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The localization of $tRNA_4^{Glu}$ genes from Drosophila melanogaster by "in situ" hybridization

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ABSTRACT

Transfer RNA_4^{Glu} was isolated from Drosophila melanogaster by affinity chromatography. The tRNA was iodinated "in vitro" with Na[^{125}I] and hybridized "in situ" to salivary gland chromosomes from Drosophila. Subsequent autoradiography allowed the localization of the genes for $tRNA_4^{Glu}$ to the right arm of the second chromosome and to the left arm of the third chromosome in the regions 52 F, 56 EF and 62 A.

INTRODUCTION

The elucidation of the arrangement, structure and function of genes in eucaryotic organisms is an important step towards the understanding of the molecular biology of development. A huge amount of information has been accumulated in the last decades on the localization of Drosophila genes involved in developmental processes by classical genetic means[1,2]. The elegant technique of "in situ" hybridization developed by Pardue and Gall[3] and John et al.[4] allows the identification of a genetic locus without possessing mutants of this particular gene. The only prerequisite is a pure primary gene product or the gene and flanking sequences. The loci for ribosomal RNAs, for some tRNAs and for histone and other proteins have been identified by this methodology in a variety of organisms[3-8].

Besides their central role in protein synthesis, tRNAs have been shown to have many other functions in procaryotes, e.g. in suppression, as co-repressors, in stringent control etc.[9]. In higher organisms the situation is less clear. For yeast the involvement of tRNA in suppression has convincingly been demonstrated by Capecchi et al. and Gesteland et al.[10,11]. In Drosophila it has been claimed that tRNA-enzyme interactions are responsible for the mechanism of suppressor of sable, su(s)[2], but these results have

been questioned by Mischke et al.[12,13]. The demonstration of tRNA involvement in nonsense suppression has been unsuccessful so far (Kubli, unpublished results)[14]. In Drosophila a class of developmental mutants called Minutes have been interpreted by Atwood as tRNA mutants[15]. White has analysed the isoacceptor patterns of several Minutes by RPC-5 chromatography[16]. He found no difference in comparison with the wild type pattern with the exception that $M(3)h^{y}$ has a reduced $tRNA_3^{Tyr}$ peak. These results must be interpreted carefully, however, since the isoacceptor pattern can vary with the food composition and the temperature of rearing[17]. A general attempt to correlate the Minute loci with the genes for tRNA has failed[18]. However, Grigliatti et al. have found the genes for $tRNA_5^{Lys}$ to coincide with M(2)40c[19].

The solution of some of these problems will be greatly facilitated by the knowledge of the loci for the tRNA genes. The existence of polytene salivary gland chromosomes and of a sophisticated genetic knowledge makes Drosophila melanogaster the organism of choice for the study of such questions by "in situ" hybridization. The knowledge of the loci will permit the production of mutants; the analysis of the effects of duplications and deletions of the corresponding regions may give insights into regulatory mechanisms.

It has been shown by Grigliatti et al. that tRNAs from Drosophila melanogaster can be resolved into 99 peaks by reversed phase chromatography[5]. The isolation of a pure species by conventional column chromatography is therefore very laborious. We have used the method of affinity chromatography developed by Grosjean et al. for the purification of a specific tRNA[20]. In this paper we describe the isolation and "in situ" hybridization of $tRNA_4^{Glu}$ from Drosophila melanogaster.

MATERIALS AND METHODS

Drosophila stocks and preparation of salivary gland chromosomes: A "Sevelen" stock of Drosophila melanogaster was reared at 25°C in plastic boxes on standard cornmeal-agar-yeast diet .The flies were collected 5 d old, frozen in liquid nitrogen and stored at -20°C. The salivary glands for the "in situ" hybridization were dissected from larvae bearing the mutation giant (gt, 1-0,9). This mutation prolongs the life span of the larvae and allows one or two extra duplications of the DNA, increasing therefore the polyteny of the salivary gland chromosomes. The salivary gland chromosomes were pre-

pared according to Atherton and Gall and Spradling et al.[21,8].

Preparation of tRNA and synthetases; aminoacylation of tRNA:
The tRNA was extracted according to the phenol method described by Kirby[22]. The RNA was dissolved in 0,1 M SSC brought to 3 M potassium acetate with 4,6 M potassium acetate, pH 6 and stored at 0°C for at least 3 hours[23]. After centrifugation 2½ volumes of cold ethanol were added to the supernatant. The precipitate was applied to a G-100 Sephadex-column and the tRNA fraction precipitated with cold ethanol.

Aminoacyl-tRNA synthetases were prepared according to the procedure of Twardzik et al.[24]. The enzymes were used immediately or stored no longer than two weeks at -70°C. For the aminoacylation we followed the procedure published by White and Tener[25].

Sepharose 4B and affinity chromatography: Drosophila melanogaster tRNA was prefractionated on Sepharose 4B as described by Holmes et al.[26]. The column (2x30 cm) was eluted at a flow rate of 40 ml/h. The $tRNA^{Glu}$ containing fractions were dialysed, the material ethanol precipitated and chromatographed on a yeast $tRNA^{Phe}$ column according to Grosjean et al.[20]. 100 A_{260} of prefractionated tRNA was applied at a flow rate of 10 ml/h. The anticodon of the immobilized yeast $tRNA^{Phe}$ forms a stable complex with the anticodon of the Drosophila $tRNA_4^{Glu}$ at 4°C. The $tRNA^{Glu}$ was eluted at a flow rate of 10 ml/h by raising the temperature to 37°C.

RPC-5 chromatography: The $tRNA^{Glu}$ eluted from the affinity column was further purified by RPC-5 chromatography. Five A_{260} $tRNA_4^{Glu}$ were applied to a 0,63x30 cm high pressure column at 37°C[27]. A 200 ml salt gradient (0,45-0,65 M NaCl) was applied and the $tRNA_4^{Glu}$ peak eluted at 0,5 M NaCl. Fractions of 1,2 ml were collected every 0,8 minutes.

Two-dimensional polyacrylamide gel electrophoresis and fingerprints:
[^{125}I] $tRNA_4^{Glu}$ was fractionated on two dimensional gels according to Blatt and Feldmann[28]. The $tRNA_4^{Glu}$ spots were fingerprinted before and after electrophoresis according to standard procedures[29].

Iodination: The procedure of Prensky was followed in detail[30]. Na[^{125}I] was purchased from NEN Boston Mass. (Cat. No. NEZ-033 L). The iodinated tRNA was separated from the free iodine by DEAE-cellulose chromatography. The [^{125}I] tRNA was precipitated with poly(A) and redissolved for storage in H_2O or in hybridization buffer[30,31].

"In situ" hybridization: The conditions of Pardue and Gall as modified by Szabo et al. were followed[35,31]. The endogenous RNA was digested with RNase A (100 µg/ml). The DNA was denatured with 0,07 M NaOH at room temperature during 2,5 min. The hybridization was done in buffer containing 2xSSC, 50% formamide and 5 µm KI at 40°C during 15 hours. The tRNA concentration was 0,57 µM, 25 µl was applied per slide. In competition experiments Drosophila melanogaster 5S RNA was used in a 300-fold excess. The 5S RNA was extracted from 10% slab gels. The unspecific hybrids were removed by RNase A treatment (20 µg/ml). The slides were coated with Kodak NTB emulsion and exposed for 55 d at 4°C. The chromosomes were stained with Giemsa (Standard, Fluka, Buchs, Switzerland) diluted 1:20 with 10 mM phosphate buffer, pH 6,8.

RESULTS

Purification of $tRNA_4^{Glu}$: The elution profile of Sepharose 4B chromatography of Drosophila tRNA is shown in Fig. 1a. The acceptor activity of [^{14}C]-glutamic acid is depicted in Fig. 1b. Most of the $tRNA^{Glu}$ is concentrated in peaks V and VI. For comparison the charging for histidine is also shown. $tRNA^{His}$ elutes as a sharp peak in fractions VII-IX. The material from fractions V and VI was loaded on a yeast $tRNA^{Phe}$ affinity column. The elution profile (Fig. 2a) shows two peaks. Peak I contains tRNAs with non-complementary anticodons to yeast $tRNA^{Phe}$ while the second peak contains $tRNA^{Glu}$. When crude [^{14}C] -Glu-tRNA was cochromatographed with our purified [^{3}H] -Glu-tRNA on RPC-5 this $tRNA^{Glu}$ species eluted at the same position as $tRNA_4^{Glu}$ of Grigliatti et al.[5]. For further purification the $tRNA^{Glu}$ was applied to a RPC-5 column. The elution profile (Fig. 2b) shows a small shoulder on the left side of the main peak. This material may correspond to peak 3 (=$tRNA_3^{Glu}$) in the RPC-5 profile of Grigliatti et al.[5]. The shaded fractions were pooled and precipitated.

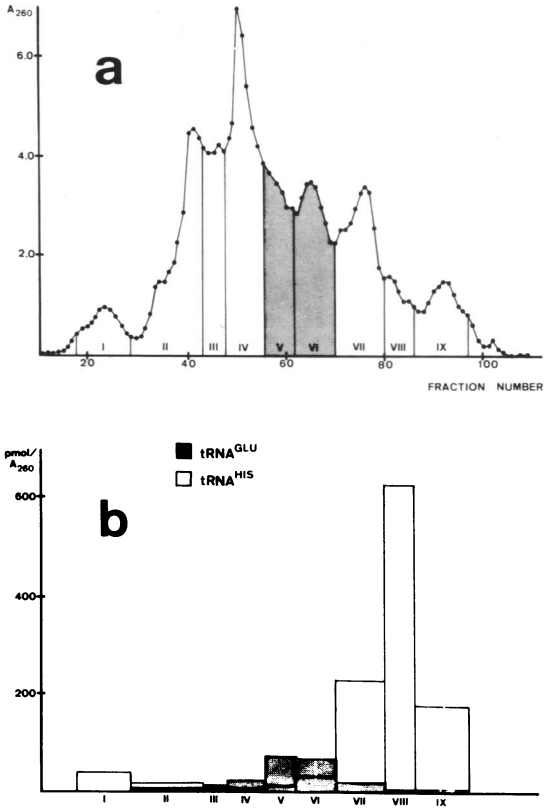

Fig. 1: a) Sepharose 4B chromatography of Drosophila melanogaster tRNA.
b) Charging profile for tRNA^{Glu} and tRNA^{His}.

Purification criteria: Minor contaminants in the purified RNA can lead to erroneous results if the DNA coding for the impurity is highly redundant[31] A careful characterization of the purity of the isolated tRNA is therefore important. We have applied three criteria: aminoacylation, fingerprint analysis and 2-dimensional polyacrylamide electrophoresis.

The charging levels obtained for tRNA^{Glu} after the affinity column and RPC-5 chromatography were 145 and 525 pmoles/A_{260} respectively. This is far below the expected value (1600 pmoles/A_{260}) for a pure tRNA. Drosophila melanogaster aminoacyl-tRNA synthetases, however, are unstable enzymes and maximum charging levels are seldom obtained. A fingerprint of iodinated tRNA$_4^{Glu}$ is shown in Fig. 3. Besides four faint spots, five major

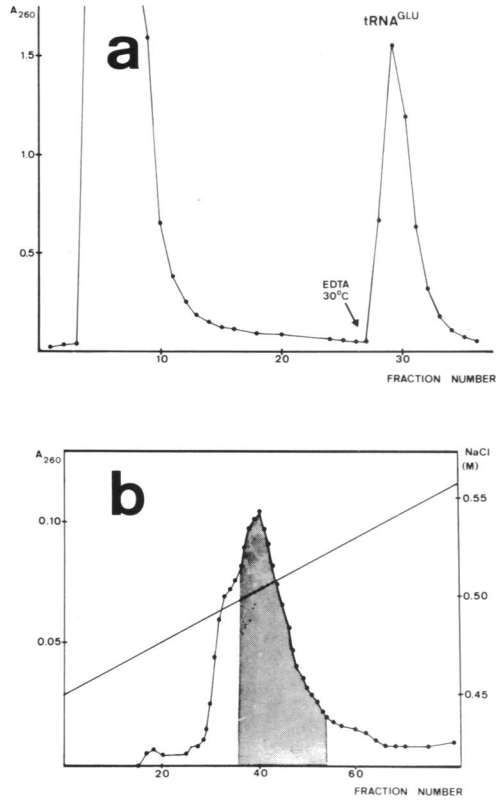

Fig. 2: a) Affinity chromatography of partially purified Drosophila tRNA.
b) RPC-5 chromatography of partially purified tRNA^{Glu}.

ones can be seen. They all correspond to the pancreatic fingerprint of uniformly labeled [^{32}P]- tRNA^{Glu} [32]. Since only cytosines will be iodinated, this agrees with the expectation for a fingerprint of pure iodinated tRNA. The differences in intensities are due to the fact, that exposed cytosines will be labeled to a greater extent than cytosine in the secondary structure of the tRNA[33].

Two-dimensional polyacrylamide electrophoresis resolves the iodinated tRNA$_4^{Glu}$ into two spots of different intensity (Fig. 4). The fingerprints of the two tRNAs are identical and do not differ from the fingerprint of the iodinated tRNA$_4^{Glu}$ before electrophoresis (Fig. 3). The minor component may therefore be an iodination artifact, or a minor tRNA^{Glu} isoacceptor with the same anticodon and similar nucleotide sequence[34].

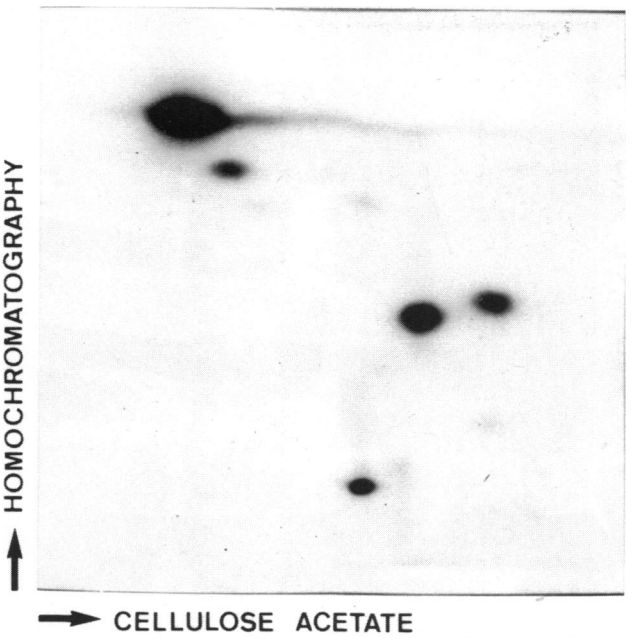

Fig. 3: Pancreatic RNase fingerprint of [^{125}I]tRNA$^{Glu}_{4}$.

Iodination and "in situ" hybridization: The applied iodination procedure gave specific activities of $2 \cdot 10^{8}$ dpm/µg tRNA. This corresponds to an average incorporation of 1.25 iodine molecules per tRNA. The "in situ" hybridization procedure of Pardue and Gall was then followed with two changes in the procedure[35]. Firstly, poly(A) was used as carrier, and secondly, the chromosomes were treated at 75°C for two hours with 2xSSC for better conservation of the chromosomal morphology[31,8]. This resulted in a strong reduction of unspecific hybridization and also lessened somewhat the grains at the tRNA loci. As can be seen from Fig. 5 labeling with [^{125}I]- tRNA$^{Glu}_{4}$ was found on the right arm of the second and on the left arm of the third chromosome in three regions: 52 F, 56 EF and 62 A. Grain counts were made on chromosomes on different slides (Table 1). With a graphical method it was shown, that the label over all three loci is statistically significant within a confidence limit of $p < 0{,}001$[36].

Special care was taken to exclude falsification of the results by a possible hybridization of contaminating molecules. The hybridization conditions were adapted to the procedure of Szabo et al.[31]. A low $R_o t$ value was used to eliminate the hybridization of a contaminant RNA represented

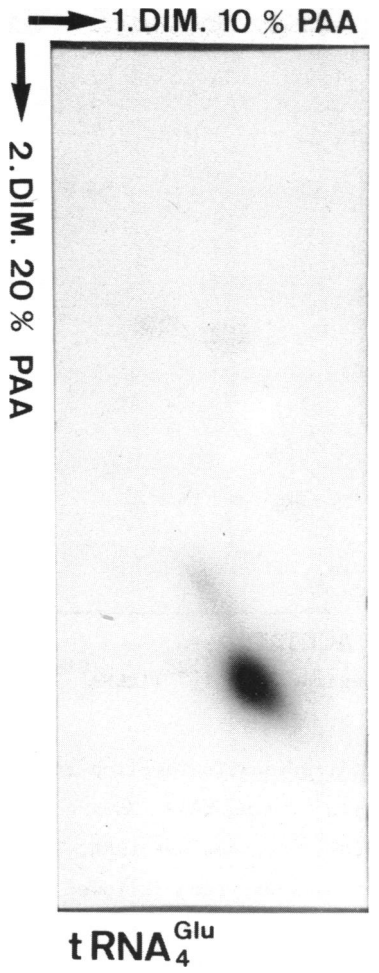

Fig. 4: Autoradiography of a two-dimensional polyacrylamide gel of [^{125}I] tRNA$_4^{Glu}$.

in the genome by a highly redundant sequence. Grigliatti et al. found a strong labeling at the 5S RNA locus that could be reduced by the addition of cold 5S RNA to the iodinated tRNA$_5^{Lys}$ preparation[19]. Since in our experiments the locus of 5S RNA genes was also labeled, we added a 300 fold excess of cold 5S RNA to our tRNA$_4^{Glu}$ preparation. It can be seen from Tab. 1 that there is no reduction in the grain number by the addition of competitor 5S RNA. In fact, there is a slight increase for the regions 52 F and 56 EF. This might be an effect of reduced RNase action on the iodinated tRNA$_4^{Glu}$ by the addition of the cold 5S RNA. The results of the

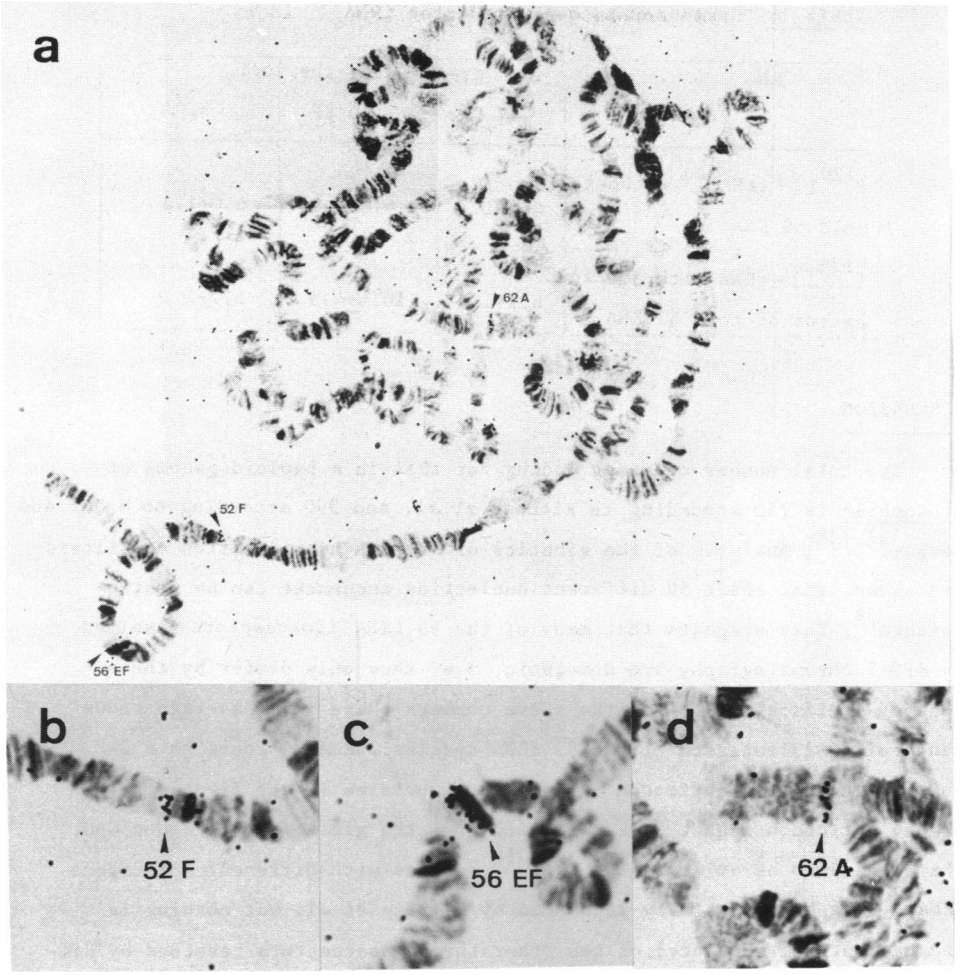

Fig. 5: a) Hybridization of [^{125}I] $tRNA_4^{Glu}$ to salivary gland chromosomes of the mutant giant. Labeling is found at the regions 52 F, 56 EF and 62 A.

b) Region 52 F enlarged.

c) Region 56 EF enlarged.

d) Region 62 A enlarged.

2-dimensional polyacrylamide electrophoresis and the fingerprints of the [^{125}I] $tRNA_4^{Glu}$ support the conclusion, that there is no contaminating 5S RNA in the $tRNA_4^{Glu}$ preparation. It follows therefore, that the genes for $tRNA_4^{Glu}$ are localized in the regions 52 F, 56 EF and 62 A.

Table 1: Grain counts over the three $tRNA_4^{Glu}$ loci

RNA	grains over the loci		
	52 F	56 EF	62 A
[^{125}I]- $tRNA_4^{Glu}$ without cold 5S RNA	4.2±1.7	4.8±1.2	6.0±1.3
[^{125}I]- tRNA with 300 fold excess of cold 5S RNA	6.1±1.6	10.4±4.5	5.7±2.0

DISCUSSION

The total number of genes coding for tRNA in a haploid genome of Drosophila is 750 according to Ritossa et al. and 590 according to Weber and Berger[37, 38]. Analysis of the kinetics of RNA-DNA hybridization on filters has shown, that about 59 different nucleotide sequences can be distinguished[38]. This suggests that many of the 99 tRNA isoacceptors resolved by RPC-5 chromatography are homogenic, i.e. they only differ by the extent of modification. Given the above numbers there is an average redundancy of 10-13 for each of the 59 tRNA species. $tRNA_4^{Glu}$ contains a 2-thiouridine in the anticodon[5]. Since 2-thiouridine in the first position of the anticodon cannot base pair with G of the glutamic acid codon GAG, there have to be at least two $tRNA^{Glu}$ species with different anticodons. $tRNA_4^{Glu}$ has been partially sequenced by Altwegg et al. but nothing is known about the sequences of the other three isoacceptors resolved by RPC-5 chromatography[32]. Hence we do not know how many different $tRNA^{Glu}$ genes are present in the Drosophila genome.

Our "in situ" hybridization data show that $tRNA_4^{Glu}$ genes are localized in three regions: 52 F, 56 EF and 62 A. This means that the $tRNA_4^{Glu}$ genes are not clustered at one site in the Drosophila genome. This is in contrast to the findings of Grigliatti et al. and Delaney et al. who found one locus for $tRNA_5^{Lys}$ at 48 F - 49 A and one locus for $tRNA_3^{Val}$ at 84 D3-4[5,6]. The dispersion of genes for the same tRNA isoacceptor in the genome opens interesting questions about the control mechanisms for these genes. Analysis of one plasmid containing Drosophila tRNA genes has shown that more than one tRNA sequence can be found in close proximity[39]. Clarkson and

Kurer have demonstrated that Xenopus tRNA genes specific for methionine and valine are not intermingled, however, $tRNA_1^{Met}$ fragments seem to contain at least one other kind of tRNA species[40]. It would be of great interest to know if these genes are controlled together and possibly activated during specific phases of the development of these organisms.

The labeling at the region 56 EF is of special interest, since this is the site where the genes for 5S RNA have been localized[41]. The labeling of $[^{125}I]-tRNA_4^{Glu}$ at this site is very unlikely due to contaminating 5S RNA. This label is also not due to contaminating highly redundant DNA, since the only satellite DNA localized in the euchromatic part of Drosophila melanogaster chromosomes is in the left arm of chromosome 2[42]. Although tRNA genes have not been found on 5S RNA containing plasmids there is more than enough DNA available in these regions to code for 160 5S RNA genes and a few tRNA genes[43]. However, the sensitivity of the "in situ" hybridization technique and the available restriction maps for the 5S genes do not allow us to decide if the $tRNA_4^{Glu}$ genes are intermingled with the 5S RNA genes or are merely in close proximity.

Although there are no correlations established between genetic loci and the cytological map near 52 F, there are four mutants of interest in this region: M(2)d, M(2)S7, l(2)me, Su(f)[1]. Unfortunately the mutants M(2)d and Su(f) have been lost. White has analysed the isoacceptor pattern for all 20 amino acids in the mutant M(2)S7 and found no differences[16]. l(2)me is a recessive lethal mutant which stops growing after 3 days of larval development[44]. Most of the homozygous larvae never pupate and die 7-8 days after hatching from the egg[45]. Zust et al. have proposed the hypothesis that a deletion for tRNA genes might be involved in the lethal effect of this mutant[46]. Preliminary experiments have shown that the isoacceptor pattern of $tRNA^{Glu}$ is affected in this mutant[47]. A deletion has not been demonstrated by cytological analysis. But if the deletion is very small this may not be possible. A combined restriction-hybridization approach may indicate, if there are any changes in the $tRNA^{Glu}$ genes in l(2)me.

In a previous attempt Steffensen and Wimber have tried to localize the tRNA genes by hybridizing crude ^3H-labeled tRNA to salivary gland chromo-

somes[18]. Since the specific activity of the tRNA was low, the loci had to be obtained by statistical methods and have therefore to be interpreted with caution. These authors found grains over the regions 53 A and 56 EF, the latter being attributed to contaminating 5S RNA molecules. The region 53 A, however, is very close to 52 F where label was found in our experiments. It might therefore represent the same tRNA locus. Since only the X and the second chromosome were screened by Wimber and Steffensen, the region 62 A was not included.

Lindsley et al. have produced a set of Drosophila aneuploids by combining elements of two Y-autosome translocations with displaced autosomal breakpoints[48]. By analysing deficiencies covering the whole genome, 41 Minute phenotypes were found. Among them are deficiencies covering the region of two $tRNA_4^{Glu}$ loci determined in this work (56 EF and 62 A). Although this does not prove that Minutes are caused by tRNA deletions, it supports the hypothesis of Atwood[15]. Comparison of the $tRNA^{Glu}$ isoacceptor patterns and analysis of the DNA coding for $tRNA_4^{Glu}$ in these mutants may give further insights into this problem.

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