In the course of studies on genes for small nuclear RNAs, seven λ phage clones containing sequences homologous to 5 S RNA were plaque purified from a rat genomic library. The seven clones were found to be from six different genomic loci. When the 5 S RNA hybridized to these clones was digested by T1 RNase, only clone 5S-2 protected the RNA completely. Moreover, clone 5S-2 which has five nucleotide substitutions in the internal control region was transcribed 10 times more efficiently than a bona fide Chinese hamster 5S gene. The other clones were less efficiently transcribed than a bona fide 5S gene or not transcribed at all. The number of gene variants for 5 S RNA in the rat genome was approximately 3000. In contrast to the clustering of 5S genes and gene variants found in Xenopus, Drosophila, hamster, mouse, and human cells, the 5S gene variants in the rat genome are dispersed and most contained conserved 3′-flanking sequences. These naturally occurring 5S gene variants may be useful in binding transcription factors that affect 5S genes.

The genome organization of 5S genes has been studied in a number of eukaryotes such as yeast, Drosophila, Xenopus, and mouse (1-4). In most cases, the 5S genes are arranged in a tandem array having short repeat lengths with the bulk of the genes coding for the predominant form of 5 S RNA found in ribosomes. In addition to the developmentally regulated embryonic and somatic 5S genes (5, 6), Xenopus also contains pseudogenes for 5S (7). The first pseudogenes described were not transcribed in vitro, but a number of eukaryotes such as yeast, Drosophila, Xenopus, and human cells also contain pseudogenes for 5S (5, 6). The first pseudogene described was characterized by transcription in vitro. The 5S pseudogenes for 5S (7). The first pseudogenes described were not transcribed in vitro, but a number of eukaryotes such as yeast, Drosophila, Xenopus, and human cells also contain pseudogenes for 5S (5, 6). The first pseudogene described was characterized by transcription in vitro.

The dried filters were autoradiographed using Kodak XAR-5 film and autoradiographed using Kodak XAR-5 film and autoradiographed using Kodak XAR-5 film. The RNA fingerprinting of 5S RNA was routinely obtained.

**RESULTS**

About 50,000 phage plaques were screened with 5 S RNA as a probe. The hybridization and washings were carried out as described under "Materials and Methods" at 42 °C. Four hundred positive signals were observed after a 3-day exposure of the filters. The 5 S Charon 4A library used for screening in this study contains 15-20 kilobase pairs of rat genomic DNA as inserts (22). Based on 6 × 10^6 base pairs in the rat haploid genome, 3 × 10^6 to 5 × 10^6 counts/μg of 5 S RNA were routinely obtained.

**Screening of Genomic Library**—The screening of rat liver genomic library in a phage Charon 4A was carried out by the method of Benton and Davis (15). The partial ecoli genomic library made from rat liver DNA in λ phage Charon 4A was kindly provided by Dr. T. Sargent and Dr. J. Bonner (Division of Biology, California Institute of Technology, Pasadena, CA). Hybridizations with increasing stringency at 42, 52, or 58 °C were carried out in 50% formamide, 4 × SET (0.15 M NaCl, 1 mM EDTA; 0.03 M Tris-HCl, pH 8.0), 2 × Denhardt's reagent, 0.1% sodium dodecyl sulfate, and 10 μg/ml yeast transfer RNA. The nitrocellulose filters were hybridized for 16 h and washed two times each for 15 min with 3 × SSC, 1.5 × SSC, and 0.5 × SSC containing 0.1% sodium dodecyl sulfate at 42, 52, or 58 °C. The dried filters were autoradiographed using Kodak XAR-5 film and DuPont Lightning Plus screens at 70 °C.

**Preparation and Analysis of 5S RNA**—The dot hybridizations were carried out by the method of Kafatos et al. (16). The RNase protection experiments were carried out as described by Weiner (17). Thioacetamide treatment of the rats was carried out as described by Ro-Choi et al. (18). Briefly, rats weighing about 200 g were injected intraperitoneally with 1 mg of thioacetamide (10 mg/ml in 0.9% NaCl) at 0 h and another 1-mg dose of thioacetamide at 12 h. 10 mCi of [32P]orthophosphate was injected at 24 h, and labeling was carried out in vivo for 3 h. 5S RNA was isolated from these rat livers and analyzed by fingerprinting.

**Fingerprinting and Sequencing**—RNA fingerprinting was carried out according to Brownlee et al. (20). The DNA sequencing was carried out by the method of Maxam and Gilbert (21).

**MATERIALS AND METHODS**

**Chemicals**—RNA ligase and polynucleotide kinase were from Pharmacia P-L Biochemicals. All restriction enzymes were obtained from New England Biolabs. The isotopes were obtained from Amersham Corp. The nucleotide triphosphates and components for the buffers were obtained from Sigma.

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Dispersed 5S Gene Variants in the Rat Genome

were plaque-purified at 56 °C. Seven phage clones were chosen at random and studied further.

Six of the Seven Clones Are from Different Loci of the Rat Genome—The seven phage DNAs were digested with several restriction enzymes separately and fractionated on 1% agarose gels, transferred to nitrocellulose by the method of Southern and hybridized to 5 S RNA. Fig. 2 shows the results obtained with the phage DNAs digested with EcoRI. Six of the seven clones had different size EcoRI fragments that hybridize to 5 S RNA. Clones 6A and 6B appear to be from the same locus since the restriction enzyme digestion patterns were similar and fragments of the same size hybridized to 5 S RNA. Similar results were obtained when these DNAs were digested with three other restriction enzymes (results not shown). These results also showed that each of the six different clones contain only one 5S gene in the 15-20 kilobase pairs of DNA insert. In addition, when rat spleen DNA (SP.DNA) was completely digested with EcoRI and hybridized with 3'-end-labeled 5 S RNA, the DNA fragments that hybridized did not correspond to any of the six different clones that were isolated. The longer exposure of this lane showed some hybridization in regions other than the main band. One possible explanation for these results is that the main band corresponds to the 5S gene repeat and none of the 5S clones isolated were from this gene repeat.

5S Sequences in the Cloned DNAs Were Not Identical to the 5 S RNA Found in Adult Rat Liver Cells—The extent of homology between 3'-end-labeled 5 S RNA found in adult rat liver and the six different 5S clones was analyzed (Fig. 3, panel A). 5 S RNA specifically hybridized to these clones. However, when these RNA-DNA hybrids were digested with T1 RNase for 15 min, intact 5 S RNA was only recovered with one clone. Only in the case of clone 5S-2 was intact 5 S RNA found (Fig. 3, panel B). However, when the 5S-2 DNA:5 S RNA hybrid was digested with T1-RNase for 1 h, instead of for 15 min, all the 5 S RNA was cleaved (results not shown). These results show that of the six different clones isolated,

![Fig. 1. Analysis of small RNAs that hybridized to different 5S cloned DNAs.](image1)

![Fig. 2. Southern blot analysis of 5 S DNAs digested with restriction endonuclease EcoRI.](image2)
Dispersed 5S Gene Variants in the Rat Genome

FIG. 3. Protection of hybridized 5 S RNA against T1 RNase digestion. 5 μg of each of DNA from different phage preparations corresponding to 5S clones 1 to 6B was immobilized on nitrocellulose dots and hybridized to a mixture of four 3'-end-labeled RNAs. The hybridization was carried out as described by Kafatos et al. (16). The RNAs bound to the filters before (panel A) and after 15-min T1 RNase treatment (panel B) were analyzed by electrophoresis on polyacrylamide gels.

only clone 5S-2 may contain 5S sequences identical to rat liver 5S RNA.

To test whether any of these 5S gene variants are transcribed in vivo, several experiments were carried out. 1) The uniformly labeled 4-8 S RNA was hybridized to the 5S-2 DNA at 56 °C, and the hybridized RNAs were digested with T1 RNase (see Fig. 3 for details). If the 5S-2 gene variant is transcribed in the cells, the transcript from this gene variant would be expected to form a T1 RNase-resistant hybrid with cloned 5S-2 DNA. Analysis of the protected 5 S RNA by fingerprinting showed that this 5 S RNA was the same as the bulk 5 S RNA (results not shown). 2) Earlier studies showed that when the rats were injected with thioacetamide, the RNA synthesis, including that of 5S RNA, increases 5-10-fold (18). To test whether any of these 5S gene variants are activated and transcribed under these drug-induced conditions, the 5 S RNA, synthesized in livers of rats treated with thioacetamide, was analyzed by fingerprinting. In this case also, the 5 S RNA was the same as in normal rat liver, and no minor oligonucleotides corresponding to 5S-1 or 5S-2 gene variants were detected. Therefore, no evidence is available to indicate that these 5S gene variants contribute to 5 S RNA population of adult rat liver.

Two of the Six 5S Clones Were Transcribed in Vitro—To determine whether any of these 5S gene variants can be transcribed in vitro, the phage or plasmid DNAs containing clones 5S-1 and 5S-2 were used for transcription in vitro (Fig. 4, lanes 3-6) using Novikoff hepatoma whole cell Weil extracts (19). Clones 5S-3, 5S-4, 5S-5, and 5S-6A or -6B did not support 5 S RNA synthesis (compare PBR322, lane 12, or no DNA added, lane 1). Clone 5S-2 gave a single 5 S size RNA and clone 5S-1 produced two RNA bands of 5 S size. These results show that of the six different 5S gene variants isolated, four (clones 3-6) did not support 5 S RNA synthesis and two (clones 5S-1 and 5S-2) were transcribed with different efficiencies. The amount of 5 S RNA synthesized was quantitated by excising the 5 S RNA bands and determining the amount of radioactivity in each band. Based on these determinations, in three different experiments, the 5S-2 DNA was 10 times more efficiently transcribed when compared to Chinese hamster 5S gene. The 5S-1 gene was transcribed less efficiently (30%) when compared to a bonafide 5S gene.

5 S-sized Transcripts Are Derived from 5 S DNA in Clones 1 and 2—To ascertain whether the 5 S size transcripts were derived from 5 S-related DNA or from some other region of the insert, the in vitro transcripts were analyzed by fingerprinting. Fig. 5 shows the fingerprints of 5S transcripts obtained from clone 5S-1 (panel B) and clone 5S-2 (panel C) compared to transcripts obtained from Chinese hamster 5S gene (panel A). The nucleotide sequences of Chinese hamster 5 S RNA and rat 5 S RNA are identical, and, therefore, the fingerprints are same. The fingerprints of 5 S RNA from clone 5S-1 and 5S-2 contained many oligonucleotides common to 5 S RNA but also showed many different oligonucleotides (Fig. 5). The T1 RNase fingerprint of the 5 S RNA obtained from 5S-1 DNA was found to be consistent with the 5S-1 DNA sequence. For example, the oligonucleotide 20 (AACG,) is missing in 5S-1 RNA, since there are two nucleotide substi-

FIG. 4. Transcription of 5 S DNAs in an in vitro system. The in vitro transcription was carried out as described by Sharp et al. (10) using whole cell extracts prepared from Novikoff hepatoma cells. The extracts were prepared by the method of Weil et al. (19), and [α-32P]UTP was used as precursor. 3 μg each of plasmid or phage DNA was added in each case and incubated for 1 h at 30 °C. The RNA transcripts were analyzed on a 5% polyacrylamide gel containing 7 M urea. Lane 1, no DNA added; lane 2, Chinese hamster 5 S DNA characterized by Hart and Folk (4); lane 3, clone 1 in PBR322; lane 4, clone 2 in PBR322; lanes 5-11, 5S gene variants 1, 2, 3, 4, 5, 6A, and 6B in phage DNA, respectively; lane 12, PBR322; lane 13, λ phage DNA.
Fig. 5. Fingerprinting of 5 S RNA and 5 S RNA transcripts made in vitro. The RNA transcripts designated 5 S in Fig. 4 were isolated and fingerprinted after T1 RNase treatment as described by Brownlee et al. (20). [α-32P]GTP was used as the RNA precursor for these experiments. A, 5 S RNA from Chinese hamster 5 S gene (4); B, 5 S-1, 5 S RNA from clone 1. The two bands observed in the case of clone 1 in the region of 5 S RNA (see Fig. 4) gave similar fingerprints. C, 5 S-2, 5 S RNA from clone 2. The oligonucleotides were numbered according to Forget and Weissman (34). The oligonucleotides 53', 54', 55', and 56' in 5 S-2 RNA and oligonucleotides 37' and 54' in 5 S-2 RNA had altered mobility compared to 5 S RNA (panel A). Other oligonucleotides with altered mobility are not indicated.

Fig. 6. A, restriction map around the 5 S DNA-1, 5 S DNA-2, and 5 S DNA-6. Some of the restriction enzyme sites found in and around the 5 S DNA in clones 1, 2, and 6 are shown. The solid bar on the DNA strand indicates the sequencing strategy used to obtain the sequence. B, nucleotide sequences of 5 S DNA-1 and 5 S DNA-2 and the flanking regions. Nucleotide sequences of clones 5S-1, 5S-2, and 5S-6 compared with the rat 5 S RNA sequences (26). The sequences in 5 S DNAs identical to 5 S RNA are indicated by dashes, and only substituted nucleotides are indicated. Stars indicate nucleotide deletions. The 5'-flanking sequences are indicated as -1 to -130, and the 3'-flanking sequences are indicated +1 to +75. The sequencing was carried out by the method of Maxam and Gilbert (21). The internal control region of 5 S DNA, corresponding to nucleotides 47 to 85 (27, 28), is underlined.

In this region of 5 S-1 DNA, in addition, several oligonucleotides like 53, 54, 55, and 56 had altered mobilities, as would be predicted from the nucleotide sequence of 5 S-1 DNA. Similarly, the oligonucleotides 8 (AAGG) and 17 (ACCG) are missing in the fingerprint of 5 S-2 RNA, as expected because of nucleotide substitutions in 5 S-2 DNA at positions 49 and 93 (see Fig. 6B). Many other oligonucleotides, like 37 and 54, had altered mobility as expected. The 5 S RNA transcripts from 5 S-1 and 5 S-2 DNAs contained pppGp (oligonucleotide 1'), the first nucleotide in 5 S RNA. These data show that the 5 S-sized transcripts were initiated and completed properly from 5 S-1 and 5 S-2 gene variants.

5 S RNA Sequences in Clones 1 and 2 Are Highly Homologous to but Not Identical to 5 S RNA—The 5 S DNA-contain-
Seven clones containing sequences homologous to 5 S RNA were purified and analyzed. Of these, six were found to be from different loci of the rat genome. These DNAs hybridized to 5 S RNA when a mixture of whole cell 4–8 S RNA was used. The sequence analysis of two transcribable 5 S DNAs showed that clone 5S-1 contained eight substitutions, but there were none in the sequence corresponding to the internal control region nucleotides 47 to 85 (27, 28). Clone 2, however, had five substitutions in this region. Interestingly, the 5S-2 clone was transcribed 10 times more efficiently than in authentic Chinese hamster 5S clone (4). Therefore, characterization of these naturally occurring 5S gene variants may be useful in understanding the interactions between 5S genes and the transcription factors.

In many species, the bona fide 5 S RNA genes were shown to be repeated tandemly (1–4). In some species, the pseudo-genes for 5 S RNA are found clustered as part of the tandem repeat (7–10). The differing restriction enzyme digestion patterns obtained for the six 5 S DNA clones and the observation that the 15–20-kilobase pair inserts contained only one 5 S homologous sequence shows that these 5S gene variants are dispersed in the rat genome and are probably not part of a tandem repeat.

Active and inactive forms of 5 S RNA genes were recently reported by Emerson and Roeder (11, 12) in mouse and human genomes. The analysis of the six clones in an in vitro transcription assay showed that two were transcribed and four were not. These present results are similar to those obtained by Emerson and Roeder (11, 12) and show that the rat genome contains both active and inactive 5S gene variants. However, the 5S gene variants reported by Emerson and Roeder (11, 12) were from the 5S gene cluster, and the gene variants characterized in the present study appear to be from loci dispersed in the rat genome and not from the 5S gene cluster.

The aim of this study was to isolate a true 5 S RNA gene from the rat liver genomic library. After screening the rat genomic equivalent once with authentic 5 S RNA, the isolated clones contained only 5S gene variants but no real 5S gene. The reason for this result is not clear. One possibility is that the partial EcoRI digestion of the rat genomic DNA did not produce suitable 5S gene-containing DNA fragments for cloning into the A phage. When the rat genomic DNA was digested with EcoRI enzyme, fractionated on 1% agarose gel, and hybridized with labeled 5 S RNA, the most intense signal observed corresponded to DNA fragments of more than 20 kilobase pairs in length (Fig. 2, lane SP.DNA). It is possible that the 5 S RNA genes are clustered in these long EcoRI fragments and were under-represented in the genomic bank screened. Recent studies by Emerson and Roeder (11, 12) and earlier studies by Jacq et al. (7) and Sharp et al. (10) showed that 5S gene variants are interspersed with real 5S genes in mouse, human, Drosophila, and Xenopus genomes. The present study shows that some 5S gene variants are dispersed in the rat genome, separated from real 5S genes. However, these results do not rule out the presence of 5S gene variants that may be clustered with the real 5S genes.

The origin and function of these 5S gene variants are not clear. Since the 3'-flanking sequence was conserved between two 5S clones and was probably conserved in three other 5S clones, these gene variants are unlikely to have arisen by RNA-mediated pseudogene formation and integration into the genome (23, 30). Direct repeats, a characteristic of many RNA-mediated pseudogenes, were not found flanking these 5S sequences. Therefore, these 5S gene variants may have had a common progenitor molecule and drifted through evolution.
A possibility is that of these gene variants some may be developmentally regulated and expressed. The 5S genes expressed in oocyte and adult tissues of Xenopus are different (5, 6), and a similar phenomenon may be present in the case of rat. It is also possible that these genes are expressed in adult tissues but contribute only a small percentage of total 5S RNA. Although no evidence is available for such minor 5S RNA species in rat cells, this phenomenon is well documented for 5S RNA in other species such as fungi (31, 32).

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An interesting possibility is that, in addition to having a different set of protein-coding genes, the Chinese hamster 5S gene and several other species may be present in the case of rat. It is also possible that these genes are expressed in adult tissues but contribute only a small percentage of total 5S RNA. Although no evidence is available for such minor 5S RNA species in rat cells, this phenomenon is well documented for 5S RNA in other species such as fungi (31, 32).

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