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Molecular cloning, sequence analysis and *in vitro* expression of a rat tRNA gene cluster

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ABSTRACT

A rat genomic DNA fragment containing a tRNA gene cluster was isolated from a lambda phage library. Hybridization and nucleotide sequence analysis revealed the presence of a 83 bp $tRNA^{Leu}_{CUG}$ gene and a 72 bp $tRNA^{Asp}_{GUG}$ gene. Both genes possessed intact coding regions and putative transcription termination signals at their respective 3' ends. In vitro transcription analysis of the two subcloned genes in a HeLa cell S-100 system demonstrated the specific synthesis of a number of RNAs by RNA polymerase III. Studies carried out in the presence of α-amanitin showed that the larger RNAs are precursors for the final processed transcripts of the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes, respectively. Further nucleotide sequence analysis of the cluster revealed the presence of $tRNA^{Gly}$ and a $tRNA^{Glu}$ pseudogenes with missing areas within their coding regions which are essential for transcription by RNA polymerase III. Within the region of DNA between the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes is a sequence which is 65% homologous to a region of the rat B1 element. The significance of this latter structure within the gene cluster is unknown.

INTRODUCTION

The combination of molecular cloning, rapid DNA sequence analysis and in vitro transcription techniques has led, in the past few years, to a better understanding of the chromosomal arrangement and mechanism of expression of transfer RNA genes in a number of cell types. Those tRNA genes analyzed so far have been found to possess two properties: reiteration and dispersion throughout the genome. Although most eukaryotes contain approximately 40 to 60 major tRNA species, the reiteration frequency of each unique tRNA gene has been found to vary between about 10 copies per cell per tRNA in yeast and Drosophila (1,2) to about 200 copies per cell per tRNA in Xenopus laevis (3). It has been determined that members of reiterated tRNA gene families are either dispersed throughout the genome, as in yeast (4,5) or clustered at different chromosomal loci, as in Drosophila (6). Frequently, several different tRNA genes (some present in multiple copies) are present at one locus, often having

opposite polarities (for a review of tRNA gene structure in eukaryotes, see Reference 7).

Much information has accumulated on the structure, arrangement and expression of eukaryotic tRNA genes from yeast, nematode, silkworm, fruit fly and frog, but little is known about the tRNA genes of vertebrates. To date, transfer RNA genes have been isolated from the genomes of humans (8), mice (9,10), and different rat strains (11-14). Data obtained from analysis of these cloned DNAs has not led to the formulation of any rules for either the mechanism of generation of these gene clusters or their organization within the vertebrate genome. In this study, we screened a rat genomic DNA phage library with total tRNA isolated from rat liver and obtained a 3.2 kb cluster which, after partial DNA sequencing, revealed the presence of $tRNA^{Leu}_{CUG}$ and $tRNA^{Asp}_{GAC}$ genes and $tRNA^{Glu}$ and $tRNA^{Gly}$ pseudogenes. In vitro transcription generated tRNA precursors which were processed into the 86 and 75 base final transcripts of the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes, respectively.

MATERIALS AND METHODS

Bacterial strains, bacteriophage and plasmids

All plasmids were maintained in E. coli HB101. Lambda phage was propagated in E. coli LE392 or DP50 supF. M13 phage clones were grown in E. coli JM103 obtained from BRL Inc. (Gaithersburg, MD). Plasmid pACYC177 was kindly supplied by Dr. David Figurski. Lambda phage Rt-61 and subcloned plasmid DNAs pRt-61, pRt-870 and pRt-460 were isolated and constructed as described in the text. Clones pRt-61, pRt-870 and pRt-460 are derivatives of plasmid vectors pBR325, pACYC177 and pBR322, respectively. The genomic library of Sprague-Dawley rat DNA was constructed as described (15) and generously provided by Drs. T. Sargent and J. Bonner. Replicative forms of M13mp7, mp8 and mp9 DNA were obtained from BRL, Inc.

Enzymes and Radioisotopes

Restriction enzymes were obtained from BRL, Inc. or Boehringer Mannheim (Indianapolis, IN). Calf intestine alkaline phosphatase, DNA polymerase I and its derived Klenow fragment, and T4 polynucleotide kinase were all obtained from Boehringer Mannheim. The exonuclease Bal-31 was obtained from New England Nuclear, (Boston, MA). All radioisotopes were obtained from Amersham Corp. (Arlington Heights, IL), with specific activities as indicated.

Isolation of tRNA

Crude rat liver tRNA was prepared from male Sprague-Dawley rat liver and separated from ribosomal RNA as previously described (16). Labeling at the 5' -end of the tRNA was accomplished by the use of T4 polynucleotide kinase and [γ-^{32}P]-ATP (spec. act. 3,000 Ci/mmol) (17). Specific labeling at the 3' -end of the tRNA was obtained by using a crude preparation of E. coli tRNA nucleotidyl transferase, prepared as described (18). The labeling reaction was carried out (19) using [α-^{32}P]ATP (spec. act. 300-400 Ci/mmol).

Isolation of phage clone Rt-61 and subcloning of tRNA genes

Approximately 75,000 individual clones from the Eco RI phage library (15) were screened by the method of Benton and Davis (20). The strongest hybridizing clone, named Rt-61 was purified by subsequent rounds of plaque purification and restriction mapped by standard techniques. Regions indicated in Figure 2 were subcloned into appropriate plasmid vectors (21) and identified by the colony screening technique of Grunstein and Hogness (22) using nick-translated fragments isolated from low melting point agarose (21,23).

Chemical cleavage sequence analysis

DNA fragments were treated with calf intestine alkaline phosphatase at 65^{o} for two hours, extracted with phenol and precipitated with three volumes of absolute ethanol. Radiolabeling was achieved by either treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP (spec. act. 3,000 Ci/mmol) (24) or Klenow fragment and [α-^{32}P]dNTP (25). Chemical cleavage reactions and denaturing gel electrophoresis were performed as described (24,26).

M13 Phage cloning and dideoxy sequence analysis

M13 phage clones were generated by the methods of Messing et al. (27, 28). Deletion clones were generated as outlined (21) using the enzyme Bal-31 and ligated into the Hinc II site of M13 mp7 RF DNA. The preparation of ssDNA from M13 phage particles was done by the method of Anderson (29). Sequencing of M13 phage ssDNA was accomplished essentially as described (30) with some modifications (27). Regions with significant secondary structure were resequenced by the dITP substitution method (31).

Secondary structure analysis of some of the DNA sequence data was analyzed by the use of two programs called BASEPR and OVRLAP (32) run on a VAX 11/780 system. The rest of the sequence information was processed on

a Hewlett-Packard HP9845C graphics computer (M. McLaughlin, unpublished results).

In vitro transcription

A cell-free extract was prepared from HeLa cells (33). Supercoiled plasmid DNA (final concentration 50 µg/ml) was incubated at 30° for 1 or 2 h in a mixture containing 50% (v/v) HeLa extract, 50 µM (each) GTP, ATP, UTP, and CTP, 10µCi of [α-^{32}P]GTP, 10 mM creatine phosphate, 7mM $MgCl_2$, 75 mM KCl, 15 mM HEPES (pH 7.9). Reactions were terminated by addition of an equal volume of 0.1 M EDTA, phenol extraction and ethanol precipitation (34). RNA transcripts were analyzed on 8% polyacrylamide gels as described above using single-stranded DNA size markers.

RESULTS

Isolation and characterization of lambda phage clone Rt-61

By screening the Eco RI lambda phage library with 5' -end labeled rat liver tRNA, we isolated and purified a recombinant bacteriophage which hybridized strongly to the radiolabeled tRNA. DNA of this bacteriophage, termed Rt-61, digested with Eco RI, showed that the total phage insert is approximately 13.2 kb long and possesses two internal Eco RI sites, thus generating the three bands of 8.0 kb, 3.2 kb, and 2.0 kb seen in lane 2 of Figure 1. Probing with 3' -end labeled tRNA indicated that all tRNA-hybridizing ability was present in the 3.2 kb fragment (Figure 1, lane 3). Digestion of the DNA with Kpn I localized the 3.2 kb fragment to the left arm of the Charon 4A phage vector (Figure 2). This Eco RI fragment was subcloned into the plasmid vector pBR325 to generate clone pRt-61 (Figure 1, lane 4). Data obtained from digestion of pRt-61 DNA with a number of different restriction enzymes followed by analytical gel electrophoresis was used to construct the restriction map at the bottom of Figure 2. The pattern of hybridization obtained with the enzymes Ava I (Figure 1, lanes 8 and 9) and Pst I (data not shown) demonstrated the presence of two distinct tRNA hybridizing regions within the clone. These two regions were subcloned as indicated at the bottom of Figure 2 to facilitate further analysis. The Pst I fragment was cloned into the unique Pst I site of pBR322, and the larger hybridizing Ava I fragment was cloned into the unique Sma I site of plasmid pACYC177.

Nucleotide sequence of clone pRT-61

The nucleotide sequence of the DNA in and around the the tRNA genes was determined by a combination of chemical cleavage (24) and dideoxy

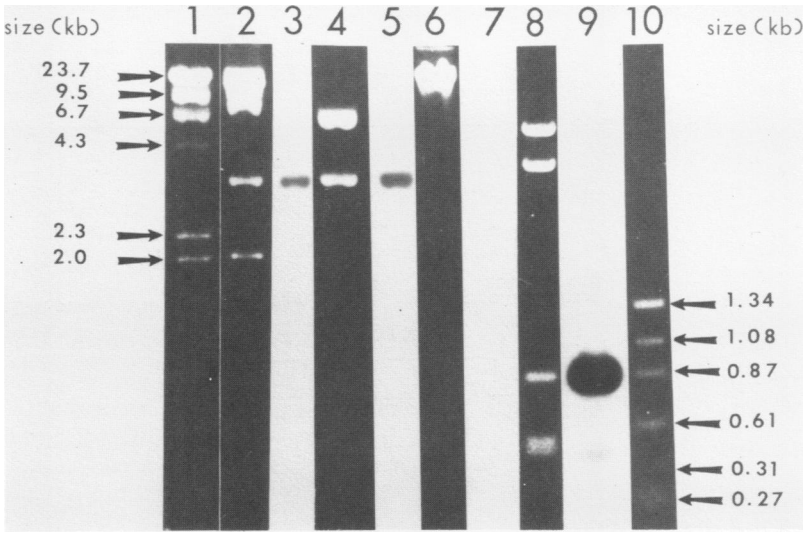

Figure 1. Southern blot analysis of clone Rt-61. Lanes 2 and 4 are Eco RI digests of phage RT-61 and the 3.2 kb RI fragment subcloned in pBR325. Lane 6 is a λ clone containing rat rRNA gene sequences as a negative control for the [^{32}P]-labeled tRNA probe. Lane 8 is an Ava I digest of the pBR325 subclone. Lanes 1 and 10 are Hind III digested λ DNA and Hae III digested ØX174 RF DNA, with corresponding sizes of fragments to the left and right, respectively. Lanes 3, 5, 7, and 9 are the autoradiographs of a Southern blot of lanes 2, 4, 6, and 8, respectively, hybridized with 3′ end-labeled tRNA from rat liver. Exposure time is 16 hours.

methods (30). Figure 3 delineates the starting points and the major routes covered for sequencing by the two techniques. The nucleotide sequence of the two genes, $tRNA^{Leu}_{CUG}$ and $tRNA^{Asp}_{GAC}$, the two pseudogenes, $tRNA^{Gly}$ and $tRNA^{Glu}$ and their flanking regions is presented in Figure 4. By viewing the genes on the map in Figure 3 in their proper orientation, it can be seen that the $tRNA^{Asp}$ gene and both pseudogenes utilize one coding strand of the DNA and the $tRNA^{Leu}$ gene the other DNA strand.

The 83 bp $tRNA^{Leu}$ and 72 bp $tRNA^{Asp}$ genes are typical of eukaryotic tRNA genes characterized to date. Neither gene codes for the terminal C-C-A sequence usually found in the final tRNA transcript, nor does either possess any intervening sequences. At the 3' -end of each gene is a tract of T residues, 9 bp from the coding region of the $tRNA^{Leu}$ gene and 10 bp from the coding region of the $tRNA^{Asp}$ gene. These sequences have been implicated in the termination of RNA polymerase III transcription (35). The proposed cloverleaf structure of each of these genes is shown in

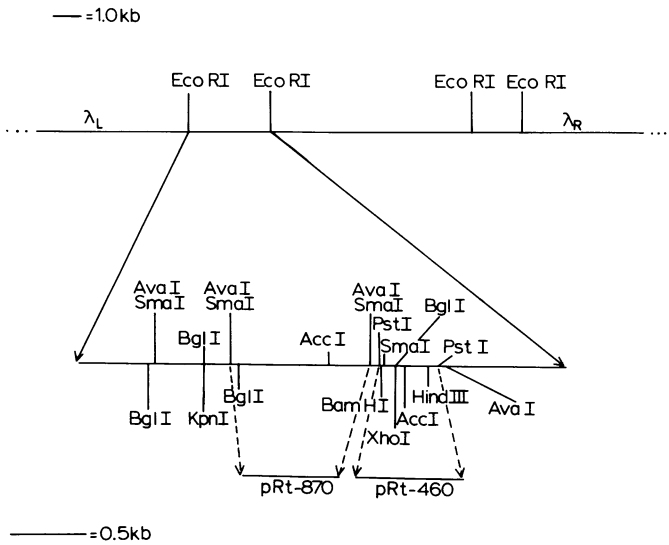

Figure 2. Restriction map of clone Rt-61. The upper line represents the structure of lambda clone Rt-61 with Eco RI sites as indicated. A size bar above the line shows the scale. The 3.2 kb Eco RI fragment was subcloned into pBR325 as described in Materials and Methods. The map of this subclone is shown on the lower line. The scale for this map is on the bottom of the figure. The two regions hybridizing to tRNA as shown in Figure 1, were subcloned and designated pRt-870 and pRt-460 as shown on the bottom of this figure.

Figure 5. It can be seen that the structures are consistent with the consensus features derived from the tRNAs and tRNA genes analyzed to date. Each gene is capable of generating a transcript which folds into a cloverleaf containing a 7 base aminoacyl stem, 5 base anticodon and pseudouridine stems and 3 or 4 base dihydrouridine stems. Both genes possess A and B block sequences, the split promoter elements of tRNA genes (36), which are localized around their dihydrouridine and pseudouridine arms.

The proposed cloverleaf structures for the $tRNA^{Gly}$ and $tRNA^{Glu}$ pseudogenes are similarly presented in Figure 5. The actual backbone sequences presented in the figures are those of two functional genes isolated from the Donryu rat genome (11). In the $tRNA^{Gly}$ sequence, the boxed nucleotides in the D arm are the 7 bp which are missing in our pseudogene. The two boxed bases at the 3' -end of the gene have been replaced by two bases (A and T) which lower the stability of the pseudouridine stem. In the same figure, the 17 bp of sequence missing

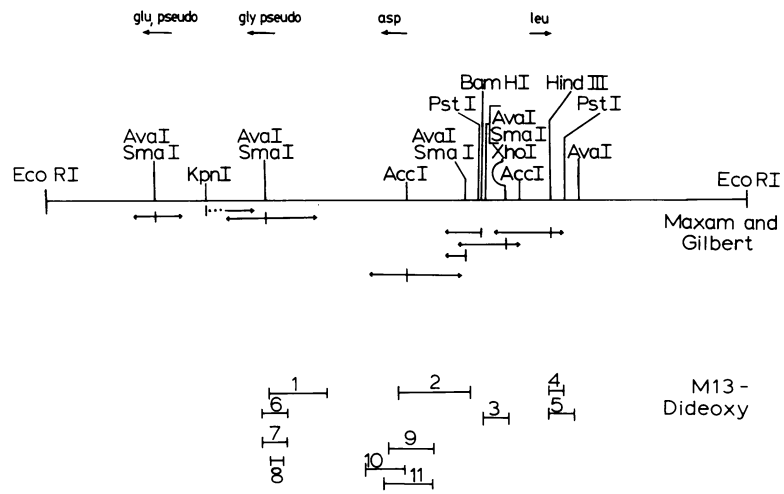

Figure 3. DNA sequencing strategy. Vertical lines depict the sites for radioactive labeling for Maxam-Gilbert sequencing. The arrows indicate the direction and extent of sequencing from a particular restriction fragment. The dotted line to the right of the Kpn I site represents a region whose sequence was not determined. Each numbered horizontal line in the M13-dideoxy section depicts a portion of an individual M13 clone that was sequenced. At the top of the figure, the locations and orientations of the genes and pseudogenes is shown.

from the 3' -end of the isolated $tRNA^{Glu}$ pseudogene are also indicated by a box, and the C to T transition is similarly indicated at position 4. It can be seen that the 7 bp deletion in the $tRNA^{Gly}$ pseudogene results in removal of 7 bp (of a total 11 bp) from the A block promoter sequence. Similarly, the truncation present at the 3' -end of the $tRNA^{Glu}$ pseudogene removes 6 out of 11 bp of that gene's B block promoter section. One would predict from these data that neither of these genes are capable of serving as templates for RNA polymerase III transcription. However, both pseudogenes possess transcription termination signals (T_4) at their 3' -end, 12 bp away from the coding region of the $tRNA^{Gly}$ pseudogene and 88 bp away from the coding region of the $tRNA^{Glu}$ sequence. This latter distance is not unprecedented in a eukaryotic tRNA gene (7). In addition, the $tRNA^{Gly}$ pseudogene possesses a potential secondary termination site 120 bp downstream from the first, similar to those found for human $tRNA^{Leu}$, $tRNA^{Lys}$, and $tRNA^{Gln}$ genes (8).

Analysis of the region surrounding the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes revealed the presence of a region possessing weak homology to the rat B1

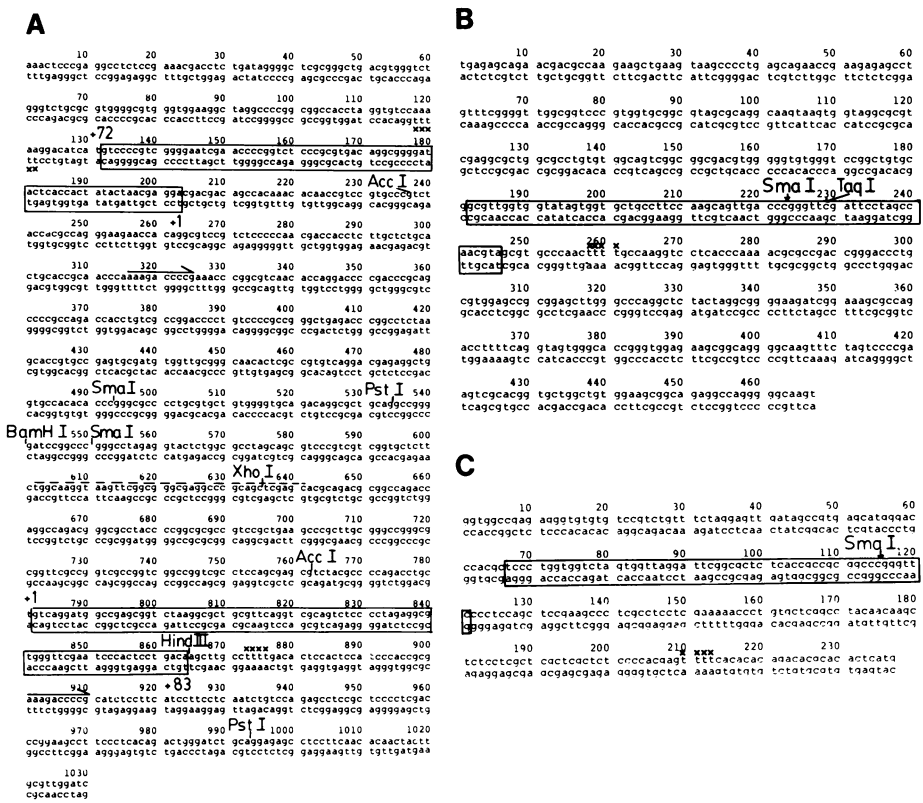

Figure 4. Nucleotide sequence of tRNA genes, pseudogenes and flanking regions. tRNA gene coding regions are enclosed in boxes, putative transcription termination signals are marked by X's, a 10 bp direct repeat is overscored by arrows above and a sequence bearing homology to the rat B1 element is denoted by a dashed line above the sequence. (A) This DNA sequence contains the $tRNA^{Asp}$ gene (from nucleotide 203 to nucleotide 132) and the $tRNA^{Leu}$ gene (from nucleotide 782 to nucleotide 864). The Pst I sites at nucleotides 529-534 and 989-994 define the insert used in constructing subclone pRt-460. (B) This DNA sequence contains the $tRNA^{Gly}$ pseudogene (from nucleotide 182 to nucleotide 246). (C) This DNA sequence contains the $tRNA^{Glu}$ pseudogene (from nucleotide 67 to nucleotide 121).

element (37). This sequence is overlined by hatched lines in Figure 4 and the homology is depicted in Figure 6. The possible function of this B1-like sequence in the genome is unclear (38).

In vitro transcription products from pRT-61

Size fractionation of the radiolabeled RNAs synthesized by a HeLa cell S-100 in the presence of clone pRt-61 revealed the specific synthesis

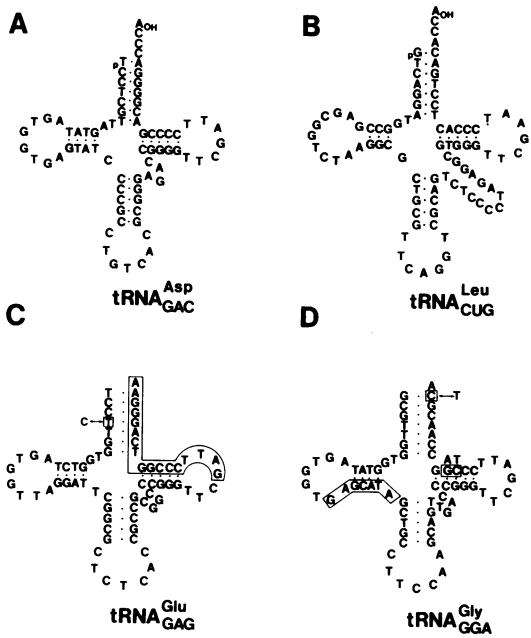

Figure 5. Cloverleaf structures of the non-coding DNA strand for the two genes and two pseudogenes. (A) $tRNA^{Asp}_{GAC}$ gene. The uncoded 3′ -CCA end has been added. (B) $tRNA^{Leu}_{CUG}$ gene. The uncoded 3′-CCA has been added. (C) pseudogene. Differences between the sequence reported previously (14) and that reported in this communication are depicted. Nucleotide differences found in our sequence are drawn outside the cloverleaf with arrows to their corresponding positions. Bases enclosed in boxes with no corresponding base outside the cloverleaf are deleted in our pseudogene but are those found in Sekiya et al. (14). (D) $tRNA^{Gly}_{GGA}$ pseudogene. As in (C), the deletions are enclosed in boxes with no corresponding bases and substitutions have our results drawn outside the cloverleaf.

of a number of transcripts, ranging in size from 108 bases down to 75 bases (Figure 7A). At this point, the 3.2 kb insert was subcloned as diagrammed in Figure 2 to generate pRt-460, containing the $tRNA^{Leu}$ gene, and pRt-870, containing the $tRNA^{Asp}$ gene. Separate transcription analysis of these two plasmids gave us the results shown in Figure 7B, lanes 1 and 3.

A number of larger RNAs and a transcript of 75 bases were synthesized in the presence of pRt-870 DNA (Figure 7B, lane 1). Incubation of this mixture of another hour in the presence of high enough levels of α-amanitin to inhibit de novo RNA polymerase III transcription resulted in the disappearance of the higher molecular weight RNAs (Figure 7B, lane 2).

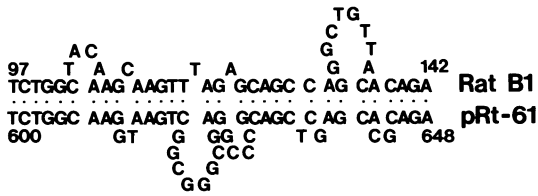

Figure 6. Sequence homology between a rat B1 element and a portion of the 3.2 kb Eco RI insert sequenced in Figure 4A, nucleotides 600-648. The rat B1 element sequence is that reported by Blin et al. (36).

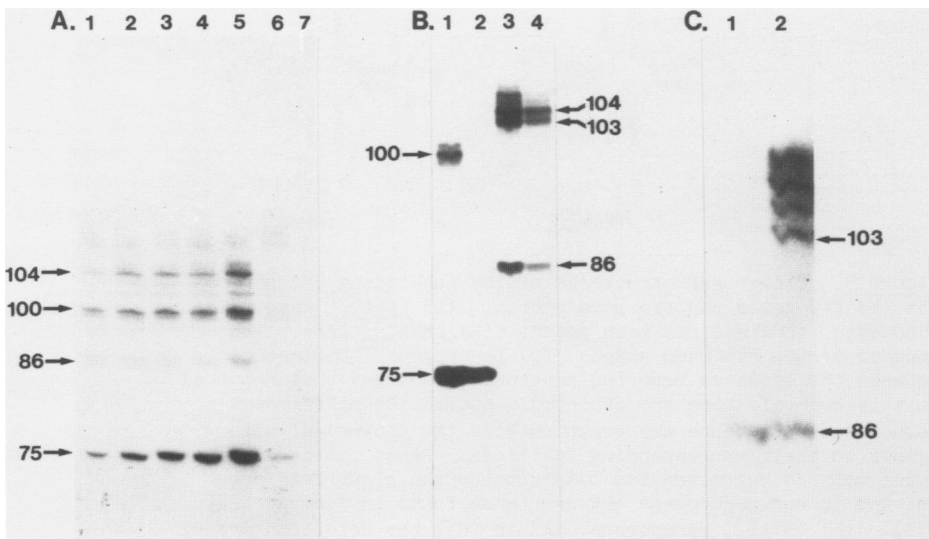

Figure 7. In vitro transcription analysis of pRt-61 and subclones. (A) Time course of in vitro transcription of pRt-61 by HeLa cell lysate S-100. Reactions (50 µl) were incubated at 30° for varying periods of time. Lanes 1-4: 40, 60, 90, and 120 minutes in the absence of α-amanitin; lane 5: 120 minutes (1.2 µg/ml α-amanitin); lane 6: 120 minutes (243 µg/ml α-amanitin); lane 7: minus DNA control. (B) In vitro transcription of subcloned tRNA genes. In lanes 1 and 2, the DNA template is pRt-870 (containing the $tRNA^{Asp}$ gene); lane 3 and 4, pRt-460 (containing the $tRNA^{Leu}$ gene). Synthesis was carried out for 1 hr at 30°, then α-amanitin (final concentration = 200 µg/ml) was added (lanes 2 and 4) and incubation continued at 30° for an additional 1 hr. (C) In vitro transcription of pRt-460 ($tRNA^{Leu}$ gene). Lane 1: 30°, 1 hr, then α-amanitin (200 µg/ml final concentration) added and incubation continued at 30° for 4 hr. Lane 2: 30°, 2 hr, no α-amanitin.

This result led us to conclude that the 99 to 101 base RNAs were precursor transcripts which were being processed to the 75 base RNA. The addition of a C-C-A sequence to the 3' -end of the anticipated 72 base $tRNA^{Asp}$ transcript would yield this size product (Figure 4).

A similar pattern of RNA transcripts was obtained utilizing pRT-460 as the template DNA (Figure 7, lane 3). Further incubation of the transcription reaction mix for one hour with high levels of α-amanitin, however, did not result in a disappearance of the higher molecular weight RNAs (lane 4). However, transcription for one hour followed by the addition of high levels of α-amanitin and a further incubation for four hours, left no detectable amount of the higher molecular weight RNAs (Figure 7C, lane 1) but a comparable amount of 86 base RNA (lane 2). representing the processed $tRNA^{Leu}$ gene transcript.

DISCUSSION

Nucleotide sequence in and around the reiterated gene cluster

Examination of the structure of our gene cluster demonstrated that it resembled that of a number of similar clusters isolated by Sekiya et al. (14). A comparison between these sequences revealed that although the sequence homology was high (comparable to the values obtained between their gene clusters), our clone was yet another member of this multicopy family, of which there are approximately ten copies in the rat genome (14). The most conserved coding region out of all four genes is the $tRNA^{Asp}$ gene. The sequence obtained by us is identical to all seven of the other $tRNA^{Asp}$ genes isolated from the rat. This high sequence conservation is maintained in the $tRNA^{Leu}$ genes. One of the other two characterized $tRNA^{Leu}$ genes (Rosen et al., Intern. tRNA Workshop, Hakone, 1983) has three nucleotide changes at positions 48, 54, and 76 (C to A, G to A in the extra arm and T to A at the base of the aminoacyl stem. The other $tRNA^{Leu}$ sequence possesses solely the G to A transition in the extra arm (Sekiya et al., Intern. tRNA Workshop, Hakone, 1983).

This sequence conservation breaks down upon analysis of the other two coding regions. RTldge, the gene cluster isolated from the Donryu strain of rat (11) contains intact $tRNA^{Gly}$ and $tRNA^{Glu}$ genes. All four of the clusters isolated from the Sprague-Dawley strain of rat (three from the same laboratory and ours) possess $tRNA^{Gly}$ pseudogenes, and two out of four possess $tRNA^{Glu}$ pseudogenes (14). All five $tRNA^{Gly}$ pseudogenes are missing the same bases at the 3' -end of the A box. Both clone RT1-2 and

Rt-61 have the C to T transition at position 61, whereas only Rt-61 has the G to A transition at position 62. Three out of five of the $tRNA^{Gly}$ pseudogenes (including Rt-61) has a change of C to T at the top of the aminoacyl stem. The variation in structure, however, is most dramatic in the $tRNA^{Glu}$ sequences. Two out of four of the $tRNA^{Glu}$ pseudogenes from Sprague-Dawley rat isolated by Sekiya et al. (14) are missing 11 bp of the 3' -end. The pseudogene isolated in our laboratory is missing this 11 bp and an additional 6 bp (shown in Figure 5). All five $tRNA^{Glu}$ sequences from Sprague-Dawley rat have a T to C transition at position 4. In summary, out of the five copies of the reiterated cluster isolated from the Sprague-Dawley rat genome, three possess $tRNA^{Glu}$ pseudogenes, whereas all five possess $tRNA^{Gly}$ pseudogenes.

An analysis of all the available sequences for 5' and 3' flanking regions in this reiterated gene cluster allowed us to come up with a few generalizations. The homology in the non-coding regions is highest within the first 100 bp or so on either side of the genes. Beyond this point, homology starts to decline. In addition, the 3' non-coding region of the genes is more highly conserved than the 5' non-coding region. This trend for preferred 3' non-coding region conservation has been seen in another multi-copy gene family, the primate globins (39).

Comparison of the 5' and 3' flanking regions of all four tRNA genes within the cluster failed to reveal any large degree of homology between them. The G-C content of these flanking regions, 68%, is much higher than the average for the genome, 43% (40). As a result, there are a number of small direct and indirect repeat sequences within the DNA surrounding the genes. The largest direct repeat sequence is 10 bp long and is indicated by arrows in Figure 4A.

Certain areas of the gene cluster possess DNA sequences which consist of simple nucleotide repeats. For example, in three different positions in and around the two pseudogenes, there are regions of alternating purines and pyrimidines. At the 5' -end of the $tRNA^{Gly}$ pseudogene at position 136 (Figure 4B) lies a $(TG)_3$ sequence. At position 14 just 5' to the $tRNA^{Glu}$ pseudogene (Figure 4C) is the sequence $(GT)_4$. At the end of the $tRNA^{Glu}$ pseudogene at position 214 is the sequence $(CA)_4(GA)(CA)(CG)(CA)_2$-$(CT)(CA)(TG)$. These types of sequences all have one feature in common; they are all capable of forming a left-handed helix, or Z-DNA structure, under suitable conditions (41). A 9 bp tract of alternating purine/pyrimidine residues has been found at the 5' -end of an inactive X.

laevis $tRNA^{Met}$ gene (42) which was later shown by deletion analysis, to play a role in the inactivity of the gene (43). Retention of the potential Z-DNA-forming alternating purine-pyrimidine sequences within the other sequenced Sprague-Dawley clones was not found to be the rule. Point mutations at the 5' -end of all four of the other $tRNA^{Gly}$ pseudogenes resulted in a loss of the 6 bp tract of alternating $(TG)_3$ sequence (13). However, the $(GT)_4$ sequence at the 5' -end of the $tRNA^{Glu}$ sequence was conserved in both genes and pseudogenes. Point mutations in the region of the DNA which contains the largest tract of alternating purines and pyrimidines, the 3' -end of the $tRNA^{Glu}$ gene, have obliterated this sequence in the other four sequenced clones at the 3' -end of the $tRNA^{Glu}$ pseudogenes or genes.

In vitro RNA synthesis

The sizes of the processed RNA transcripts obtained upon prolonged incubation of the $tRNA^{Leu}$ and $tRNA^{Asp}$ genes in the presence of HeLa S-100 and α-amanitin are consistent with the predicted final products as deduced from their respective coding regions. The $tRNA^{Asp}$ and $tRNA^{Leu}$ gene sequence have a 72 and 83 base coding region, respectively (Figure 4). The addition of a C-C-A triplet at the 3' -end of the processed tRNA transcripts has been seen in in vitro transcription directed by X. laevis oocyte cell extracts (42). The presence or absence of this activity in HeLa cell extracts remains undetermined to date (44). Assuming that the ssDNA markers used to determine RNA transcript size were accurate, this would indicate that C-C-A addition does take place in HeLa cell S-100 extracts.

The longer chase requirement for processing of the $tRNA^{Leu}$ precursors as opposed to the $tRNA^{Asp}$ precursors under the same conditions indicates a much slower rate of processing for the former. Although it has been suggested that a perfect base pairing of the 5' and 3' ends of the precursor tRNA may be involved in the reduced rate of processing (45), it is not the case here, since neither $tRNA^{Leu}$ nor $tRNA^{Asp}$ precursors possess perfect base pairing in the aminoacyl stem. Thus, another mechanism must be involved in the processing cleavage reactions.

Analysis of the data seems to indicate that neither tRNA pseudogene is capable of in vitro transcription. This result was expected since data obtained in other laboratories indicate that in vitro transcription is critically dependent upon intact A and B box structures (46,47). In each of the pseudogenes isolated here, fully one-half of either the A box or B

box of the promoter has been deleted (Figure 5). However, since we have not proven the identity of the transcripts by RNA sequence analysis, we cannot rule out the possibility that the pseudogenes are transcriptionally active.

Taken together, the results presented here are in agreement with previously obtained data on the structure and in vitro transcription of eukaryotic tRNA genes. As more tRNA gene clusters will be analyzed, one might expect that certain general rules in the organization and expression of the mammalian genes will emerge.

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Abbreviations: tRNA, transfer RNA; RF, replicative form; bp, base pairs; kb, kilobase pairs; ssDNA, single-stranded DNA.

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