Glutamate tRNA genes are adjacent to 5S RNA genes in *Drosophila* and reveal a conserved upstream sequence (the ACT-TA box)

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ABSTRACT

In *Drosophila melanogaster* at least six transfer RNA genes are located adjacent to the 3' end of the 5S RNA gene cluster. Three of these have been sequenced and identified as coding for glutamate tRNA₄. In the chromosome they are arranged as tandem repeats on the same DNA strand and transcribed in the same direction as is 5S DNA, towards the centromere.

We have also identified a sequence, the ACT-TA box, that is highly conserved among the polymerase III transcribed genes. Usually the sequence is located at 37±8 base pairs upstream from the first nucleotide of the structural gene. A similar sequence is also observed upstream of yeast and silkworm tRNA genes and the mitochondrial tRNA genes of mouse and humans.

INTRODUCTION

The location of transfer RNA genes in the genome of *Drosophila melanogaster* has been studied extensively by means of *in situ* hybridization of radiolabelled transfer RNA's to polytene chromosomes. These studies have helped establish that the 600-800 copies of the various tRNA genes are localized at approximately 54 chromosomal loci; that genes for a specific tRNA isoacceptor may occur at more than one locus; and that a single locus may contain genes for several different tRNA species (for review see 1). Several different purified tRNA species hybridize to position 56EF on the polytene chromosome, the locus that also contains the cluster of 165 tandemly repeated 5S RNA genes (2). Hayashi et al. (3,4) found strong and consistent labelling at this site for tRNA₂^{lys}, tRNA₃^{met} and tRNA₃^{gly} and Elder et al. (5) reported hybridization of tRNA₂^{phe} and tRNA₃^{met}. Kubli and Schmidt (6) found that tRNA₄^{glu} consistently hybridized to 56EF and Schmidt and Kubli (7) also demonstrated hybridization of tRNA^{his} at this locus. Further verification of the presence of tRNA genes at 56EF has come from *in situ* hybridization studies with probes derived from cloned fragments containing tRNA genes. Gergen et al. (8) detected hybridization at 56EF using a plasmid bearing both tRNA₂^{lys} and tRNA^{ile} genes, and Hershey and Davidson (9) found that a cloned

DNA fragment carrying two tRNA^{Gly} genes hybridized exclusively to this locus. Thus at least six different tRNA genes appear to be located at 56EF.

In order to determine the structural relationship of tRNA genes to the cluster of 5S RNA genes, recombinant DNA clones containing sequences that flank 5S DNA were isolated. We have determined that the 5S DNA is arranged in the chromosome with the orientation, telomere...5' 5S DNA 3'...centromere and that there are at least six tRNA genes, three of which code for glutamate tRNA₄, within 10 kb downstream from the 5S DNA. No tRNA genes appear to be located within 11.5 kb upstream of 5S DNA. In addition, a conserved sequence located upstream from polymerase III transcribed genes has been identified. This sequence is also present in the tRNA genes of yeast and at least one mitochondrial tRNA gene in mouse and man.

MATERIALS AND METHODS

Preparation of DNA.

Recombinant λ phage were grown in *E. coli* (C2600), purified and DNA isolated as previously described (10). *Drosophila* DNA was prepared by the method of Procunier and Tartof (11).

Restriction Enzyme Mapping.

DNA fragments were mapped using a variety of restriction enzymes obtained from commercial sources. In some cases, mapping was accomplished with the partial digestion method of Smith and Birnstiel (12) where only one end of the DNA fragment is labelled with ³²P. In most cases, DNA fragments were generated by digestion with enzymes that recognize a six base pair sequence and then isolated by electrophoresis in low melting point agarose. They were then digested with several other enzymes, either singly or in combination. Restriction maps produced in this way were further verified by digesting the intact cloned DNA with appropriate enzymes, singly or in combination, to check that all of the expected fragment sizes were present.

Large DNA fragments were analyzed in 0.7% agarose gels in E buffer (40 mM Tris, 50 mM sodium acetate, 1 mM EDTA, pH 7); small fragments were usually run on 1.6% agarose gels in TBE buffer (50 mM Tris, 50 mM borate, 1 mM EDTA pH 8.5). Lambda DNA digested with HindIII or ϕ X174 cut with HaeIII were used as calibration standards. Transfer to nitrocellulose was performed according to the procedure of Southern (13).

Preparation and Hybridization of Radioactive Probes.

RNA was labelled with ¹²⁵I as described by Tereba and McCarthy (14). Nick translation of DNA with α ³²P-CTP was performed according to the method

of Rigby *et al.* (15).

Hybridization to RNA probes was performed in 50% formamide, 5 X SSC (1 X SSC is 0.15 M NaCl, 0.15 M sodium citrate, pH 7), 20 mM phosphate buffer, pH 7. Following overnight incubation at 37°, filters were washed 2 times with 2 X SSC, digested for 1 hour with RNase (30γ/ml) in 2 X SSC at 37° and finally rinsed two times with 2 X SSC.

Filters hybridized with ³²P labelled DNA probes were pretreated for 2 hours at 37° in a solution consisting of 5 X SSC, 20 mM phosphate buffer, pH 7, 5 X Denhardt's solution (1 X Denhardt's Solution is 0.02% Ficoll, 0.02% PVP and 0.02% bovine serum albumin; 16) and 200γ/ml sheared and denatured carrier salmon sperm DNA. The DNA probes were denatured by heating at 100° for 4 minutes and quick cooled before adding to a hybridization solution consisting of 50% formamide, 20 mM sodium phosphate, pH 7, 5 X SSC, 1 X Denhardt's Solution. Following overnight hybridization at 37°, the filters were washed two times with 2 X SSC, 0.1% SDS at room temperature and two times with 0.1 X SSC, 0.1% SDS at 50°.

DNA Sequencing.

DNA fragments were labelled at their 5' ends using γ³²P-ATP and polynucleotide kinase. Uniquely labeled strands were then prepared by digesting the labeled DNA with an appropriate restriction enzyme or by strand separation on polyacrylamide gels. The DNA was then sequenced according to the methods of Maxam and Gilbert (17).

RESULTS

Sequences Flanking the 5S RNA Gene Cluster.

Clones bearing 5S RNA genes were selected from a Charon 4-Drosophila (Canton S) recombinant DNA library (18) using ¹²⁵I labeled 5S RNA. Since the 5S RNA gene repeats do not contain recognition sites for the restriction enzymes EcoRI, HindIII, SalI or SmaI (19), digestion of each clone with these enzymes made it possible to distinguish between those carrying only 5S DNA and those containing both 5S DNA and a flanking sequence. In this way, four clones containing 5S DNA and a flanking sequence were obtained.

Digestion with a variety of restriction enzymes followed by agarose gel electrophoresis was used to construct the maps shown in Figure 1. The clones fall into two classes. The restriction map of one of the clones, λDm5S-U, overlaps the map of 12D8, a plasmid isolated by Artavanis-Tsakonas *et al.* (20) that contains 5S DNA and its 5' flanking sequence. The maps of the other three clones λDm5S-C, G, and O, overlap each other and differ

Figure 1. Restriction maps of clones containing sequences flanking 5S DNA. λDm5S-U contains sequences 5' to the 5S gene cluster and overlaps the map of 12D8 isolated by Artavanis-Tsakonas et al. (20). Clones λDm5S-O, G, and C bear sequences 3' to the 5S RNA genes. The amount of 5S DNA in each clone is not to scale (see text). The numbers above the maps indicate the trRNA positive regions. In the expanded map, the arrows above indicate the location and orientation of the three sequenced genes. The arrows below the map show the fragments sequenced and the direction of sequencing. Only those HhaI, HaeIII, AluI and RsaI sites relevant to the sequencing scheme are shown. The restriction enzyme code is indicated in the figure.

completely from λDm5S-U. These, therefore, represent sequences 3' of the 5S gene cluster. None of the maps overlap with the restriction map of λDm5S-6, a clone that hybridizes to 56EF and contains trNA^{81y} genes.

The location and size of the 5S DNA sequences within the clones was established by probing Southern blots of restriction enzyme fragments with either ¹²⁵I labelled 5S RNA or ³²P labelled 12D1, a plasmid whose insert contains only *Drosophila* 5S genes (20). As shown in Figure 1, the clones contain long stretches of non-5S DNA and relatively few 5S RNA genes. Calculating from the size of the 5S DNA repeat (375 bp; 19) and knowing the length of the *Drosophila* insert, we estimate that there are approximately 8 5S genes in λDm5S-C, 3 in λDm5S-G, 4 in λDm5S-O, and 6 in λDm5S-U. There is approximately 10 kb of flanking sequence in λDm5S-C and 11.5 kb of flanking sequence in λDm5S-U. In all four clones, the 5S DNA adjoins the right arm of the lambda chromosome.

The orientation of the λDm5S-C and λDm5S-U clones relative to the centromere of chromosome II was deduced with the aid of two deletion mutants generated by segmental aneuploidy (21). T(Y;2), Df L139-L62 begins within the 5S DNA and extends towards the centromere whereas T(Y;2), Df L62-L141 begins within the 5S DNA and extends away from it (11). DNA samples from the wild type and heterozygous mutant genotypes were digested with SmaI or EcoRI and transferred to nitrocellulose filters. The SmaI digested DNA was hybridized

to the ^{32}P labelled 400 bp *Sma*I fragment from $\lambda\text{Dm5S-C}$ (3' flanking). The *Eco*RI digested DNA was hybridized to the ^{32}P labelled 1.8 kb *Eco*RI fragment of $\lambda\text{Dm5S-U}$ (5' flanking). The autoradiograms are shown in Figure 2. With the *Sma*I fragment from $\lambda\text{Dm5S-C}$ as probe, a 50% reduction in the signal was evident in DNA from flies of genotype +/Df L139-L62 (Figure 2a). When the *Eco*RI fragment of $\lambda\text{Dm5S-U}$ was used as probe, hybridization was reduced by half only in +/Df L62-L141 flies (figure 2b). This indicates that the 5S gene cluster is arranged in the orientation, telomere...5' 5S DNA 3'... centromere.

The sequences flanking 5S DNA were examined for the presence of tRNA genes by hybridizing them to ^{125}I labelled 4S RNA. Since total transfer RNA preparations are contaminated with 5S RNA, some hybridization to 5S DNA fragments was anticipated. Therefore, separate hybridizations with ^{125}I 5S or 4S RNA were performed and only fragments hybridizing solely to 4S RNA were scored as potential sites of tRNA genes. When $\lambda\text{Dm5S-U}$ is digested with *Eco*RI, hybridization with 4S RNA occurs only to those fragments that also hybridize to 5S RNA. However, when $\lambda\text{Dm5S-C}$, G and O are digested with *Eco*RI and *Sal*I the 4.2 fragment that is produced hybridizes only to 4S RNA (Figure 3). Thus, tRNA genes are located only in the 3' flanking region of the 5S

Figure 2. Chromosomal orientation of 5S DNA. Five micrograms of wild type or mutant DNA were digested with (a) *Sma*I, or (b) *Eco*RI, subjected to electrophoresis and transferred to nitrocellulose filters. DNA digested with *Sma*I was hybridized to the ^{32}P labelled 400 bp *Sma*I fragment from $\lambda\text{Dm5S-C}$ and the DNA digested with *Eco*RI was hybridized to the ^{32}P labelled 1.8 kb *Eco*RI fragment from $\lambda\text{Dm5S-U}$. For each panel, lane 1 contains wild type DNA (+); lane 2, Df L139-L62 ($\Delta 1$); lane 3, Df L62-L141 ($\Delta 2$).

Figure 3. Analysis of the recombinant λ clones for regions containing tRNA genes. DNA from λ Dm5S-O, G and C was digested with EcoRI and SalI; λ Dm5S-U, which contains no SalI, site was digested solely with EcoRI. The left panel shows the electrophoretic pattern of restriction digests separated on a 0.7% agarose gel stained with ethidium bromide. The middle panel illustrates an autoradiogram of the Southern blot hybridized with ^{125}I labelled 4S RNA. The right panel is an autoradiogram of the Southern blot hybridized with ^{125}I labelled 5S RNA. The ladder effect observed near the 5S DNA positive bands in the right panel is due to microheterogeneity in the number of 5S DNA repeats within each clone. The large (>12 kb) 5S DNA positive band in the λ Dm5S-U lane is a result of incomplete enzyme digestion. Wild type λ DNA digested with HindIII served as a molecular weight standard.

DNA cluster and none appear to be located in the 11.5 kb adjoining the 5' end of the 5S gene cluster.

Further studies with different enzyme combinations established that tRNA genes are located in at least six regions of λ Dm5S-C. The locations of tRNA positive sites in λ Dm5S-C are shown in Figure 1 by numbers above the map. Region 1 is contained within a 210 bp SmaI fragment approximately 1.5 kb from the 5S gene cluster. Region 2 is located within the 540 bp SmaI fragment to the left of the SalI site, and region 3 is localized to the 400 bp SmaI, HaeIII fragment within the 1.3 kb SmaI sequence. There are two positive sites within the 2.7 kb SalI, SmaI fragment (regions 4 and 5) and one within the 230 bp HindIII, BamHI fragment of the 1.5 kb SmaI, SalI sequence (region 6). We have also observed weak signals at the 400 bp and 750 bp bands in autoradiograms of SmaI digested λ Dm5S-C.

Homology Among tRNA Genes that Flank 5S DNA.

To determine whether the regions containing tRNA genes in λ Dm5S-C

represent similar sequences, the 210 base pair *Sma*I fragment (region 1) was isolated from λ Dm5S-C, 32 P labelled by nick translation and hybridized to λ Dm5S-C digested with *Sma*I. As shown in Figure 4, regions 2 and 3 also hybridize with region 1.

Sequencing Studies.

The procedure of Maxam and Gilbert (17) was used to sequence regions 1, 2 and 3. We also sequenced that part of the chromosome on the 3' side of the *Sma*I sites of regions 1 and 2. The fragments sequenced and the direction of sequencing are indicated in Figure 1. The complete sequence of region 2 and partial sequences of regions 1 and 3, as shown in Figure 5, were checked by reading both strands or by independent sequencing runs. With the aid of the computer program "HAIRPN" (22), a portion of the sequence from region 2 could be arranged in a cloverleaf configuration typical of transfer RNA. As shown in Figure 6, this sequence contained the anti-codon, UUC that is complementary to the codon GAA and thereby identifies this gene as coding for tRNA^{Leu}₄ (23). As indicated by the hybridization studies (Figure 4), there are two other copies of this gene in sequences derived from regions 1 and 3. The three tRNA^{Leu}₄ genes are arranged in tandem on the same DNA strand and are

Figure 4. Region 1 is repeated in the λ Dm5S-C clone. λ Dm5S-C was cleaved with *Sma*I and hybridized with 32 P labelled DNA from region 1 of λ Dm5S-C. Bands labeled 1, 2 and 3 correspond to their respective regions as indicated in Figure 1.

REGION	-80	-70	-60	-50
1	AGAAGGTGTT	AATAGTTATA	ACTGTTAGCA	GAAACTTTGG
2	GGTACATTAT	ACGTTAAATA	CGTTCAGGTT	GGTAATTAAAT
3	ACAAGTGAAT	GTAAATCACT	TAAAGCTGAT	TAATGTGCTT
	-40	-30	-20	-10
1	GACTGAAACT	AATATAGGAG	ATTGACTAAA	TAGGTATGTG
2	TCAATAAGAA	AACCGCGTTT	TCACAACAGG	TTTGTGACG
3	GCACTTAAAA	TACAGACGTA	AATCGGACTT	CGCTACAGTA
	10	20	30	40
1	TCCCATATGG	TCTAGTGGCT	AGGATATCTG	GCTTTCACCC
2	TCCCATATGG	TCTAGTGGCT	AGGATATCTG	GCTTTCACCC
3	TCCCATATGG	TCTAGTGGCT	AGGATATCTG	GCTTTCACCC
	50	60	70	80
1	AGAA	CGATTC	CCGGTATGGG	AGCAACCAA
2	AGAAGGCCCG	GGTTCGATTC	CCGGTATGGG	ATAATATTG
3	AGAA			
	90	100	110	120
1	CTTTTGTGTG	AGGTACTTTA	CATAATTTAT	TATTTGTTAC
2	TTTTCCTTAC	AAAAGAAACA	AAGATCACTT	TATATCCCT

Figure 5. The nucleotide sequences of the three tRNA^{glu} genes of λDm5S-C and their flanking regions (the noncoding strand is shown). Structural gene sequences are boxed. Sequences for region 1 between nucleotides 44 and 55 and region 3 beyond nucleotide 44 were not obtained. The SmaI recognition sequence of region 2 (Figure 1) begins at 47 bp.

transcribed in the same direction as the 5S RNA genes. They contain no intervening sequences following the anti-codon loop and, like other eukaryotic tRNA genes, do not encode the ultimate CCA of the mature transfer RNA.

Sequence homology outside the structural gene is low. As has been

Figure 6. A cloverleaf arrangement of the glutamate tRNA gene as deduced from the sequence data of Figure 5.

observed in other tRNA genes, the 3' flanking regions are AT rich and characterized by strings of T's 11 base pairs (region 1) and 10 base pairs (region 2) beyond the structural gene. However, in the 5' flanking portion of region 1, the sequence ACTAAATA at -16 bp is almost exactly repeated at -33 bp as ACTAATA. Likewise, in region 3 the sequence, ACTTAAATA appears at -38 bp. This prompted us to inspect other tRNA genes of Drosophila for a similar upstream sequence.

The ACT-TA Box

Table 1 is a compilation of sequences that resemble the upstream ACTAAATA sequence obtained by inspecting 27 tRNA genes of Drosophila. Twenty-five of the twenty-seven tRNA genes contained one, and sometimes two or three, such sequences. At the bottom of Table 1 the number of times a base (or no base) occurs at a given position is indicated, and from this, the consensus sequence, ACT(A/T)_nTA is derived. The middle portion of the sequence is variable with n = 0 to 5 bp and A's being about twice as frequent as T's. We shall refer to this consensus sequence as the ACT-TA box.

As shown in Figure 7, for 20 of the 25 tRNAs the ACT-TA box begins between 29 and 45 bp upstream from the first nucleotide of the structural gene. The X's above the bars in Figure 7 represent those ACT-TA boxes present more than once in a given gene. If they were removed from consideration, then the clustering of ACT-TA boxes is even more striking. The remaining five tRNAs contain ACT-TA boxes that are distributed at distances upstream that are roughly twice that of the others, namely, between -73 and -95 bp.

It should be noted that it is possible that some of the tRNA genes may be non-functional or function aberrantly, a difficulty that the organism can live with because these genes are redundant. This, in turn, may also account for some of the heterogeneity among ACT-TA boxes and for their apparent absence in two genes.

The 5S RNA genes of Drosophila (29) also contain an ACT-TA box at position -45 bp with the sequence, ACTGAATA. However, no similar sequence could be found in the somatic or oocyte 5S RNA genes of Xenopus laevis or Xenopus borealis. It appears then, that in Drosophila at least, the ACT-TA box seems to be highly conserved among RNA polymerase III transcribed genes.

Inspection of several tRNA genes from other organisms also reveals the presence of an ACT-TA box. As illustrated by the examples given in Table 2, in Saccharomyces cerevisiae the box is present but located significantly closer to the structural gene, on average at about -17 bp. In Bombyx mori,

Table 1. The sequence and location of the ACT-TA box upstream of *Drosophila* tRNA genes.

tRNA gene	chromosome location	clone region number	Sequence	bp from gene	Ref.
glu	56EF	1	ACTAAATA	-16	
			ACTAATA	-33	
		2	ATTCAATA	-42	
			ACGTTAAATA	-70	
		3	ACTTAAATA	-38	
			ACTTA	-63	
		1	ACTTTCTA	-40	
			ACAAATA	-60	
arg	42A				24
ile	42A	2	ACTAATA	-30	24
lys	42A	2	ACTGAAT	-45	24
			ACATTA	-84	
			ACTTATA	-110	
		3	none apparent		
		4	ACTAATA	-74	
		4	ATGAATA	-43	
asn	42A				24
arg	42A	11F	none apparent		25
lys	42A	35D	ACTAGCGTA	-39	25
			ACTTA	-109	
arg	42A	35D	ACTAATA	-35	25
gly	56F		ATCAAAATA	-44	9
			ACTATATA	-64	
glu	62A	2	ATCAATTA	-41	26
		3	ATACATA	-40	
		4	ACTATA	-36	
		5	ATCTTA	-73	
met	61D	1	ACTTTGTA	-12	27
			ACTTG	-36	
		2	ACTTTGTA	-12	
			ACTTTG	-43	
		3	ACTTTATA	-12	
			ACTTTG	-43	
		A	ACTTA	-95	
			GCTATTTA	-41	
			ATAGAAATT	-93	
ile	50AB	D	ACTCTCAA	-34	28
		E	ACATAATA	-90	
leu	50AB	A	ACTACGGA	-29	28
		B	ACTTTTA	-36	

Table 1 (continued)

Base	Position in Sequence									
	1	2	3	4	5	6	7	8	last-1	last
A	35	0	5	14	16	9	4	2	2	31
T	0	7	26	13	9	2	0	0	33	2
G	1	0	2	2	1	3	1	0	1	3
C	0	29	3	3	1	3	0	0	0	0
none	0	0	0	4	9	19	31	34	0	0
Consensus	A	C	T	A/T	A/T	A	A	A	T	A

it is present at -33 bp in the ala₄ tRNA gene. We have also noted its presence upstream from the proline tRNA genes in the mitochondrion of mouse and man. In each case the sequence is present once at -20 and -45 bp, respectively.

DISCUSSION

We have shown that at least six tRNA genes, three of which code for tRNA^{Alu}₄, lie within a few kilobases downstream of the 5S RNA gene cluster of *Drosophila melanogaster* located at the cytogenetic position 56EF. This entire array is oriented in the chromosome such that the genes are transcribed from the same strand, and in the same direction, toward the centromere of chromosome II. No tRNA genes appear to be located within 11.5 kb upstream of the 5S DNA. This arrangement differs from another region of the

Figure 7. Upstream position of the ACT-TA box for 25 tRNA genes of *Drosophila*. Those bars marked with an X indicate copies of the box in a given gene in addition to the one copy between -29 and -45 bp.

Table 2. The ACT-TA box upstream from tRNA genes in various organisms.

tRNA gene (organism)	clone region number	Sequence	bp from gene	Ref.
phe (yeast)	2	CCTATAAATA	-14	30
	5	ACTAATA	-18	
	15	ACTAATA	-18	
ser ₂ (yeast)	B	ACTCAAGA	-15	31
	G	ACTAATCA	-17	
	J	ATCATTA	-11	
glu ₃ (yeast)		ATGCAACACATA	-24	32
		ATGAATA	-72	
		TCTCAATA	-88	
leu (yeast)		ACAAAATT	-22	33
ala ₂ (silkworm)		ACTTTATA	-33	34
		ACTGAAAATA	-58	
pro (mouse mitochondria)		ACTATGTA	-20	35
		ACTAGCTTA	-80	
pro (human mitochondria)		AGTCAATA	-45	35

genome, 42A, where 18 tRNA genes representing four different families of amino acid acceptors are clustered (24,25). In this case, the tRNAs are transcribed from both strands. Like the cluster at 56EF, however, the tRNA genes at 42A are irregularly spaced and their flanking sequences show little homology to one another.

It is curious that there is such considerable divergence among the sequences flanking the three tRNA₄^{lu} genes. This contrasts sharply with the homogeneity of spacer sequence among the 165 copies of 5S DNA just a few kilobases away. Whatever mechanisms may be functioning to maintain spacer homogeneity among 5S RNA genes be they selection, unequal exchange or something else, they appear not to be operative at the neighboring tRNA₄^{lu} genes and imply that there is something special about the 5S DNA cluster in this regard.

Inspection of the sequences upstream of the tRNA₄^{lu} genes has led to the identification of the ACT-TA box. This sequence is highly conserved among the polymerase III transcribed genes of *Drosophila*, both in sequence and in position relative to the start of the structural gene. A similar ACT-TA box has been identified in the tRNA genes of yeast, silkworm and the mitochon-

drial tRNA genes of mouse and humans. The ACT-TA box bears some resemblance to the TATA box of polymerase II genes in both AT richness and location. However, in vitro transcription of Drosophila tRNA and 5S RNA genes does not require the ACT-TA sequence (36,37) whereas the TATA box is necessary for accurate initiation in vitro (38,39). It is possible, however, that the ACT-TA box may modulate transcription in a positive or negative manner in vivo, a notion that can be tested experimentally.

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