Genes and pseudogenes in a reiterated rat tRNA gene cluster

Ada Rosen, Sara Sarid* and Violet Daniel

Departments of Biochemistry and *Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT

A 13.4 kb rat genomic DNA fragment containing two related tRNA gene clusters was isolated from a λ recombinant and analyzed for gene arrangement and nucleotide sequence. One cluster was found to contain a tRNA^Leu^ gene while the second contained a tRNA^Leu^ 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^Glu^, tRNA^Gly^ and tRNA^Glu^; the second cluster contained in addition to tRNA^Leu^ pseudogene, the tRNA^Asp^, tRNA^Glu^ 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. \([a-\text{32P}]\) nucleotide triphosphates, \([\gamma-\text{32P}]\)ATP and (5'-32P)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'-32P-labeled tRNA probe and the isolation of the \(\chi\)Ch4-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 \([\gamma-\text{32P}]\)ATP and T4 polynucleotide kinase or by filling the 3'-recessed ends using the required \([a-\text{32P}]\)deoxynucleotide and reverse transcriptase.

**Genomic DNA Blot Hybridization**

Five \(\mu\)g 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 [32p] labeled RNA molecules. Filters were then washed with 2xSSC at 68°C, dried and autoradiographed.

RESULTS

Arrangement of tRNA Genes in λCh4-rC12 Recombinant DNA

The DNA of bacteriophage λCh4-rC12 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-rC12 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 prC12-1 and prC12-3 (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 [32p]γ-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 prC12-1 and prC12-3 clones. It separates two distinct tRNA hybridizing regions within prC12-1 (0.3 and 2.0 kb) but only one hybridizing region 2.0 kb is detected in prC12-3. The HindIII-BamHI and BamHI-EcoRI fragments from both prC12-1 and prC12-3 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 \(3'-32p\) t-RNA are marked with heavy bars.
B) Location of tRNA genes in the 2.3 kb HindIII-EcoRI fragments subcloned as prC12-1 and prC12-3. Small arrows indicate tRNA gene orientation.
C) Restriction maps of subclones prC12-1 and prC12-3. 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 prC12-1 and prC12-3 has been determined by the Maxam-Gilbert method (22) and is presented in Fig. 2. It appears that prC12-1 contains a tRNA^Leu^ gene and prC12-3 has a pseudogene of tRNA^Leu^ 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 Pst I (Fig. 1A, 1C).

The tRNA^Leu^ 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 CTTTTC arrangement, is located 9 bp downstream from tRNA^Leu^ coding region. The tRNA^Leu^ 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
Fig. 2: Nucleotide sequence of DNA fragments containing the tRNA\textsubscript{Leu} gene and the tRNA\textsubscript{Leu} pseudogene. Nucleotide sequences of the coding and flanking regions derived from prC\textsubscript{12-1} (a) and prC\textsubscript{12-3} (b) are compared using the antisense strand. The coding sequence is underlined, and the arrow indicates the gene orientation.

sequence of the tRNA\textsubscript{Leu} and pseudo tRNA\textsubscript{Leu} genes arranged in a clover-leaf structure is shown in Fig. 3. While tRNA\textsubscript{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\textsubscript{Leu} gene and tRNA\textsubscript{Leu} pseudogene. Circled bases indicate the substituted bases in tRNA\textsubscript{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
dihydouridine 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 tRNASp, tRNA Gly, and tRNAGlu 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 tRNAGlu genes in prC12-3 cluster differ from the respective genes in the prC12-1 cluster by single base substitutions. In tRNA Gly gene an A replaces G in position 22 and in tRNAGlu a G replaces C in the third position of the coding sequence (Fig. 5), tRNASp, tRNA Gly and tRNAGlu 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 CTTTTTG 13 bp from the coding region of tRNASp gene in prC12-1 and prC12-3 cluster respectively; CTTTTG 10 bp and in ATTTTTG 11 bp from the tRNA Gly in prC12-1 and prC12-3 respectively; CTTTTG 48 bp and 45 bp from the tRNAGlu gene in prC12-1 and prC12-3 respectively.

Identification of tRNA Leu and tRNASp, tRNA Gly, tRNAGlu Gene-Containing Fragments in Rat Chromosomal DNA

Rat genome DNA cleaved with EcoRI or a mixture of EcoRI and BamHI was electrophoresed on an agarose gel, transferred to a nitrocellulose filter and hybridized with nick-translated BamHI-EcoRI fragment (2.0 kb containing tRNASp, tRNA Gly, tRNAGlu genes) or with HindIII-BamHI fragment (0.3 kb containing tRNA Leu gene) derived from prC12-1. The results shown in Fig. 6 indicate that the fragment containing tRNASp, 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\text{12-1} and lanes C and D were hybridized with the 0.3 kb HindIII-BamHI fragment (containing tRNA\text{Leu}) of prC\text{12-1}.

\text{tRNA}\text{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 \text{tRNA}\text{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 \text{tRNA}\text{Leu}-containing [HindIII-BamHI] probe correspond with the 3.2 kb EcoRI fragment of the 13.4 kb rat DNA in clone \lambda \text{Ch }-\text{C}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\text{12-1} (a), prC\text{12-1} HB (b) and prC\text{12-3} (c). Transcription of 0.5 \mu g of each plasmid DNA was carried out as described in Methods. The \Gamma a-\text{32P} 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 \text{tRNA}\text{Leu} gene-containing HindIII-BamHI (0.3 kb) fragment of prC\text{12-1}. Band 4 contains three \text{tRNA} species in the transcript of prC\text{12-1} and two \text{tRNA} species in the transcript of prC\text{12-3} (Fig. 8).
Fig. 8: Two-dimensional polyacrylamide gel electrophoresis of \( \alpha^{32}P \)-UTP labeled transcription products of prC\(_{12-1}\) DNA (A) and prC\(_{12-3}\) DNA (B)

**In vitro tRNA synthesis**

Transcription in vitro of prC\(_{12-1}\) DNA by Xenopus oocyte extract and size fractionation of the products by 10% polyacrylamide gel electrophoresis reveal a number of transcripts between 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 - BamHI of prC\(_{12-1}\) containing the tRNA\(^{\text{Leu}}\) gene (A), BamHI - Bgl I of prC\(_{12-3}\) containing the tRNA\(^{\text{Asp}}\) gene (B), Ava I-Ava I 180 bp fragment of prC\(_{12-3}\) containing 46 bp of the tRNA\(^{\text{Gly}}\) gene (C) and Bgl I-Ava I 100 bp fragment of prC\(_{12-1}\) containing 46 bp of tRNA\(^{\text{Glu}}\) gene (D) as described in Materials and Methods.
110, 100 and 85 nucleotide long, all hybridize with the 0.3 kb Hind III–BamHI DNA fragment (Fig. 9). These bands contain therefore tRNA\textsuperscript{Leu} sequences and represent precursors and mature form of this tRNA species. Evidence was obtained that the sequence CTTTG, 9 bp downstream from tRNA\textsuperscript{Leu} coding sequence, functions as a terminator signal in the in vitro system. A subclone of the tRNA\textsuperscript{Leu} gene region, prC\textsubscript{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\textsuperscript{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\textsuperscript{Leu} gene must have some difficulty in processing since only traces of mature size tRNA\textsuperscript{Leu} were observed. No RNA molecules derived from the tRNA\textsuperscript{Leu} pseudogene were obtained by transcription in vitro of the tRNA gene cluster from prC\textsubscript{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\textsubscript{12-1} (Fig. 8A, spots 4, 5 and 6) and only two tRNA species for the transcript derived from prC\textsubscript{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\textsubscript{12-1} and prC\textsubscript{12-3} clones containing individual tRNA genes. The results presented in Fig. 9 show that transcription of the tRNA gene cluster of prC\textsubscript{12-1} produces tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Gly} and tRNA\textsuperscript{Glu} (spots 4, 5 and 6) while the similar cluster of prC\textsubscript{12-3} yields only two tRNA species tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Gly} (spots 7 and 8).

**DISCUSSION**

The two tRNA gene clusters in the 13.4 kb rat DNA fragment of clone λCh4–C\textsubscript{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\textsuperscript{Leu}, tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Gly}, tRNA\textsuperscript{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-ρC\textsubscript{12}, clone (Fig. 1).

found only in our clone λCh4-ρC\textsubscript{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-ρC\textsubscript{12}, subcloned as prC\textsubscript{12-1} was found to contain in a 2.0 kb BamHI-EcoRI fragment (Fig. 1) three intact, transcribable tRNA genes tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Glu}, and tRNA\textsuperscript{Glu}, in this order. The sequence of this fragment was found to be highly homologous to a 2.1 kb BamHI-EcoRI fragment (RT1dg clone) of the Donryu rat of Sekiya et al. (15). The sequence of the tRNA\textsuperscript{Glu} gene isolated by us (Fig. 5) presents a T to C transition at position 4 as reported for all the tRNA\textsuperscript{7} 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\textsubscript{12-1} clone, which seems to be identical with the RT1dg 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 strain it was found that while tRNA\textsuperscript{Asp} gene sequence was highly conserved, three repeats possess tRNA\textsuperscript{Glu} pseudogenes and all five contain tRNA\textsuperscript{Gly} pseudogenes (17, 27). We have isolated from the prC\textsubscript{12-1} clone a fourth tRNA gene coding for tRNA\textsuperscript{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\textsuperscript{Leu} (29) differing from it in three positions (20, 21 in D loop and 77 in the aminoacyl stem). One other characterized rat tRNA\textsuperscript{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\textsuperscript{Leu} sequence appears to be present in all the reiterated clusters containing the tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Gly} and tRNA\textsuperscript{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\textsubscript{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\textsubscript{Leu}-containing DNA fragment are present always together with the genes for tRNA\textsubscript{Asp}, tRNA\textsubscript{Gly} and tRNA\textsubscript{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\textsubscript{Leu} and tRNA\textsubscript{Asp} genes. Another member in the multicopy family is the second cluster in our \(\lambda\text{Ch4-rC}_{12}\) recombinant phage subcloned as prC\textsubscript{12-3}. This repeat was found to contain three tRNA genes, coding for tRNA\textsubscript{Asp}, tRNA\textsubscript{Gly} and tRNA\textsubscript{Glu}, and a tRNA\textsubscript{Leu} pseudogene. It should be observed that, with the exception of tRNA\textsubscript{Asp} gene, all tRNA sequences in prC\textsubscript{12-3} cluster are affected by point mutations. While tRNA\textsubscript{Glu} and tRNA\textsubscript{Gly} have a single base substitution (Fig. 5), the tRNA\textsubscript{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\textsubscript{Leu} pseudogene the three base substitutions at positions 10, 15 and 16 in the A block sequence decrease the homology to the consensus sequence (31) and may have an adverse effect on the transcription of this gene. In fact no transcription products of the tRNA\textsubscript{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 inaccurate 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\textsubscript{12-1} 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\textsubscript{12-3} 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 prC12-3 cluster as compared to prC12-1 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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