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The Localization of Transfer RNA$_5^{Lys}$ Genes in *Drosophila melanogaster*

(5S RNA/^{125}I-iodination/*in situ* hybridization)

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ABSTRACT **Transfer RNA$_5^{Lys}$ was isolated and purified from *Drosophila melanogaster*. It was iodinated *in vitro* with sodium [^{125}I]iodide and hybridized *in situ* to the polytene salivary gland chromosomes from Drosophila. Autoradiographs exposed three to four weeks allowed the localization of this tRNA to the right arm of chromosome 2 between bands 48F and 49A.**

In eukaryotic organisms, mutations in cistrons coding for ribosomal RNA that are known to be redundant have yielded considerable information (1). The fact that the repeat sequences are homogeneous poses a fundamental question of how the duplicates retain their fidelity with one another. Transfer RNA molecules represent another class of gene transcripts that can be isolated with a high degree of purity and that have been estimated to be duplicated to the extent of at least 8 gene copies per iso-accepting tRNA in Drosophila (2). Mutations of tRNA genes have yielded considerable data on tRNA precursors and gene structure in *Escherichia coli* (3), It appears that the tRNA genes, at least of some phages of *E. coli*, are clustered and may be transcribed as a transcriptional unit (4, 5). It will be of interest to see if such situations occur in eukaryotes where tRNA changes during development have been implicated in possible translational control mechanisms (6). The chromosomal localization of single species of tRNA molecules in Drosophila by hybridization *in situ* would indicate the extent of redundancy and the chromosomal position of the cistrons. In addition to providing a direct test of Atwood's (7) suggestion that mutations of the *Minute* class represent sites of tRNA cistrons, a localization will permit the use of genetic techniques (8) for the recovery of mutations in this region. This paper reports the first cytological localization of the genes coding for a lysine tRNA.

MATERIALS AND METHODS

The Samarkand wild-type strain of *Drosophila melanogaster* was raised in large hatching cages at 22°, with a standard growth medium. The larvae were fed daily with yeast paste from 1 day after hatching until the late third larval instar. After anesthetization, adult flies were harvested with a vacuum cleaner and immediately frozen on dry ice. They were subsequently stored at either −70° or −26°.

The tRNA was isolated by phenol extraction (9) and DEAE–cellulose chromatography (10). The aminoacyl-tRNA synthetases were prepared from adults by a modification of the methods described by Twardzic *et al.* (11). The methods used are described fully elsewhere (12).

Purification of Drosophila tRNA$_5^{Lys}$. Crude tRNA (1–2 g) was suspended in 0.35 M NaCl–10 mM $MgCl_2$ and applied to a benzoylated DEAE–cellulose (BD–cellulose) column (2.5 × 110 cm). The column was eluted by a 10-liter linear gradient from 0.4–1.0 M NaCl containing 10 mM $MgCl_2$. The flow rate was 300 ml/hr, with fractions of 23 ml each being collected. Each fraction was assayed for amino-acid acceptance, and the peak containing tRNA$_7^{Ser}$ and tRNA$_5^{Lys}$ was isolated and chromatographed on a reversed phase-5 (RPC-5) column (2.4 × 60 cm) (13). The RPC-5 column was eluted with a 2-liter gradient of 0.6–0.7 M NaCl, containing 10 mM $MgCl_2$, 10 mM sodium acetate (pH 4.5), and 1 mM 2-mercaptoethanol, at 180 ml/hr (37°); 10-ml fractions were collected. The second major peak from this column contained tRNA$_5^{Lys}$ in virtually pure form. It was isolated and further purified by chromatography on an RPC-5 column under similar conditions at pH 8.0.

Preparation and Purification of 5S RNA. 5S RNA was prepared from a crude tRNA extract by chromatography on a Sephadex G-100 column (2.5 × 195 cm) in 10 mM Tris·HCl (pH 7.5) buffer containing 40 mM NaCl and 10 mM magnesium acetate. Fractions containing 5S RNA were pooled, and the RNA was precipitated with 2 volumes of 95% ethanol at −20° overnight. The RNA was isolated, suspended in a buffer containing 0.45 M NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 10 mM sodium acetate (pH 4.5), and applied to an RPC-5 column (0.9 × 60 cm). The RNA was eluted at 50 ml/hr with a 700-ml gradient (0.55–0.65 M NaCl) in the preceding buffer; 3.5-ml fractions were collected. Fractions containing 5S RNA were pooled and extensively dialyzed against distilled water; the RNA was isolated by freeze-drying.

Gel Electrophoresis. Different preparations of RNAs were analyzed on 10% polyacrylamide disc gels (14) and run for 2–2.5 hr at 200 V at 4°.

Iodination of RNA. RNA preparations were iodinated *in vitro* by a modification of the technique reported by Commerford (15). The reactions were carried out at 60° for 20 min in sodium acetate at pH 4.0 instead of pH 5.0. Carrier-free sodium [^{125}I]iodide (1–2 mCi; New England Nuclear Corp.) was used for each reaction mixture containing 4 μg of RNA in a total reaction volume of 20–25 μl. Increasing the $TlCl_3$ concentration to 2.4 mM approximately doubled the ^{125}I incorporation. The heating step in the presence of Na_2SO_3, which removes the loosely bound iodine, was performed at 75° for 30 min (15–17). Care was taken not to overexpose the tRNA to Na_2SO_3, as it can cause conversion of cytosine to uracil (18, 19).

Two techniques for separating the iodinated RNA from the reaction mixture were most successful in reducing the

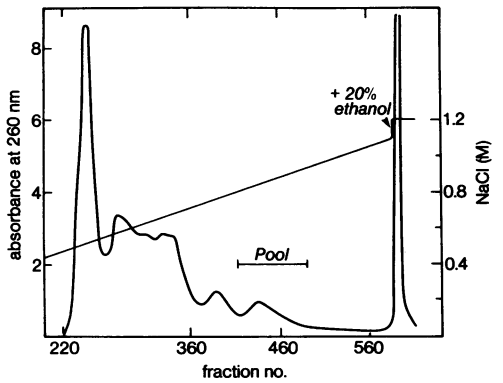

FIG. 1. Chromatography of Drosophila tRNA on BD-cellulose. The peak preceding fraction number 460 (peak 7) contains $tRNA_5^{Lys}$, and was pooled as indicated. See *Materials and Methods* for details.

background in hybridization experiments. The first was chromatography on a Sephadex G-10 column (0.8 × 25 cm) in low salt buffer containing 1 mM Na_2SO_3, followed by extensive washing of the tRNA with 0.25 M NaCl in Tris·HCl buffer (pH 7.5) on a small DEAE-cellulose column (0.8 × 3 cm). The RNA was removed by addition of 1 M NaCl, dialyzed against distilled water, lyophilized, and suspended in 0.60 M NaCl–0.060 M sodium citrate, pH 7.0 for hybridization. The second technique involved chromatography on a small hydroxylapatite column in phosphate buffer followed by the Na_2SO_3 treatment (15), then rechromatography on a second small hydroxylapatite column and extensive dialysis against 0.15 M NaCl–0.015 M sodium citrate (17).

We determined the yield of iodinated tRNA by adding 100 μg (about 2 A_{260} units) of carrier *E. coli* tRNA at the first chromatographic step and calculating the percent recovery. The specific activity of the final tRNA could thus be estimated; it ranged between 5 × 10^7 and 1 × 10^8 dpm/μg of tRNA, the variability being at least partly dependent on the age of the ^{125}I. At the higher specific activity, there is approximately one ^{125}I-labeled cytosine per tRNA molecule. The following Drosophila RNAs have been iodinated: (*i*) crude tRNA preparations containing 4S RNA, 5S RNA, and fragments of

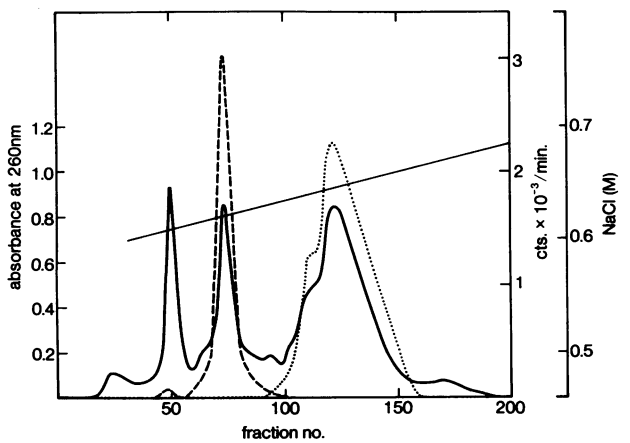

FIG. 2. Chromatography of pooled fractions from BD-cellulose column on a preparative RPC-5 column. *Solid line*, A_{260}; *dashed line*, cpm [^{14}C]lysyl-tRNA; *dotted line*, cpm [^{14}C]-seryl-tRNA. See *Materials and Methods* for details.

18S and 28S ribosomal RNAs; (*ii*) purified 5S RNA; (*iii*) $tRNA_7^{Ser}$, $tRNA_5^{Lys}$, $tRNA_2^{Phe}$, and $tRNA_{1\gamma}^{His}$ (2).

In Situ Hybridization. Salivary glands, containing polytene chromosomes, were isolated, fixed, and squashed by standard cytological techniques (for a recent review, see ref. 20). The chromosomes used were obtained from larvae bearing the mutation. *giant* (*gt*, 1–0.9), which prolongs the larval lifespan, thus allowing one or two extra rounds of DNA replication, thereby increasing the polyteny of the salivary gland chromosomes (21, 22). This 2- or 4-fold increase in the amplification of chromosomal DNA should facilitate the detection by hybridization *in situ* of genes of a lower tandem redundancy or small size, such as those for the tRNAs. The chromosomes were prepared for hybridization by the techniques of Gall and Pardue (20). The hybridization was in 0.30 M NaCl–0.030 M sodium citrate or in 0.60 M NaCl–0.060 M sodium citrate (0.04 μg of RNA in 50 μl per slide) for 18 hr at 65°. The slides were then dipped in Ilford K.5 liquid emulsion, dried, and stored at 4° for various lengths of time. The emulsion was developed in D-19 (Kodak) for 3 min.

RESULTS

$tRNA^{Lys}$. The results of chromatography of Drosophila tRNA on BD-cellulose are shown in Fig. 1. The peak preceding fraction 460 contained mainly $tRNA_7^{Ser}$ and $tRNA_5^{Lys}$; upon chromatography on a preparative RPC-5 column at pH 4.5 (Fig. 2), it was resolved into four peaks. The second major peak contained the $tRNA_5^{Lys}$. When this peak was isolated and chromatographed on an RPC-5 column at pH 8.0, it eluted as one symmetrical peak, and had an amino acid acceptance of greater than 1730 pmoles/A_{260} unit. In subsequent tests on polyacrylamide disc gels under conditions where a 1% contaminant could be detected, the $tRNA_5^{Lys}$ migrated as a single sharp band. Therefore, by usual biochemical standards, this $tRNA_5^{Lys}$ is pure.

5S RNA. The elution profile of 5S RNA from the Sephadex G-100 column was similar to that reported earlier (23). On an RPC-5 column, 1 minor and 2 major peaks could be detected, and the two major peaks were isolated. The 5S RNAs isolated from RPC-5 columns migrated as pure species on polyacrylamide disc gels.

Hybridization. The utility of ^{125}I-labeled RNA of high specific activity and the increased DNA content of chromosomes from the *giant* strain for hybridization experiments *in situ* is well documented (17, 24). Ten- to thirty-day autoradiographic exposures of chromosomes incubated with ^{125}I-labeled $tRNA_5^{Lys}$ showed a significant amount of label over two areas of the genome (Fig. 3). There were numerous grains over 56EF, the 5S RNA region (*arrow a*, Fig. 3). In addition, the 48F–49A region of the right arm of chromosome 2 (2R) was consistently labeled with 25–30 grains after a 25-day exposure (*arrow b*, Fig. 3).

There are three possible explanations for these results: (*i*) the grains over 56EF result from 5S RNA contamination in the $tRNA_5^{Lys}$ preparation; (*ii*) the $tRNA_5^{Lys}$ genes are located at both 48F–49A and 56EF; and (*iii*) there are sufficient sequence homologies between $tRNA_5^{Lys}$ and 5S RNA to allow cross-hybridization.

We attempted to eliminate one or more of these possibilities by competitive hybridization studies. Dilution competition

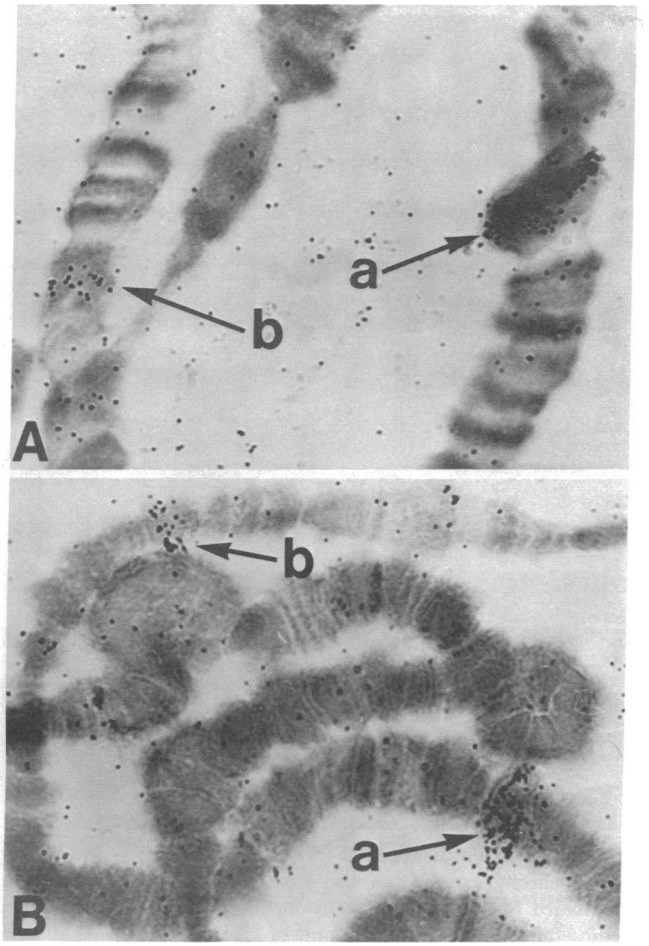

FIG. 3. Hybridization of purified $tRNA_5^{Lys}$ to salivary gland chromosomes from the *giant* mutant. (*A* and *B*) Two examples of hybridization with ^{125}I-labeled $tRNA_5^{Lys}$ (specific activity about 8×10^7 dpm/μg) showing labeled material at region 56EF (*a*) and region 48F–49A (*b*). These preparations were exposed for 27 days. Total magnification ×3240.

experiments between ^{125}I-labeled 5S RNA and various amounts of unlabeled 5S RNA were performed as a control for the competition experiments between labeled tRNA and unlabeled 5S RNA. Purified 5S RNA was labeled with ^{125}I to a specific activity of about 3×10^7 cpm/μg and allowed to compete for binding sites on the salivary chromosomes with unlabeled 5S RNA at concentrations of 0, 5, 10, 25, and 50 times that of the input ^{125}I-labeled 5S RNA. After incubation and washing, the emulsion was exposed for 40 hr. The results are shown in Figs. 4 and 5.

The amino-acid acceptor activity and polyacrylamide gel electrophoresis pattern of the $tRNA_5^{Lys}$ preparation suggest that there is less than 1% contaminating material. Therefore, enough unlabeled purified 5S RNA was added to a preparation of ^{125}I-labeled $tRNA_5^{Lys}$ to make unlabeled 5S RNA 10% of the total RNA present. This would be at least 10 times the maximum amount of possible contamination. This solution was used for hybridization *in situ*, and the slides were exposed for 30 days. There was at least a 2-fold reduction of label at the 56EF region (from greater than 55 grains to about 29 grains) with little or no reduction of label at the 48F–49A region (from 20 grains to 18 grains) (Fig. 6). Subsequently, a dilution competition hybridization was performed with solutions of

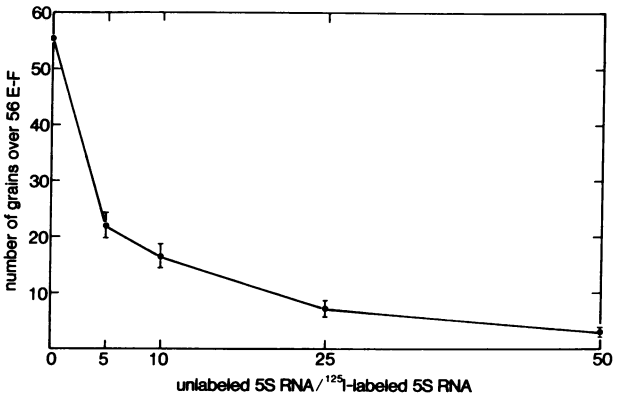

FIG. 4. Results of competition hybridization between unlabeled 5S RNA and ^{125}I-labeled 5S RNA to salivary gland chromosomes from the *giant* mutant, at increasing ratios of unlabeled 5S RNA to labeled 5S RNA. The slides were incubated for 15 hr at 65° and exposed for 40 hr. Each point represents the mean of the number of grains over the 56EF region of 50 chromosomes. The 95% confidence limits were not included for the first value since the grains are too numerous to be counted accurately. The first point, therefore, represents the average minimum number of grains we can detect.

^{125}I-labeled $tRNA_5^{Lys}$ containing unlabeled 5S RNA at 0.0, 0.02, 0.1, 0.25, and 0.5 times the amount of labeled $tRNA_5^{Lys}$. The results were consistent with the preceding result and suggest that the $tRNA_5^{Lys}$ is slightly contaminated with 5S RNA. When unlabeled RNA of any type (including *E. coli* tRNA) was added to preparations of ^{125}I-labeled tRNA, the background often increased; we have no explanation for this phenomenon.

Extended exposure (49 days) of chromosomes hybridized with purified ^{125}I-labeled 5S RNA (6×10^7 cpm/μg) showed grains only at the 56EF region and no grains at the 48F–49A region. Hence, there is no homology between 5S RNA and the DNA in 48F–49A. Thus, the grains detected at 48F–49A (Fig. 3) are not a reflection of 5S RNA contamination and are, therefore, presumed to be a reflection of $tRNA_5^{Lys}$ hybridization.

DISCUSSION

Hybridization has been attempted with four purified species of tRNA (24). The most intriguing results have been those obtained with $tRNA_5^{Lys}$. This $tRNA_5^{Lys}$ preparation has been rigorously tested and shown to be very pure by several criteria. However, when hybridized to the salivary gland chromosomes, it consistently gave a significant number of grains over the 5S RNA locus, 56EF. Assuming that $tRNA_5^{Lys}$ genes are not located at 56EF, the sample obviously contains a contaminating species of RNA that will hybridize to this region. We feel this result should serve as a caution to accepting any *in situ* localization result too readily.

As it is difficult to purify any nucleic acid to 100% homogeneity, it is always possible that a preparation contains a contaminant. If this contaminating RNA is transcribed from a gene with significant tandem redundancy, it would produce more grains than the RNA being localized and lead to completely erroneous results. Therefore, the assignment of any locus by this technique should remain putative, until genetic studies produce a more definitive answer. The final proof, even in the well-documented localization of 5S RNA in Drosophila

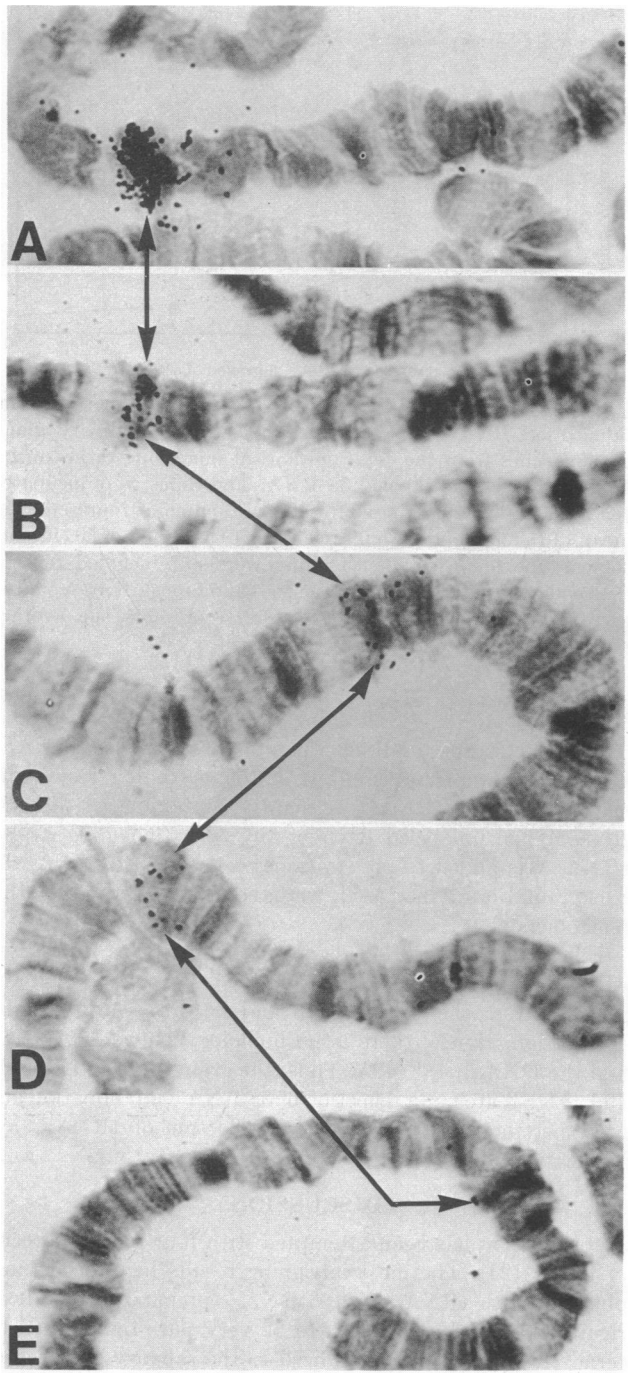

FIG. 5. Competition hybridization between unlabeled 5S RNA and ^{125}I-labeled 5S RNA to salivary gland chromosomes from the *giant* mutant. Purified ^{125}I-labeled 5S RNA (specific activity about 3×10^7 dpm/μg) hybridized as described in *legend* of Fig. 4. Ratio of unlabeled 5S RNA to ^{125}I-labeled 5S RNA; (*A*) 0; (*B*) about 5; (*C*) about 10; (*D*) about 25; and (*E*) about 50. Total magnification ×3240.

(25), will rest with approaches such as those initiated by Nix (8).

In addition to the label at 56EF, hybridization *in situ* with ^{125}I-labeled $tRNA_5^{Lys}$ consistently produced a significant number of grains at the 48F–49A region. The number of grains found over this region at various exposure times closely approximates the number expected from calculations based

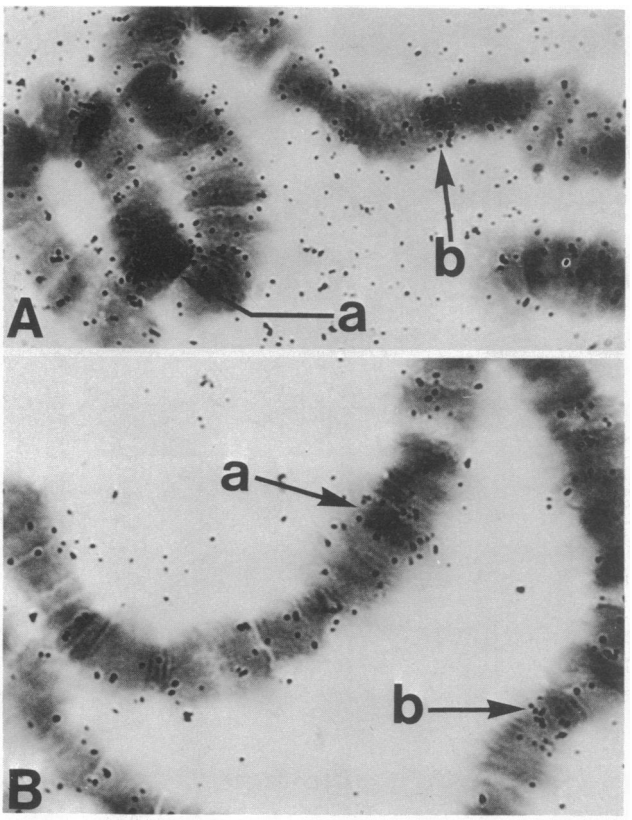

FIG. 6. Hybridization of ^{125}I-labeled $tRNA_5^{Lys}$ to salivary gland chromosomes from the *giant* mutant in the presence of unlabeled 5S RNA. Slides were incubated for 18 hr at 65° and exposed for 30 days. The amount of 5S RNA is at least 10 times the suspected 5S RNA contamination (see *text*). Segments of the right arm of two different second chromosomes are shown. *Arrow a*, 56EF region; *arrow b*, 48F–49A region. When unlabeled RNA of any type is added, the background is often increased. Total magnification ×3240.

on tRNA genes with a 10-fold tandem redundancy. Therefore, this might represent the region of the chromosome in which the $tRNA_5^{Lys}$ genes are located.

Competition experiments were used to determine whether the label at the 56EF region was due to a contamination of our $tRNA_5^{Lys}$ preparation. Addition of unlabeled 5S RNA to the ^{125}I-labeled $tRNA_5^{Lys}$ at a concentration at least 10 times the maximal level of any contaminant reduced the number of grains at 56EF by about 2-fold. This result agrees with the control curve obtained from competition experiments *in situ* between ^{125}I-labeled 5S RNA and various amounts of unlabeled 5S RNA, where a 10-fold excess of unlabeled 5S RNA reduced the grain count at least 3-fold (see *legend* of Fig. 4). We conclude, therefore, that the grains over the 56EF region in hybridization experiments with ^{125}I-labeled $tRNA_5^{Lys}$ are a result of 5S RNA contaminants in the preparation. In addition, very long exposures (49 days) of chromosomes hybridized with purified ^{125}I-labeled 5S RNA resulted in no label over the 48F–49A region, while the 56EF region was black with grains. Therefore, the label over the 48F–49A region in slides hybridized with ^{125}I-labeled $tRNA_5^{Lys}$ is not due to 5S RNA, its breakdown products, or a contaminant that is present in both the 5S RNA and the $tRNA_5^{Lys}$ preparations.

Finally, the grains at 48F–49A are not a result of contamination of the $tRNA_5^{Lys}$ with fragments of highly redundant

satellite DNA, as the only *Drosophila melanogaster* satellite DNA localized to a euchromatic area is in the left arm of chromosome 2 (26). Therefore, all of the evidence is consistent with the conclusion that the genes for $tRNA_5^{Lys}$ are located within the 48F–49A region of chromosome 2. The argument made earlier in the *Discussion*, that this may be a region complementary to some unknown contaminant in our preparation, cannot be eliminated, though it does seem unlikely.

It is interesting to compare our localization of purified $tRNA_5^{Lys}$ with the results Steffenson and Wimber (27) obtained using [^{3}H]uracil-labeled crude 4S and 5S RNA fractions. Their 4S RNA fraction produced little or no label in the area of 48F–49A. However, they concluded the 5S fraction did show a statistically significant amount of label at 49B, after a 6-month exposure.

In their original study of segmental aneuploidy of the Drosophila autosomes, Lindsley *et al.* (28) concluded that there was no *Minute* mutation associated with the 48F–49A segment of the polytene chromosome. However, in a recent and more intensive study, Lindsley and Day (personal communication) have found that heterozygosity for a deficiency spanning the interval from 48E to 50A produces a fly with vestigial wings and all of the characteristics of a *Minute* phenotype. From previous studies (29), it was concluded that a series of deletions extending from 49B to 50A produces a *vestigial* but not a *Minute* phenotype. It, therefore, appears that there is a *Minute* locus in the region 48E to 49B, the precise location in which we find hybridization with ^{125}I-labeled $tRNA_5^{Lys}$. This correlation is further supported by the genetic localization of *Minute(2)40c* at 65, just two map units to the left of *vestigial* (30). Hence, it is near bands 48F–49A on the cytological map; unfortunately, this mutation no longer exists. While we do not take this correlation as proof of Atwood's hypothesis that *Minute* mutations are lesions in the tRNA genes, it does lend credence to his suggestion.

The extent of labeling detectable after 25–30 days is consistent with considerable duplication of the genes coding for $tRNA_5^{Lys}$ around 48F–49A, possibly as tandem repeats. This does not eliminate the possible existence of single gene copies for $tRNA_5^{Lys}$ elsewhere in the genome. With the genetic tools available in Drosophila, we can now expect extensive genetic characterization of clusters of tRNA cistrons.

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