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Genes and pseudogenes in a reiterated rat tRNA gene cluster

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ABSTRACT

A 13.4 kb rat genomic DNA fragment containing two related tRNA gene clusters was isolated from a rat λ recombinant and analyzed for gene arrangement and nucleotide sequence. One cluster was found to contain a $tRNA^{Leu}_{CUG}$ gene while the second contained a $tRNA^{Leu}_{CUA}$ pseudogene with multiple base substitutions. The $tRNA^{Leu}$ gene was found to possess an intact coding region and a functional transcription termination signal at the 3' end as demonstrated by in vitro transcription and processing of precursors to mature size tRNA. The first tRNA gene cluster was found to contain in addition to $tRNA^{Leu}$, three other transcribable genes coding for $tRNA^{Asp}_{GAC(U)}$, $tRNA^{Gly}_{GGA(G)}$ and $tRNA^{Glu}_{GAG}$; the second cluster contained in addition to $tRNA^{Leu}$ pseudogene, the $tRNA^{Asp}$ $tRNA^{Gly}$ and $tRNA^{Glu}$ genes. Examination of flanking sequences of the corresponding tRNA genes in the two clusters shows no homology at the 5' ends and partial conservation of sequences at the 3'-end region. Genomic rat DNA blot hybridizations show that the $tRNA^{Leu}$ gene is distributed together with the $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ on a 10 fold repeat of 3.2 kb EcoRI fragment.

INTRODUCTION

In eukaryotes, 40-60 major tRNA species are transcribed from a multiple copy family of genes which are dispersed throughout the genome. The reiteration frequency of each specific tRNA gene varies between an average of 10 copies per haploid genome in yeast (1, 2) and Drosophila (3) to about 200 copies in Xenopus laevis (4). It has been shown that members of reiterated tRNA gene families may be clustered at several chromosomal loci as in Drosophila (5, 6) or scattered throughout the genome as in yeast (7, 8). Duplication of regions containing tRNA genes have been observed: in Drosophila (9, 10), a simple duplication of a $tRNA^{Gly}$ gene or a pair of $tRNA^{Glu}$ genes, and in Xenopus, a 300 fold repeat of a 3.18 kb sequence containing four tRNA genes (11). Relatively little information is available about the organization of tRNA genes in mammals, though several tRNA genes have been isolated from human (12-14) rat (15-18) and mouse (19, 20) genomes. Recent reports by us (16) and others (15, 17) indicate that rat tRNA genes are present as

solitary genes or in small clusters scattered on the chromosome. The number of tRNA genes in rat was estimated to be similar to that in human genome (12) and to contain 10 to 20 copies of each tRNA gene species (16).

We have previously described the isolation, from a rat gene library, of a 13.4 kb fragment containing two related clusters of four different tRNA genes (16). We report here on the organization and sequence of tRNA genes in these two clusters. One cluster contains genes coding for $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ and the sequence of a fourth tRNA gene coding for $tRNA^{Leu}$ with opposite polarity. The second cluster contains three tRNA genes, $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$, and a $tRNA^{Leu}$ pseudo gene. The frequency of distribution of these tRNA gene clusters in the rat genome was studied and a tentative model for their tandem arrangement was derived.

MATERIALS AND METHODS

Enzymes and Reagents

Restriction enzymes were purchased from New England Biolabs, T4 DNA ligase and T4 RNA ligase from PL Laboratories and T4 polynucleotide kinase from Boehringer-Mannheim. [α-^{32}P] nucleoside triphosphates, [γ-^{32}P]ATP and (5'-^{32}P)pCp were purchased from Amersham. Reverse transcriptase was obtained from Molecular Genetic Resources, Tampa FL.

Bacterial Strains and Bacteriophage

The construction of the genomic library of Sprague Dawley rat DNA, the screening of the library with [5'-^{32}P]pCp-labeled tRNA probe and the isolation of the λCh4-rC_{12} clone have been previously described (16). E. coli strain DP50 sup F was used as the host for the propagation of the recombinant phage and E. coli strain HB 101 for the propagation of plasmid DNAs derived by subcloning of rat DNA fragments in pBR322.

DNA mapping and Sequencing

Mapping of restriction enzymes cleavage sites in cloned DNAs was done by end labeling and cleavage methods (21). DNA sequencing was done by the method of Maxam and Gilbert (22) after labeling the DNA fragments at the protruding 5'-ends with [γ-^{32}P]-ATP and T4 polynucleotide kinase or by filling the 3'-recessed ends using the required [α-^{32}P] deoxynucleotide and reverse transcriptase.

Genomic DNA Blot Hybridization

Five μg rat chromosomal DNA cleaved with one or two restriction enzymes was subjected to electrophoresis on 1% agarose gel then transferred to nitrocellulose filter (23), and hybridized with nick-translated (24) DNA fragments according to Wahl et al. (25).

In vitro Transcription of Rat tRNA Genes

Xenopus germinal vesicle extracts were prepared as described by Schmidt et al. (26) and the transcription carried out as previously described (16). The synthesized RNA molecules were phenol extracted and fractionated by electrophoresis on 10% polyacrylamide 7 M urea gel using single stranded DNA size markers. For the identification of the RNA species, the RNA bands were purified by electrophoresis in the second dimension on a 20% polyacrylamide-7 M urea gel. The radioactive RNA spots were extracted from the gel by shaking with 0.5 M NaCl, 0.1 M Tris pH 7.4 and served as probes for DNA dot hybridization. In order to locate the template of the synthesized RNA species, DNA restriction fragments were spotted on nitrocellulose filters, which were layed sequentially on Whatman 3 MM papers soaked in 0.5 M NaOH, in 0.5 M Tris HCl pH 7.4 and finally in 2xSSC. The filters were then dried, baked at 80°C for 2 hours and hybridized in 2xSSC at 68°C for 18 hrs with the *in vitro* synthesized [^{32}P]labeled RNA molecules. Filters were then washed with 2xSSC at 68°, dried and autoradiographed.

RESULTS

Arrangement of tRNA Genes in λCh4-rC$_{12}$ Recombinant DNA

The DNA of bacteriophage λCh4-rC$_{12}$ containing a rat DNA insert of about 13.4 kb yields upon digestion with EcoRI three fragments: 3.2, 4.6 and 5.6 kb long. Two of these fragments, 3.2 and 5.6, contain tRNA genes as determined by hybridization with 3'-end labeled unfractionated rat tRNA probe (Fig. 1A). For a detailed analysis of this clone we have digested the λCh4-rC$_{12}$ DNA with both EcoRI and HindIII and observed that all tRNA genes are found in a repeat of 2.3 kb EcoRI-HindIII fragment separated by 8 kb of DNA (Fig. 1A). The two 2.3 kb EcoRI-HindIII fragments have been subcloned in pBR322 to generate clones prC$_{12-1}$ and prC$_{12-3}$ (Fig. 1B). Data obtained from digestion of the two subclones with a number of restriction enzymes followed by fragment separation and Southern blot hybridization with [^{32}p]-pCp-labeled unfractionated tRNA probe enabled the construction of the maps presented in Fig. 1C. It was found that BamHI removes about 300 bp from the 2.3 kb EcoRI-HindIII fragment in both prC$_{12-1}$ and prC$_{12-3}$ clones. It separates two distinct tRNA hybridizing regions within prC$_{12-1}$ (0.3 and 2.0 kb) but only one hybridizing region 2.0 kb is detected in prC$_{12-3}$. The HindIII-BamHI and BamHI-EcoRI fragments from both prC$_{12-1}$ and prC$_{12-3}$ were further subcloned at the respective sites in pBR322 to facilitate sequence analysis.

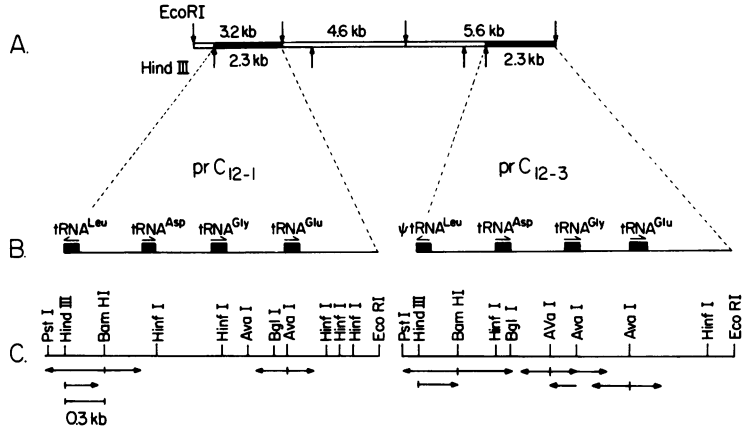

Fig. 1: Structure of 13.4 kb rat DNA fragment in λCh4-rC12 recombinant DNA

A) EcoRI and HindIII restriction sites are indicated by arrows and the regions which hybridize with [3'-^{32}P]-tRNA are marked with heavy bars.
B) Location of tRNA genes in the 2.3 kb HindIII-EcoRI fragments subcloned as prC_{12-1} and prC_{12-3}. Small arrows indicate tRNA gene orientation.
C) Restriction maps of subclones prC_{12-1} and prC_{12-3}. The arrows indicate the sequencing strategy. The Pst I site marked to the left of HindIII site is outside the subcloned 2.3 kb DNA fragments and was used for DNA sequencing from the 3.2 and 5.6 kb EcoRI fragments described in A.

Nucleotide Sequence of Subcloned Fragments Carrying tRNA Genes

The DNA sequence of the HindIII-BamHI fragments of prC_{12-1} and prC_{12-3} has been determined by the Maxam-Gilbert method (22) and is presented in Fig. 2. It appears that prC_{12-1} contains a $tRNA^{Leu}_{CUG}$ gene and prC_{12-3} has a pseudogene of $tRNA^{Leu}_{CUA}$ both oriented in the same direction. Since HindIII cleaves immediately at the end of the structural genes, the flanking 3'-end sequences were obtained by 5'-end labeling of the 0.9 kb EcoRI-HindIII and HindIII-HindIII fragments (derived from the initial 3.2 and 5.6 kb EcoRI fragments respectively) and subsequent cleavage with PstI(Fig. 1A, 1C).

The $tRNA^{Leu}_{CUG}$ gene, similar to other tRNA genes in higher eukaryotes characterized to date, lacks the CCA sequence of mature tRNA and does not possess an intervening sequence. A track of T residues in a CTTTTG arrangement, is located 9 bp downstream from $tRNA^{Leu}$ coding region. The $tRNA^{Leu}_{CUA}$ pseudogene has 15 base substitutions in the coding sequence as compared to the $tRNA^{Leu}$ gene and 9 bp downstream carries a track of Ts in the CTTTTC sequence. Analysis of the regions surrounding the $tRNA^{Leu}$ and the pseudo $tRNA^{Leu}$ genes shows homology only in the 3'-end flanking region. The DNA

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                                                                    60
a  3'  TCTACGTCCAGACACTCCCTTCCGAAGCCTCAGCCCAGAAAGCGCCACCCTACCTCACGC
b  3'  CCGCTACTTGTCCAACTCCTCCTCCGTTCTGTACGCCAGAGAACACCACCCCACTCCACG
                                                                   120
       CACCTCACAGTTTTCCGTTCGAACAGTCCTCACCCTAAGCTTGGGTGCGGAGGTCCCCTC
       CTACCTGACCTTTTCCGTTCGAACATTCCTCACCCTAAGCTTGGGTGTGGAGCTCCCCCT
                                                                   180
       TGACGCTGGACTTGCGTCGCGGAATCTGGCGAGCCGGTAGGACTGTCGTCCAGACCCCGC
       TGACGCTGGATTTGCGTTTCTGAATCTGGAAAGCCAGTAGACCCGGCCTCCGCTGAGAGC
                                                                   240
       ATCTGGCGGCGGAGCGTCCGCGCCTGGCGCGGTTCGCGCGTTCGCCCGAAGTCGAATGCC
       GTGTCCGCTGGCCGCGGGTTCACCCTTGTTCGTCGGAACTGGCCTGCCGCGCGGCAAGCC
                                                                   300
       GCGCGGCCCGTCCGCGCAGCCGGAGCAGACCAGCGCAAACCCACGAGCTCGACGCCCAGA
       CACGTCTAGACTTGCGGAGACCCACGAGTTCGACGCCCGTAGCAGGCTGCTTGAGTTGGC
                                                                   360
       GCGGCTGAAATGAAACGGTCTTCTCGTGGCTGCTGCCCTGCGACGATCCGCGGTCTCATG
       ACGAGACGAGCGAGCCATGGTCGCACAGCTGCTGGCCTGCGACGATCCGCGTTCTCACGG

       GAGTCCAGGCCCGGCCT  5'
       AGGTCAAGGCCAAACCC  5'
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Fig. 2: Nucleotide sequence of DNA fragments containing the tRNA^{Leu} gene and the tRNA^{Leu} pseudogene. Nucleotide sequences of the coding and flanking regions derived from prC12-1 (a) and prC12-3 (b) are compared using the antisense strand. The coding sequence is underlined, and the arrow indicates the gene orientation.

sequence of the tRNA^{Leu} and pseudo tRNA^{Leu} genes arranged in a clover-leaf structure is shown in Fig. 3. While tRNA^{Leu} gene is expected to form a transcript able to fold in the clover leaf configuration it is less probable that a putative transcript of the pseudogene can assume such a stable secondary structure. The base substitutions in the aminoacyl,

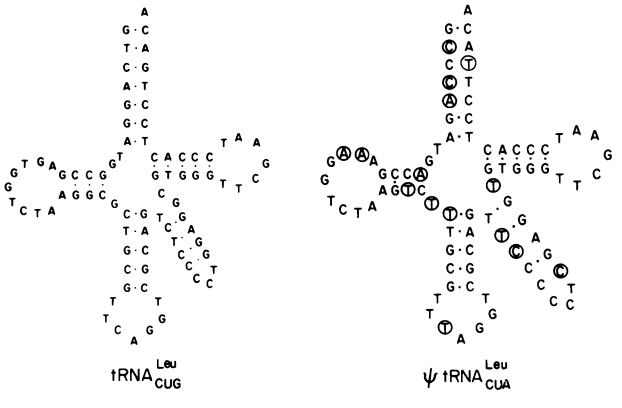

Fig. 3: Clover leaf structure derived from DNA sequences of the tRNA$^{Leu}_{CUG}$ gene and tRNA$^{Leu}_{CUA}$ pseudogene. Circled bases indicate the substituted bases in tRNA^{Leu} pseudogene.

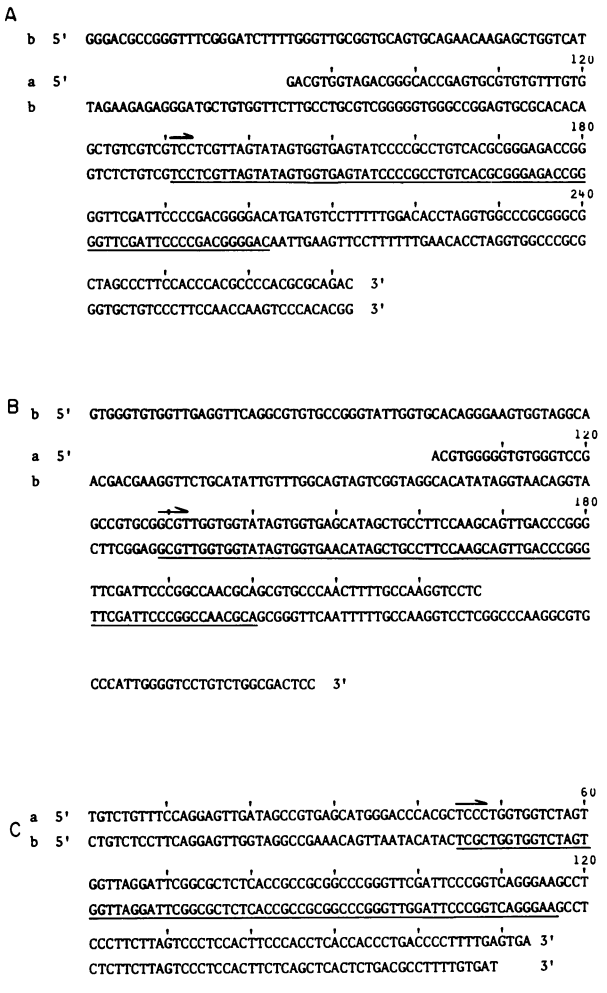

Fig. 4: Nucleotide sequence of tRNA genes and surrounding regions in BamHI-EcoRI fragment of prC_{12-1} (a) and prC_{12-3} (b).
A) DNA sequence containing the $tRNA^{Asp}$ gene
B) DNA sequence containing the $tRNA^{Gly}$ pseudogene
C) DNA sequence containing the $tRNA^{Glu}$ gene

dihydrouridine and extra-arm stems have reduced the base pairing from 7, 3 and 5 to 3, 1 and 3 respectively (Fig. 3).

Sequence analysis of the 2.0 kb BamHI-EcoRI fragments of prC_{12-1} and prC_{12-3} shows the presence of a $tRNA^{Asp}_{GAU(C)}$, $tRNA^{Gly}_{GGA(G)}$ and a $tRNA^{Glu}_{GAG}$ gene in that order (Fig. 4). These genes are oriented in opposite direction as compared to the $tRNA^{Leu}$ gene,

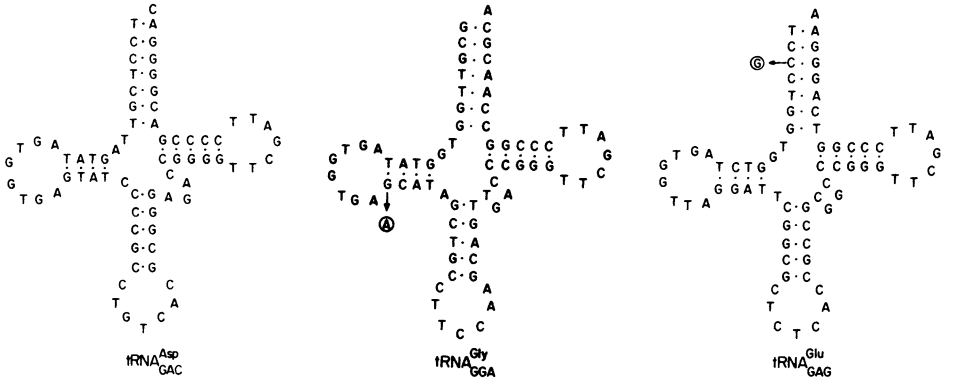

Fig. 5: Clover leaf structure for $tRNA^{Asp}_{GAC(U)}$, $tRNA^{Gly}_{GGA(G)}$ and $tRNA^{Glu}_{GAG}$ genes The circled A indicates the base substitution in $tRNA^{Gly}$ gene and the circled G indicates the base substitution in $tRNA^{Glu}$ gene from prC_{12-3} cluster.

and pseudogene. The clover leaf structure of $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ genes deduced from the DNA sequence is shown in Fig. 5. These genes do not contain intervening sequences nor the coding sequence for the 3'-terminal CCA of mature tRNAs. The $tRNA^{Gly}$ and $tRNA^{Glu}$ genes in prC_{12-3} cluster differ from the respective genes in the prC_{12-1} cluster by single base substitutions. In $tRNA^{Gly}$ gene an A replaces G in position 22 and in $tRNA^{Glu}$ a G replaces C in the third position of the coding sequence (Fig. 5), $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ genes in both clusters possess intact A and B block promoter sequences which should be able to produce transcripts folding into regular tRNA clover leaf structure. At the 3' end of each gene is a track of T residues in CTTTTTG 10 bp and CTTTTTTG 13 bp from the coding region of $tRNA^{Asp}$ gene in prC_{12-1} and prC_{12-3} cluster respectively; CTTTTG 10 bp and in ATTTTTG 11 bp from the $tRNA^{Gly}$ in prC_{12-1} and prC_{12-3} respectively; CTTTTG 48 bp and 45 bp from the $tRNA^{Glu}$ gene in prC_{12-1} and prC_{12-3} respectively.

Identification of $tRNA^{Leu}$ and $tRNA^{Asp}$, $tRNA^{Gly}$, $tRNA^{Glu}$ Gene-Containing Fragments in Rat Chromosomal DNA

Rat genome DNA cleaved with EcoRI or a mixture of EcoRI and BamHI was electrophoresed on agarose gel, transferred to a nitrocellulose filter and hybridized with nick-translated BamHI-EcoRI fragment (2.0 kb containing $tRNA^{Asp}$, $tRNA^{Gly}$, $tRNA^{Glu}$ genes) or with HindIII-BamHI fragment (0.3 kb containing $tRNA^{Leu}$ gene) derived from prC_{12-1}. The results shown in Fig. 6 indicate that the fragment containing $tRNA^{Asp}$, $tRNA^{Gly}$,

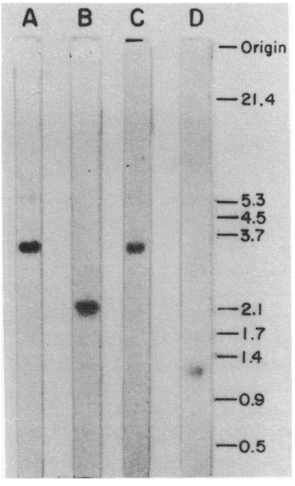

Fig. 6: Blot hybridization. Rat chromosomal DNA restricted with EcoRI (lanes A and C) or a mixture of EcoRI and BamHI (lanes B and D) was fractionated by electrophoresis on 1% agarose gel, transferred to a nitrocellulose filter and hybridized with nick-translated probes. Lanes A and B were hybridized with the 2.0 kb BamHI-EcoRI fragment of prC_{12-1} and lanes C and D were hybridized with the 0.3 kb HindIII-BamHI fragment (containing $tRNA^{Leu}$) of prC_{12-1}.

$tRNA^{Glu}$ genes hybridized mainly with a 3.2 kb and weakly with a 5.6 kb EcoRI fragment (lane A). Further restriction of genomic DNA with BamHI reduced these hybridization bands to a 2.1 kb band (lane B). The $tRNA^{Leu}$ gene-containing probe hybridized to an EcoRI fragment of 3.2 kb and to an EcoRI-BamHI fragment of 1.2 kb (Fig. 6 lanes C and D). The size of the genomic fragments hybridizing with the $tRNA^{Leu}$-containing [HindIII-BamHI] probe correspond with the 3.2 kb EcoRI fragment of the 13.4 kb rat DNA in clone $\lambda Ch\text{-}rC_{12}$. The 1.2 kb EcoRI-BamHI is derived from the 3.2 kb by restriction with BamHI (see Fig. 1A and 1C).

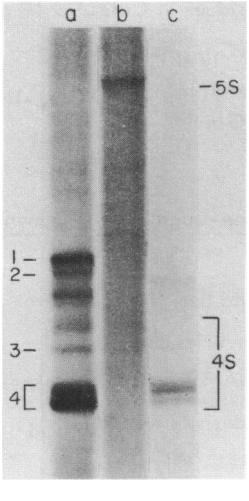

Fig. 7: In vitro transcription analysis of subclones prC_{12-1} (a), prC_{12-1} HB (b) and prC_{12-3}(c). Transcription of 0.5 μg of each plasmid DNA was carried out as described in Methods. The [α-^{32}P]UTP labeled in vitro transcription products were separated by electrophoresis on 10% polyacrylamide - 8 M urea gel. Bands 1, 2 and 3 indicate RNA molecules that hybridize with $tRNA^{Leu}$ gene-containing HindIII-BamHI (0.3 kb) fragment of prC_{12-1}. Band 4 contains three tRNA species in the transcript of prC_{12-1} and two tRNA species in the transcript of prC_{12-3} (Fig. 8).

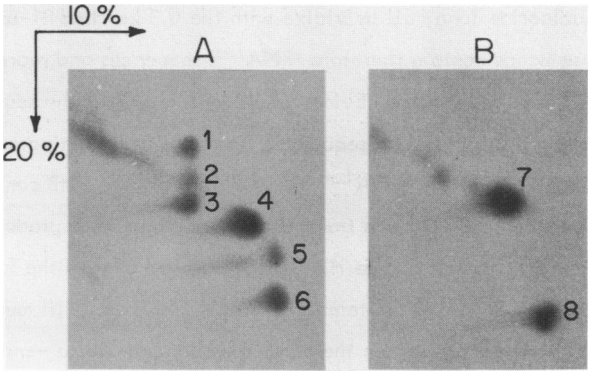

Fig. 8: Two-dimensional polyacrylamide gel electrophoresis of [α-^{32}]-UTP labeled transcription products of prC12-1 DNA (A) and prC12-3 DNA (B)

In vitro tRNA synthesis

Transcription in vitro of prC12-1 DNA by Xenopus oocyte extract and size fractionation of the products by 10% polyacrylamide gel electrophoresis reveal a number of transcripts betwee 110 and 75 nucleotides long (Fig. 7 lane a). These RNA bands were further resolved in a second dimension electrophoresis on a 20% polyacrylamide gel (Fig. 8). The RNA spots were extracted and hybridized with DNA restriction fragments of the clone spotted on nitrocellulose filters (Fig. 9). It was found that RNA bands 1, 2 and 3 (Fig. 7, lane a),

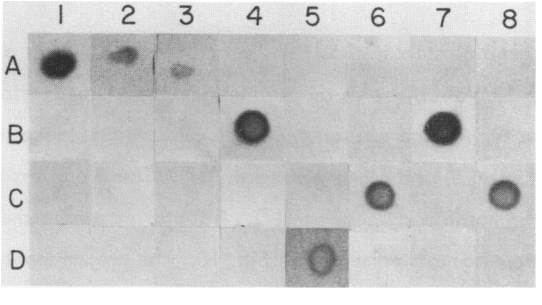

Fig. 9: Hybridization of in vitro synthesized tRNAs with DNA fragments containing individual tRNA genes. RNA spots (1 to 8) extracted from the gel described in Fig. 8 were assayed for hybridization with DNA fragments Hind III - BamH I of prC12-1 containing the tRNA^{Leu} gene (A), BamH I - Bgl I of prC12-3 containing the tRNA^{Asp} gene (B), Ava I-Ava I 180 bp fragment of prC12-3 containing 46 bp of the tRNA^{Gly} gene (C) and Bgl I-Ava I 100 bp fragment of prC12-1 containing 46 bp of tRNA^{Glu} gene (D) as described in Materials and Methods.

110, 100 and 85 nucleotide long, all hybridize with the 0.3 kb HindIII-BamHI DNA fragment (Fig. 9). These bands contain therefore $tRNA^{Leu}$ sequences and represent precursors and mature form of this tRNA species. Evidence was obtained that the sequence CTTTTG, 9 bp downstream from $tRNA^{Leu}$ coding sequence, functions as a terminator signal in the in vitro system. A subclone of the $tRNA^{Leu}$ gene region, prC_{12-1} HB containing the 0.3 kb HindIII-BamHI fragment in pBR322, was transcribed in a larger RNA product about 150 nucleotides long (Fig. 7, lane b). Since HindIII has cleaved next to the last coding nucleotide of $tRNA^{Leu}$ gene removing the terminator, RNA polymerase III must have used an alternative termination sequence within the pBR322 sequence. This 3'-end elongated transcript of $tRNA^{Leu}$ gene must have some difficulty in processing since only traces of mature size $tRNA^{Leu}$ were observed. No RNA molecules derived from the $tRNA^{Leu}$ pseudogene were obtained by transcription in vitro of the tRNA gene cluster from prC_{12-3} DNA (Fig. 7, lane c and Fig. 8B). RNA band 4, indicated in Fig. 7, lane a and lane c, contains three tRNA species for the transcript derived from prC_{12-1} (Fig. 8A, spots 4, 5 and 6) and only two tRNA species for the transcript derived from prC_{12-3} (Fig. 8B, spots 7 and 8). These tRNA transcripts were extracted from spots of the gel and hybridized with DNA fragments from prC_{12-1} and prC_{12-3} clones containing individual tRNA genes. The results presented in Fig. 9 show that transcription of the tRNA gene cluster of prC_{12-1} produces $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ (spots 4, 5 and 6) while the similar cluster of prC_{12-3} yields only two tRNA species $tRNA^{Asp}$ and $tRNA^{Gly}$ (spots 7 and 8)

DISCUSSION

The two tRNA gene clusters in the 13.4 kb rat DNA fragment of clone $\lambda Ch4\text{-}rC_{12}$ reported in this study are not unique in the rat chromosome. Examination of the structure and sequence of these tRNA gene clusters reveals a close resemblance with similar clusters isolated by Sekiya et al. (15) and Makowski et al. (27). These clusters form a family of about ten copies in the haploid rat genome (17). A tentative arrangement of the repeated clusters in the rat chromosome is presented in Fig. 10. The predominant unit (A in Fig. 10) is a 13.4 kb segment containing the tRNA genes in a 3.2 kb EcoRI fragment followed by 0.2, 8 and 2 kb EcoRI fragments. This segment must be repeated several times since five independent clones containing this arrangement have been reported in Sprague Dawley rat (17, 27). In one case the 3.2 kb EcoRI fragment in A (Fig. 10) is preceded by a 1.2 kb EcoRI segment (17) and in another, the 3.2 kb EcoRI fragment is shortened by deletions to 2.4 kb (28). The other unit (B in Fig. 10) containing two tRNA gene clusters in a 13.4 kb fragment was

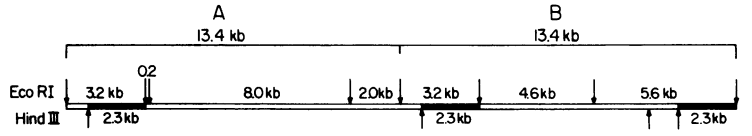

Fig. 10: Tentative arrangement of the tRNA^{Leu}, tRNA^{Asp}, tRNA^{Gly}, tRNA^{Glu} gene cluster in rat genome. Solid bars indicate tRNA gene clusters. Unit A must be repeated at least seven times (17, 27, 28) while unit B was observed only in λCh4-pC_{12}, clone (Fig. 1).

found only in our clone λCh4-rC_{12} and may be unique in the chromosome. This conclusion is supported by the low intensity of the hybridization band of 5.6 kb as compared to the dominant 3.2 kb in the EcoRI restricted rat DNA (Fig. 6, lane A).

Comparison of the sequences among the tRNA gene clusters revealed that there is sequence heterogeneity among the repeats. The first tRNA gene cluster in λCh4-rC_{12}, subcloned as prC_{12-1} was found to contain in a 2.0 kb BamHI-EcoRI fragment (Fig. 1) three intact, transcribable tRNA genes tRNA^{Asp}, tRNA^{Gly} and tRNA^{Glu}, in this order. The sequence of this fragment was found to be highly homologous to a 2.1 kb BamHI-EcoRI fragment (RTIdg clone) of the Donryu rat of Sekiya et al. (15). The sequence of the tRNA^{Glu} gene isolated by us (Fig. 5) presents a T to C transition at position 4 as reported for all the tRNA^{Glu} genes and pseudogenes isolated from five other repeats of the family derived from the Sprague Dawley rat genome (17, 27). The tRNA gene cluster in our prC_{12-1} clone, which seems to be identical with the RTIdg gene cluster isolated from Donryu strain of rat, is so far the only repeat isolated from the Sprague Dawley rat which contains three intact, transcribable tRNA genes. In five other copies of the reiterated cluster isolated from this rat stain it was found that while tRNA^{Asp} gene sequence was highly conserved, three repeats possess tRNA^{Glu} pseudogenes and all five contain tRNA^{Gly} pseudogenes (17, 27). We have isolated from the prC_{12-1} clone a fourth tRNA gene coding for tRNA^{Leu}, with opposite polarity to the other three genes (Fig. 1 and Fig. 2). This is an intact, in vitro transcribable gene (Fig. 7 lane a) whose sequence shows high similarity to that of cow mammary gland tRNA^{Leu} (29) differing from it in three positions (20, 21 in D loop and 77 in the aminoacyl stem). One other characterized rat tRNA^{Leu} gene appears to possess a G to A transition in the extra arm (27) and a gene isolated by Sekiya et al. (28) had two point mutations at position 49 and 77. The tRNA^{Leu} sequence appears to be present in all the reiterated clusters containing the tRNA^{Asp}, tRNA^{Gly} and tRNA^{Glu} genes. Hybridization of genomic blots derived from EcoRI or EcoRI and BamHI restricted rat DNA with nick translated 0.3 kb HindIII-

BamHI fragment containing $tRNA^{Leu}$ gene shows radioactive DNA bands corresponding to 3.2 and 1.2 kb chromosomal fragments respectively (Fig. 6 lanes C and D). Hybridization of the EcoRI-cleaved chromosomal DNA blot with the 2.0 kb BamHI-EcoRI probe (containing the three tRNA genes) shows a main radioactive band corresponding also to a 3.2 kb fragment (Fig. 6 lane A). These findings indicate that the sequences of the $tRNA^{Leu}$-containing DNA fragment are present always together with the genes for $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ on the 3.2 kb EcoRI repeat (Fig. 1). In one case, reported by Sekiya et al. (28) the cluster is shortened by deletion to 2.4 kb and was found to contain only the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes. Another member in the multicopy family is the second cluster in our $\lambda Ch4\text{-}rC_{12}$ recombinant phage subcloned as $prC_{12\text{-}3}$. This repeat was found to contain three tRNA genes, coding for $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$, and a $tRNA^{Leu}$ pseudogene. It should be observed that, with the exception of $tRNA^{Asp}$ gene, all tRNA sequences in $prC_{12\text{-}3}$ cluster are affected by point mutations. While $tRNA^{Glu}$ and $tRNA^{Gly}$ have a single base substitution (Fig. 5), the $tRNA^{Leu}$ pseudogene possesses 15 base substitutions in the coding region (Fig. 3). Two control elements for RNA polymerase III within the coding region of eukaryotic tRNA genes have been identified: the A block (positions 8-19) and B block (positions 52-62) (30). In $tRNA^{Leu}$ pseudogene the three base substitutions at positions 10, 15 and 16 in the A block sequence decrease the homology to the concensus sequence (31) and may have an adverse effect on the transcription of this gene. In fact no transcription products of the $tRNA^{Leu}$ pseudogene have been detected in the cell-free system which may indicate that it cannot serve as template for RNA polymerase III. However, due to multiple point mutations in the aminoacyl, dihydrouridine and extra arm stems, an unstable secondary structure and inacurate processing of a putative transcript of this gene cannot be excluded.

Analysis of the sequences for 5' and 3' flanking regions in the two tRNA gene clusters of 13.4 kb rat DNA fragment presently studied in comparison with other gene clusters of this family, allows us to make some conclusions concerning the conservation of sequences outside the coding region. First, the sequences flanking the tRNA in $prC_{12\text{-}1}$ cluster show a close homology to those in other similar gene clusters studied by Sekiya et al. (17). This homology extends on both sides of the genes, the 3' non coding region being generally more conserved than the 5' non coding region. On the other hand the sequences surrounding the tRNA genes in $prC_{12\text{-}3}$ cluster show considerable divergence from those of other clusters: little or no homology in the 5' flanking region and only partial homology, between 9 to 50 bp, in the 3'-non coding region interrupted by base substitution, deletions

and insertions of nucleotides. In addition, patches of partial sequence homologies are observed to occur also in the intergene spacers of prC_{12-3} cluster as compared to prC_{12-1} or other clusters (15, 27). The results presented here, together with the data from other different clusters containing the $tRNA^{Leu}$, $tRNA^{Asp}$, $tRNA^{Gly}$, $tRNA^{Glu}$ genes (17, 27) support the assumption that this tRNA gene family was derived from cluster duplication followed by mutations and deletions of nonessential sequences.

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