fractionation of the tRNAs which hybridize to these clones reveals that two of the clones, λMt-4A and λMt-6A hybridize to only one or two tRNAs, while one clone, λMt-4B, hybridizes to at least seven tRNAs. Two of the tRNAs were identified as $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGA}$, and others have been identified as tRNAs which are selectively encapsidated into virions of murine leukemia virus and avian reticuloendotheliosis virus. The DNA sequences of putative genes for $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGA}$, plus flanking regions, were determined. A clone of Balb/c mouse DNA which selectively hybridized to 5S rRNA was also isolated and partially characterized.

INTRODUCTION

Transfer RNA (tRNA) genes in eukaryotes are present in multiple copies per cell. Mouse, _Xenopus_, _Drosophila_ and yeast all appear to express 40-60 major sequences of mature tRNAs (1-10). DNA sequences homologous to each of those tRNAs occur within the genome with reiteration frequencies ranging from 100-200 fold in _Xenopus_ and mouse to about 10 fold in _Drosophila_ and yeast.

Relatively little information is available about the genomic organization of tRNA genes in mammals. tRNA genes may either be clustered or scattered in the genome. For example, a 2.1 kb fragment of rat DNA carries genes for $tRNA^{Asp}$, $tRNA^{Glu}$ and $tRNA^{Gly}$ (7). Another clone of rat DNA, 11.9 kb long, carries a cluster of three $tRNA^{Pro}$ and three $tRNA^{Lys}$ genes (8). Genes for $tRNA^{Lys}$, $tRNA^{Gln}$, and $tRNA^{Leu}$ are within 1.5 kb of each other on a clone of human DNA (9,10). In contrast, at least one mouse cell $tRNA^{His}$ (11) two human $tRNA^{Asn}$ genes (10), and twelve human $tRNA^{Met}_{i}$ genes (5) are scattered at different loci.

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We report here the cloning and characterization of three fragments of mouse genomic DNA which hybridize to mouse tRNAs. The three clones have distinctly different organizations of tDNA sequences. In this paper, we refer to these sequences as tRNA genes although we have not yet demonstrated that they can be expressed. One clone contains a large cluster of tRNA genes whereas the other two clones contain only one or two tRNA genes. We have also cloned a 13.2 kb fragment of mouse DNA which is capable of hybridizing to 5S ribosomal RNA (rRNA). Restriction enzyme analysis reveals that 5S rDNA sequence is not repeated in this cloned DNA fragment. As is the case with the tDNA clones, we have not yet determined if the 5S rDNA clone contains an active gene or a pseudogene.

MATERIALS AND METHODS

Enzymes and Reagents

Restriction enzymes were purchased from New England Biolabs, or Bethesda Research Laboratories. All digestions were done using conditions recommended by the supplier. T_4 polynucleotide kinase was from P-L Laboratories. T_4 DNA ligase was from New England Biolabs. T_1 ribonuclease was from Calbiochem. Formamide was from Fluka (puriss), sucrose was from Schwarz-Mann, (ultra pure RNase free). [^{32}P] orthophosphate was from New England Nuclear.

Bacterial Strains and Phage

A genomic library of Balb/c mouse DNA EcoRI partial digestion fragments cloned into λCh4A was obtained from F. Blattner and N. Newell (12).

E. coli strain DP50 SupF (a gift from F. Blattner) was used as the host strain for the work with the λ Charon 4A Balb/c library. In subcloning experiments, *E. coli* strain HB101 was used to propagate plasmid DNAs (all derived from pBR322).

All experiments involving viable phages and bacteria containing recombinant DNA were performed under P2 conditions specified by NIH Guidelines for recombinant DNA research.

Preparation of tRNA Probe to Screen the Library

The tRNA probe was prepared from a subset of chicken liver tRNAs which had been partially enriched for $tRNA^{Met}$ by chromatography on DEAE-Sephadex A-25 (13). Three mg of this tRNA sample were electrophoresed on a 16% acrylamide-7M urea gel, and full-length tRNAs were located by staining a strip of the gel with "stains-all" (14). After elution from the remainder of the gel and filtration through a Millipore filter the RNA was phenol

extracted once and adsorbed to 0.5 ml hydroxylapatite (HAP, Bio-Rad) in 10 mM $NaHPO_4$, pH 7.0 at 4°C for 2 hrs. After washing the HAP 5 times with 5 ml of 0.1M NaH_2PO_4, RNA was eluted by the addition of 0.4M $NaHPO_4$ followed by gentle agitation for two hours at 4°C. The eluted RNA was concentrated, desalted by chromatography on CF-11 (Whatman) and ethanol precipitated. The overall yield was 1.15 mg (40% of the initial 4S RNA fraction).

5S rRNA was prepared by 2D gel electrophoresis of 10 mg of mouse cell small RNAs mixed with a small amount of purified ^{32}P-labeled mouse cell 5S rRNA. The gel-purified material was eluted and further purified over hydroxylapatite and CF-11 as described above. Yield of the 5S rRNA was about 0.75 mg (0.8% of the total starting material).

The purified RNAs were fragmented by partial alkaline digestion (5 μg RNA in 80 μl 0.05M Tris-Cl, pH 9.5 at 95° for one hour) and labeled at their 5' ends by treatment with polynucleotide kinase and γ[^{32}P]ATP. After one phenol extraction and two ethanol precipitations, labeled RNA was separated from nucleotides by chromatography on Sephadex G-50 fine (Pharmacia). The RNA had a specific activity of 0.2-1.0X10^8 cpm/μg.

Screening of the Balb/c Mouse Genomic Library

Approximately 12,000 recombinant phage were plated on DP50supF *E. coli* cells on 15cm NZYDT plates. Plaques were transferred to nitrocellulose filters and screened using the procedure of Benton and Davis (15) with modifications described by Maniatis *et al*. (16). Filters were hybridized for 60 hours at 42°C in 0.5M NaCl, 0.15M Na_2PO_4, 0.005M EDTA, 50% formamide, 2-8X10^5 cpm/ml 5' end-labeled tRNA or 5S rRNA probe, and a 500-fold mass excess (over the probe) of unlabeled mouse 18S and 28S rRNA. The filters were washed three times for 15 minutes in 5X SSC (1X SSC = 0.15M NaCl, 0.015M Na citrate, pH 7) at 65°C and five times for 15 minutes in 2X SSC at room temperature. Plaques which hybridized to the probe were located by autoradiography.

Phage from the region of the plate which gave a positive signal on the autoradiogram were rescreened at lower plaque density. DNA was prepared from plate lysates by the method of Thomas and Davis (17). Digestion of DNA with restriction enzymes, agarose gel electrophoresis and transfer of fragments to nitrocellulose were as described by Southern (18).

Subcloning into pBR322 and Isolation of DNA from Plasmids

Phage DNA was digested with HindIII, EcoR1 (single digests) or HindIII/EcoR1 (double digests) and the fragments were ligated into pBR322

DNA which had been cleaved by Hind III, EcoRl or Hind III plus EcoRI. Recombinant plasmids were used to transform HB101 cells. Plasmid DNAs were prepared as described (19) except that the digestion with pronase was omitted.

Hybrid Selection

Nitrocellulose filters (1 cm X 1 cm) containing 10-20 μg of denatured cloned DNAs were incubated for 16-20 hours at 68°C in 1 ml of a solution containing 2 x 10^5 cpm uniformly ^{32}P-labeled RNAs (13), 6X SSC, 0.1% SDS and 1X Denhardt's solution (20) as described by Weiner (21). After hybridization, the filters were washed five times in 6XSSC at 68°C, then three times in 2XSSC at room temperature, and once in water at 4°C. Hybridized RNAs were eluted from the filters by incubation in 300 μl of 0.5mM EDTA, 0.1% SDS and 10 μg carrier RNA at 80-85°C for five minutes.

Polyacrylamide Gel Electrophoresis

Both uniformly labeled preparations of 4-8S total cellular RNAs and hybrid selected small RNAs were separated by two-dimensional polyacrylamide gel electrophoresis (22). When necessary, regions of the gel containing RNAs of interest were excised and used as origins for a third dimension of electrophoresis (23). The tRNAs, located by autoradiography, were eluted from the gel in 0.3M NaCl.

RNA Fingerprinting, DNA Mapping and DNA Sequencing

After ethanol precipitation, individual RNA species were characterized by RNase T_1 fingerprinting (24). Mapping of restriction enzyme cleavage sites in cloned DNA was done by standard end labeling and cleavage methods (25). DNA sequencing was done by the method of Maxam and Gilbert (26). The fragments and strands which were sequenced are illustrated in Figure 4.

RESULTS

Isolation and Mapping of tDNA clones

A Balb/c mouse genomic DNA library was screened using 5'-[^{32}P] labeled chicken liver tRNA fragments as probes. This library contained fragments from a partial EcoRl digest of mouse liver DNA (approximately 13-22 kb in length) (12) in λCharon 4A. Three clones which hybridized to the RNA were obtained by screening 12,000 plaques (about 6% of the mouse genome). These clones, designated λMt-4A, λMt-6A and λMt-4B, contained 13.7 kb, 14.2 kb, and 15.3 kb inserts, respectively.

Figure 1 shows the locations of restriction endonuclease cleavage sites

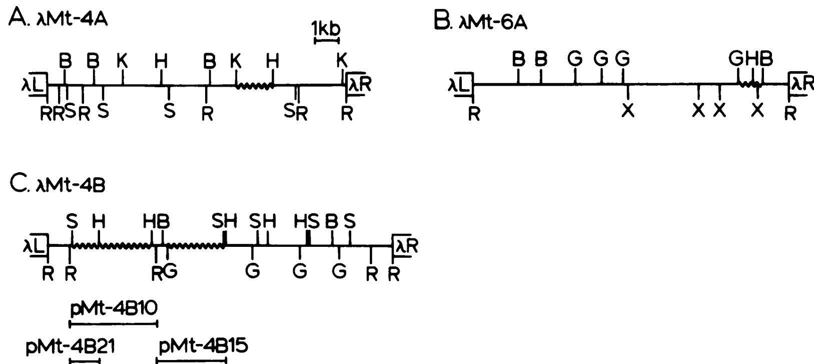

Figure 1. Restriction maps of λMt cloned DNAs.
A: λMt-4A; B: λMt-6A; C: λMt-4B. The wavy line indicates regions within the DNAs which are homologous to the tRNA probes. The orientations of the DNAs with respect to the left and right arms of lambda are indicated. The bars below the map shown in C indicate fragments of λMt-4B DNA which have been subcloned into pBR322. The following abbreviations are used: B, BamH1; H, HindIII; G, Bgl II; K, KpnI; R, EcoR1; S, SstI; X, XbaI.

in these cloned DNAs. The approximate locations of the regions in the cloned DNAs to which tRNAs hybridize are indicated in this figure. These regions were identified by Southern blot hybridization analysis using total chicken cell tRNAs as probes. An example of such an analysis is shown in Figure 2.

Fragments of clone 4B DNAs which had sequences homologous to tRNAs were subcloned into pBR322. The resulting three subclones were called pMt-4B21 (containing a 1.2 kb HindIII/R1 fragment), pMt-4B10 (containing a 3.9 kb EcoRI fragment) and pMt-4B15 (containing a 3.5 kb HindIII/R1 fragment). The locations of the subcloned DNA fragments are indicated in Figure 1.

Polyacrylamide Gel Separation of Hybrid Selected Mouse tRNAs

RNAs homologous to the mouse DNA fragments were prepared by hybridization to λMt DNAs and the selected RNAs were analyzed by two dimensional (2-D) gel electrophoresis (Fig. 3). Comparison of the 2-D gel patterns of the hybridized RNAs (Fig. 3B-D) with that of total mouse cell small RNAs (Fig. 3A) showed that the cloned DNAs selected specific subsets of molecules from the mixture of cellular tRNAs. λMt-4A DNA hybridized predominantly to two tRNAs (Fig. 3B), while λMt-6A DNA hybridized to only one labeled tRNA species (Fig. 3C). The RNAs hybridizing to DNA of clone λMt-4B presented a more complicated gel pattern with at least seven tRNA-containing spots being resolved (Fig. 3D). That number is a minimum

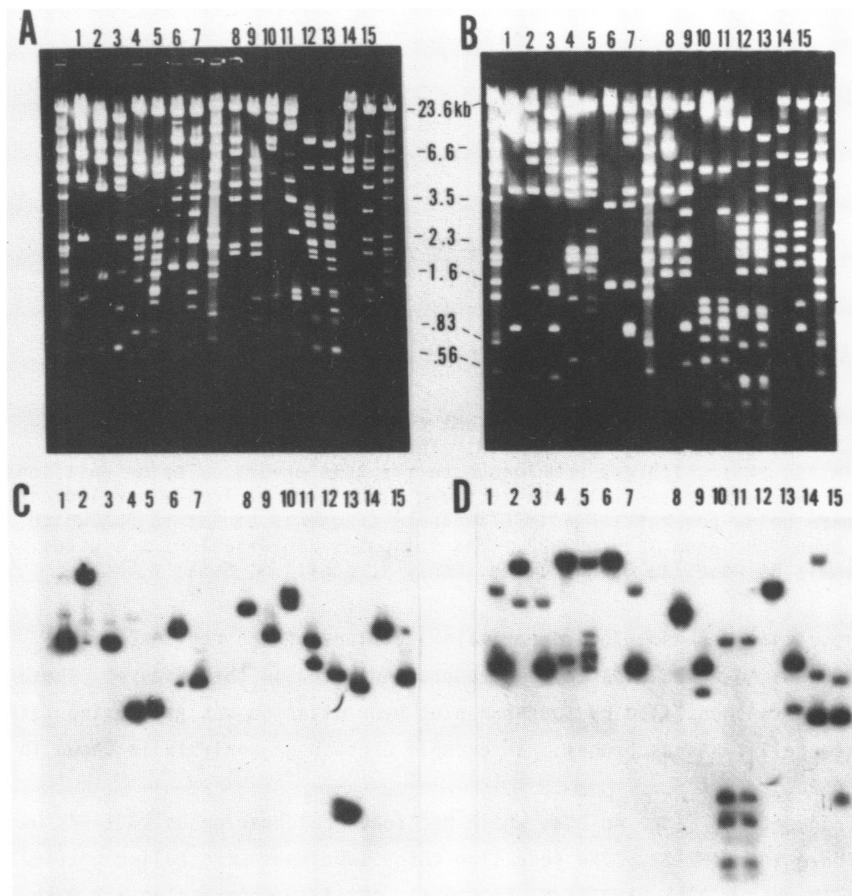

Figure 2. Restriction analysis of λMt-4A and λMt-4B.
A: Ethidium bromide stained 1.2% agarose gel containing restriction fragments of λMt-4A DNA. Slots on the sides and in the middle of the gel contained DNA size markers (λC17s7DNA digested with EcoRl plus HindIII). The DNAs in the odd-numbered slots were digested with EcoRI. In addition the DNAs were digested with Bam HI (slots 2 and 3), BglII (slots 4 and 5), KpnI (slots 6 and 7), SstI (slots 8 and 9), XbaI (slots 10 and 11), PstI (slots 12 and 13) and HindIII (slots 14 and 15). B. Ethidium bromide stained gel with restriction fragments of λMt-4B DNA. Markers and enzymes used for digestion were the same as for λMt-4A DNA (Fig. 2A). C. Autoradiogram of hybrids formed between radioactively labeled chicken cell tRNA and the fragments shown in Fig. 2A, after transfer of these fragments from the gel onto a nitrocellulose filter. D. Autoradiogram of hybrids formed to the filter containing the fragments of Fig. 2B (probe as in Fig. 2C).

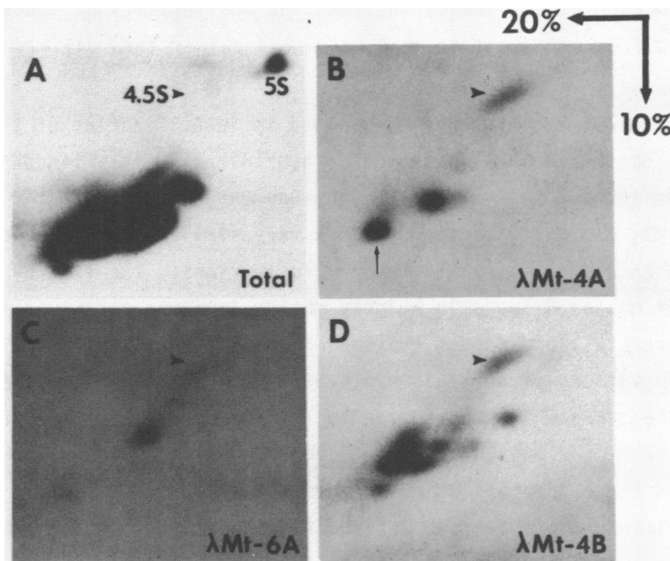

Figure 3. Two-dimensional gel analysis of mouse cell RNAs prepared by hybridization to cloned tDNAs.
Uniformly labeled small RNAs prepared by hybrid selection were separated by electrophoresis in polyacrylamide gels as indicated. A: Two-dimensional display of total uniformly labeled mouse tRNAs. B, C, D: 4-5S RNAs selected by hybridization to the DNAs of λMt-4A, λMt-6A and λMt-4B, respectively. The positions of 4.5S and 5S RNAs are indicated. RNAs eluted from the spots shown in B, C and D were analyzed by RNase T_1 fingerprinting (not shown). The vertical arrow in panel B indicates the position of $tRNA^{Gly}_{GGG}$.

estimate of the number of selected tRNAs since several of these spots contained more than one tRNA, as assayed by RNase T_1 fingerprinting (not shown). This large number of RNAs was consistent with the restriction enzyme analyses of λMt-4B DNA in which tRNA genes were found in several restriction fragments (cf. Fig. 2).

All of the cloned DNAs also selected two other mouse cell small RNAs. By their electrophoretic mobilities in both 1-D and 2-D gels, these RNAs were identified as 7S and 4.5S RNAs (27,28). This result indicated that these clones all contained B1-type middle repeat sequences (29).

Identification of tRNAs hybridizing to the DNAs of clones λ-Mt4A, λMt-6A and λMt-4B.

The purified RNAs hybridizing to the three cloned DNAs were characterized by RNase T_1 fingerprinting. As expected, the fingerprints were

distinct from each other, indicating that the multiplicity of spots on the gels resulted from different primary sequences rather than different conformations of a few molecules.

In a few cases, the fingerprints helped in identification of the tRNAs. For example, one of the RNAs selected by hybridization to λMt4A DNA was identified as $tRNA^{Gly}_{GGG}$ (G.R. Sams and J.E. Dahlberg, unpublished; 7,30). Two other tRNAs had RNase T_1 fingerprints very similar to RNAs which were specifically encapsidated in RNA tumor virus virions: the tRNA hybridizing to λMt-6A DNA has been found in Moloney murine leukemia virus virions (spot 13 of ref. 27) and one of the RNAs hybridizing to λMt-4B DNA has been found in virions of reticuloendotheliosis virus (spot 3A of ref. 31).

Two of the tRNAs which hybridized to λMt-4B DNA had T_1 fingerprints of $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGA}$. Subsequent sequencing of regions of subclone pMT-4B10 DNA confirmed those identifications as described below.

In experiments not shown, we assayed for the ability of eight tRNAS to hybridize to specific regions in the λMt-4B or λMt-4A DNAs. These tRNAs were gel-purified from uniformly ^{32}P-labelled mouse cell RNA. Those molecules corresponding to hybrid-selected tRNAs by the criteria of gel mobility and RNase T_1 fingerprinting were individually hybridized to Southern blot filters of EcoRl/HindIII double digests of the two tDNA clones. Seven of the eight tRNAs each hybridized to a unique restriction fragment from the λMt clones. All five of the tRNAs which were expected to hybridize to λMt-4B DNA did so, two to the 3.5 kb HindIII/EcoRl fragment, and three, including $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGA}$, to the 2.4 kb HindIII fragment. In contrast, only two of the three λ4A-specific RNAs hybridized to that DNA (the 4.2 kb EcoRl fragment of λMt-4A). The similarity of the RNase T_1 fingerprints of these two species indicated that they had the same primary sequence and differed only in their post-transcriptional modification. Surprisingly, purified $tRNA^{Gly}_{GGG}$ did not hybridize to λMt-4A DNA in spite of the fact that it had been hybrid selected by the DNA out of a mixture of tRNAs. The explanation for this result is unclear; perhaps $tRNA^{Gly}_{GGG}$ associated with the DNA in a ternary structure with the other tRNA seen in Fig. 3b, when both were present in the mixture of unfractionated RNA.

DNA sequencing of tRNA genes in pMt-4B10.

Both $tRNA^{Pro}_{CCG}$, and $tRNA^{Gly}_{GGA}$ contained the sequence C-C-C-G-G-G (7, 23). Thus we expected that the genes for these tRNAs should be cleaved by the

restriction endonuclease SmaI. We therefore determined the DNA sequence of the areas around the two SmaI sites in the region of tRNA hybridization in pMt-4B10. Figure 4 shows a detailed restriction map and the DNA sequencing strategy used. The DNA sequences around the two SmaI sites are shown in Figure 5. These sequences confirm that the DNA could contain the genes for the two tRNAs. The SmaI sites occurred in the expected positions in the genes.

Isolation of 5S rDNA Clone

The same mouse DNA genomic library which had been screened for clones containing tDNA sequences was also screened for clones containing sequences homologous to 5S ribosomal RNA. In this case, the probe was highly purified 5S ribosomal RNA. One clone was obtained after screening 20,000 plaques (approximately 10% of the mouse genome). This clone, designated λM5S-591, contained a single 13.2 kb EcoR1 fragment. Figure 6 shows the restriction map of this fragment and indicates the region which hybridizes to 5S rRNA.

A 2.6 kb HindIII fragment, which contained the region homologous to the 5S RNA, was subcloned into pBR322 to make a subclone we call pM5S-591. A Sau3A fragment, about 500 nucleotides long, located within the 2.6 kb HindIII fragment was the only subfragment in the 13.2 kb clone that contained 5S RNA hybridizing sequences. Thus, clone λM5S-591 did not contain a long tandem array of 5S genes. As indicated in Figure 6, this

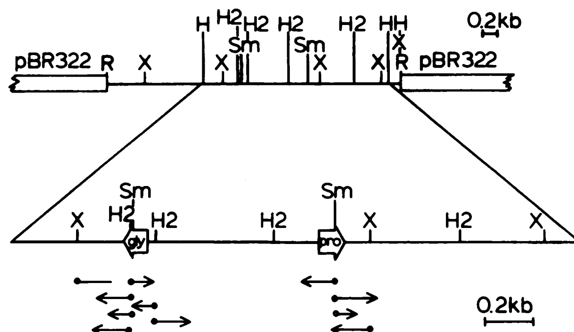

Figure 4. Restriction sites in pMt-4B10 DNA.
The expanded section shows the 2.4 kb HindIII fragment which hybridizes 3 tRNAs: tRNA^{Gly}, tRNA^{Pro} and an unidentified tRNA. Arrows below the map indicate DNA fragments which were sequenced from their 5' (●) or 3' (O) ends. The positions of the putative tRNA genes are indicated by thick arrows pointing in the direction of transcription, i.e. 5' to 3'. The restriction enzyme sites indicated are abbreviated as X, XhoI; R, EcoRI; H, HindIII; H2, and HincII; Sm, SmaI.

Figure 5. DNA sequences of putative $tRNA^{Pro}_{CCG}$ (A) and $tRNA^{Gly}_{GGA}$ (B) genes. The DNA sequences were determined by the strategy shown in Figure 4. The lines above the sequences indicate the regions corresponding to the tRNAs. These sequences are folded into cloverleaves, on the left side of the figure. Note that the 3'-terminal C-C-As are not encoded in the DNA sequences. T-rich clusters downstream of the tDNA sequences are enclosed in boxes.

region is at least 4.9 kb away from any other 5S or tRNA hybridizing sequences.

When λM5S-591 DNA was used to select small RNAs from a mixture of uniformly labeled cellular RNAs by hybridization, two molecules were obtained. By electrophoretic mobility these were identified as 7S RNA (which presumably hybridized to middle repetitive sequences, such as the B1 family, present on the clone) and 5S ribosomal RNA. This latter RNA was analyzed by RNase T_1 fingerprinting. The fingerprint was that of a mammalian cell 5S RNA (32); results of redigestion of all the oligonucleotides were consistent with that conclusion (data not shown).

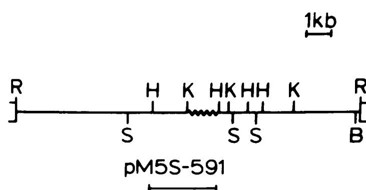

Figure 6. Restriction endonuclease cleavage sites of λM5S-591 DNA. The wavy line indicates the region within the DNA which contains sequences homologous to the 5S RNA probe. The bar below the map shows a HindIII fragment of the insert which was subcloned into pBR322 to make the subclone pM5S-591. Abbreviations are as described in the legend to Figure 1.

DISCUSSION

In this paper we report the identification and initial characterization of several clones of mouse DNA which are complementary to tRNAs and 5S rRNA. We used chicken cell tRNAs or 5S rRNA as probes to screen the mouse genomic library. As expected, there was sufficient interspecies homology to allow for hybridization of these probes to the mouse DNA, but insufficient homology between middle-repeat sequences of the two species to cause a high background of false positive signals. The mouse DNA clones did contain such sequences, in addition to the tDNA and 5S DNA sequences, as evidenced by hybridization of mouse 4.5S and 7S RNAs to the DNA (21).

The three tDNA clones which we isolated hybridized to different tRNAs. Two of the clones, λMt-6A and λMt-4A, hybridized specifically to only one tRNA each; that result was similar to what was found for mouse $tRNA^{His}$ (11), human $tRNA_i^{Met}$ (5) and $tRNA^{Asn}$ (10) genes. In contrast, one of the clones, λMt-4B, hybridized to at least seven different tRNAs. Clustering of tRNA genes has also been found in the human (9) and rat (8) genomes and in the genomes of lower eucaryotes (3,4).

We identified the potential coding regions for $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGA}$ in the DNA of pMt-4B10 by DNA sequencing. As shown in Figure 5, the two genes, if they are functional, would be transcribed in opposite directions. The DNA sequences to which the two tRNAs hybridize resemble previously described eukaryotic tRNA genes. Both tRNA coding regions are followed by clusters of T residues. The T-rich cluster downstream of the $tRNA^{Pro}_{CCG}$ gene is particularly striking. Similar clusters have been found 3' distal to other tRNA genes (3,5,7,8,9,11), and it has been suggested that these clusters could act as sites for transcription termination. In neither case is the 3' terminal CCA encoded in the DNA sequence, nor is there an intervening sequence in the

anticodon region.

The RNA coding regions of the $tRNA^{Gly}_{GGA}$ and $tRNA^{Pro}_{CCG}$ sequences found on pMt-4B10 are identical to the sequences of the rat $tRNA^{Gly}_{GGA}$ and $tRNA^{Pro}_{CCG}$ genes reported by Sekiya and coworkers (8). However, we note that there is no obvious homology between the 5' or 3' flanking sequences of the rat and the mouse genes.

Since we have only one example of a mammalian 5S rRNA hybridizing sequence, we are unable to generalize about possible clustering of these genes in the mouse genome. We can, however, conclude that 5S rDNA sequences are not all displayed in long tandem arrays in the mouse genome, since the 5S hybridizing sequence in our clone has been narrowed down to about 500 nucleotides.

None of the tDNA clones described here has been tested for the transcriptional activity of their tRNA genes. Therefore, at present, we are unable to say whether these tDNA sequences are true genes or pseudogenes which hybridize to our tRNA probes. Since the DNA sequences of two of the genes in clone λMt-4B agree completely with the known sequences of $tRNA^{Pro}_{CCG}$ and $tRNA^{Gly}_{GGG}$ we expect that they will be found to be true genes.

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+Present address: Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA

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