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CHARACTERIZATION OF RECOMBINANT PLASMIDS CARRYING *DROSOPHILA* TRANSFER RNA GENES

by

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Abstract

The purpose of this study was to characterize recombinant plasmids carrying Drosophila melanogaster tRNA genes. The two groups of recombinant plasmids studied were those which carried $tRNA_4^{Val}$ genes and those with $tRNA_{4,7}^{Ser}$ genes.

pDt92 and pDt120, both $tRNA_4^{Val}$ gene-carrying plasmids, were characterized initially to determine the number of inserts they contained and the size of the inserts. For plasmids containing multiple inserts, the insert which carried the $tRNA_4^{Val}$ gene was also determined. These characteristics were studied by HindIII digestion of the plasmid DNA, agarose gel electrophoresis, Southern transfer onto nitrocellulose filters and hybridization to $[^{125}I]$ $tRNA_4^{Val}$. It was found that both, pDt92 and pDt120 contained two inserts each of sizes 0.5kb and 1.7kb, and 2.0kb and 5.4kb respectively, with the 0.5kb and 2.0kb fragments carrying the $tRNA_4^{Val}$ genes.

pDt92 and pDt120 then were recloned so as to contain only the fragments which carried the $tRNA_4^{Val}$ genes, namely the 0.5kb and 2.0kb fragment respectively.

pDt92RC and pDt120RC plus three other $tRNA_{4,7}^{Ser}$ gene containing plasmids, pDt16, pDt17RC and pDt27RC were further characterized by the technique of in situ hybridization to study the organization of these tRNA genes on the Drosophila genome. Four of these plasmids with the exception of pDt17RC hybridized to only one site on the Drosophila chromosome. Both, pDt92RC and pDt120RC hybridized

to the 90BC site on the right arm of the third chromosome; pDt16 and pDt27RC hybridized to the 12DE site on the first or the X chromosome. pDt17RC on the other hand hybridized predominantly to the 12DE site and to a lesser extent to 23E (2L), 56D (2R), 62D (3L) and 64D (3L) sites.

These in situ hybridization results when studied together with those reported by Dunn et al. (1979b) show that genes for a single species of tRNA are located on more than one site on the Drosophila genome.

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Abbreviations Used

ATP	-	5' - riboadenosine triphosphate
CTP	-	5' - ribocytidine triphosphate
GTP	-	5' - riboguanosine triphosphate
UTP	-	5' - ribouridine triphosphate
RNA	-	ribonucleic acid
tRNA	-	transfer ribonucleic acid
$tRNA^{Val}$	-	nonacylated valine tRNA
$tRNA^{Ser}$	-	nonacylated serine tRNA
rRNA	-	ribosomal ribonucleic acid
cRNA	-	complementary RNA
DNA	-	deoxyribonucleic acid
RNase	-	ribonuclease
DNase	-	deoxyribonuclease
Arg, Asn, Asp,	-	amino acids: arginine, asparagine,
Glu, Ile, Lys,		aspartic acid, glutamic acid, isoleucine,
Met, Ser, Val		lysine, methionine, serine, valine
HindIII	-	restriction endonuclease isolated from Haemophilus influenzae Rd
pDt	-	recombinant plasmid carrying Drosophila tRNA gene
pDtRC	-	recloned pDt (Recloned pDt was designated as pDtR by Dunn et al. (1979b))
RPC	-	reverse phase chromatography
LB	-	Luria broth
EDTA	-	ethylene diamine tetra acetic acid
Tris	-	Tris (hydroxymethyl) aminomethane
2-ME	-	2-mercaptoethanol

Abbreviations Used

DTT	-	dithiothreitol
cpm	-	counts per minute
Fig.	-	figure
mCi	-	millicurie (10^{-3} curie)
min.	-	minute
ul, ml	-	microliter (10^{-6} liter), milliliter (10^{-3} liter)
ug, g	-	microgram (10^{-6} gram), gram
mM, M	-	millimolar (10^{-3} molar), molar
2L, 2R, 3L, 3R	-	left or right arm of second or third chromosome of *Drosophila*
U.V.	-	ultraviolet

INTRODUCTION

Transfer RNAs are very important biological molecules. Besides their central role in protein synthesis, tRNAs have been shown to have many other functions (Rich et al., 1976). In *Drosophila* there exists some evidence that tRNA genes are differentially regulated during development (White et al., 1973a) and Atwood (1968) has suggested that a class of mutants called *Minutes* represent lesions in the tRNA genes. The elucidation of the arrangement, structure and the function of the tRNA genes would help to study some of these aspects. The availability of extensive genetic information and of many mutants together with the existence of polytene salivary gland chromosomes make *Drosophila* an ideal system for studying tRNA gene organization and expression.

Recent studies indicate that there are approximately 590 (Weber and Berger, 1976) to 750 (Ritossa et al., 1966) genes for all species of tRNA in the haploid genome of *Drosophila*. An estimate of 400-500 genes for 4S RNA from *in situ* hybridization studies (Elder et al., 1980) may be too low. On RPC-5 columns, *Drosophila* tRNA can be resolved into 63 major and 36 minor peaks (White et al., 1973a) giving a total of 99 different species of tRNA. It has been suggested (White et al., 1973b) that several chromatographically distinct forms of isoaccepting tRNAs have the same nucleotide sequence and are probably products of the same gene i.e., these are homogeneic species resulting from different degrees of post-transcriptional modification. From an analysis of the kinetics of RNA:DNA

hybridization on membrane filters, Weber and Berger (1976) estimated that Drosophila tRNA is made up of about 59 families of kinetically different sequences. All these data suggest that there is an average of 10-13 genes (Weber and Berger, 1976; Ritossa et al., 1966) for each tRNA sequence.

The technique of in situ hybridization developed by Gall and Pardue (1969) and John et al. (1969) 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 itself. Using this technique, DNA sequences known to be highly redundant in Drosophila have been mapped on the chromosomes; 5S RNA genes occur at 56EF (Wimber and Steffenson, 1970) and those for histones at 39DE (Pardue et al., 1977). The salivary gland chromosomes from the mutant, giant, (Kaufman, 1971) with an increase in the degree of polyteny, facilitates the localization of genes of a lower tandem redundancy (i.e., tRNAs) than is available in the case of 5S, 18S and 28S rRNAs and histone genes.

Steffenson and Wimber (1971) hybridized total 4S ^{3}H-RNA to intact chromosomes of D. melanogaster and found that it hybridized to 68 sites widely distributed on the two chromosomes examined. Most recently Elder et al. (1980) hybridized ^{125}I-labelled 4S RNA to salivary gland polytene chromosomes and identified a total of 63 sites. They believe that these 63 sites represent most of the tRNA sites present in Drosophila. Many of the sites determined by

these two groups agree but there are numerous differences, presumably due to the difficulty caused by the low specific activity of the ^{3}H-labelled 4S RNA. A comparison of the 4S RNA sites with the locations of Minute mutations suggests that the Minute loci do not correspond to the structural genes for tRNA (Elder et al., 1980; Tener et al., 1980).

In situ hybridization studies with single, purified tRNA species show that a single sequence may be present in several gene copies at more than one site on the chromosomes. Evidence for this conclusion is provided by Kubli and Schmidt (1978) who localized genes for $tRNA_{4}^{Glu}$ at 52F, 56EF and 62A, by T. Schmidt et al. (1978) who localized $tRNA_{2\delta}^{Asp}$ genes at 29D and E, by Elder (1978) who showed that $tRNA^{Arg}$ hybridized to 42A and 84F and $tRNA^{Met}$ to 48AB and 72F-73A and by Dunn et al. (1979a) who localized the genes for $tRNA_{3b}^{Val}$ at 84D, 90BC and 92B. Most recent evidence is provided by Hayashi et al. (1980) who showed that essentially all the twelve purified tRNAs tested, hybridized to more than one site on the polytene chromosomes. Whether all these tRNA sites truly represent presence of the respective gene depends on the purity of the tRNA used for hybridization. It is not possible to rule out that at least some sites are the result of traces of other RNAs hybridizing to highly redundant sequences on the DNA (Grigliatti et al., 1974; Hayashi et al., 1980; Elder et al., 1980).

The development of techniques for molecular cloning of DNA (Sinsheimer, 1977) has now made it possible to isolate the tRNA genes in pure form. This methodology has allowed the study of the organization of tRNA genes in the genomes of yeast (Beckman et al.,

1977), Xenopus laevis (Clarkson et al., 1978), Drosophila (Yen et al., 1977; Dunn et al., 1979b) and Bombyx mori (Hagenbüchle et al., 1979). A recombinant plasmid, pC1T12 (Yen et al., 1977) containing a 9.34 kb Drosophila DNA fragment has been found to contain a total of eight tRNA genes irregularly spaced within the DNA and coding for a single $tRNA_2^{Arg}$, three $tRNA^{Asn}$, one $tRNA^{Ile}$ and three $tRNA_2^{Lys}$ (O. Schmidt et al., 1978; Hovemann et al., 1980). Transcripts from this plasmid hybridized to the 42A region on the Drosophila chromosome (Yen et al., 1977).

Dunn et al. (1979b) undertook to isolate tRNA genes from Drosophila on recombinant plasmid DNA molecules by the "shot gun" technique. Such cloned tRNA genes would be useful as hybridization probes to localize tRNA genes on the Drosophila genome, they could serve as material for nucleotide sequence analysis to study the structure of the tRNA genes and finally they could serve as templates for transcription studies.

The procedure used to get the recombinant plasmids was given by Dunn et al. (1979b). A total of 90 clones of recombinant plasmids containing genes for eleven different tRNAs were isolated. Before these plasmids could be used for any of the purposes listed above, they had to be characterized initially to determine the number of inserts they carried, the size of the inserts and the insert which contained the tRNA gene. Where necessary those plasmids which carried multiple inserts were recloned. Such characterization for some of the recombinant plasmids isolated was reported by Dunn et al. (1979b). Some of these plasmids then were used to

study the organization of tRNA genes on the Drosophila chromosomes by the technique of in situ hybridization (Dunn et al., 1979b).

The purpose of this study was to finish characterization of two groups of recombinant plasmids isolated by Dunn et al., (1979b); those which contained $tRNA^{Ser}_{4,7}$ genes and those with $tRNA^{Val}_{4}$ genes. In situ hybridization studies on five of these plasmids confirmed the location of their genes on the chromosomes and added to the general picture that in Drosophila, genes for a single species of tRNA occur at more than one site on the genome.

MATERIALS

I. Reagents

Description	Source
ATP	Calbiochem Behring Corp.
Agar	Difco Laboratories
Agarose powder	Bio-Rad Laboratories
Ampicillin	Ayerst Laboratories
Bromophenol blue	BDH Chemicals Ltd.
Casamino acids	Difco Laboratories
Cesium chloride (technical grade)	Kawecki Berylco Industries, Inc.
Chloramphenicol	Sigma Chemical Co.
Dextrose	Difco Laboratories
Dithiothreitol	Calbiochem
E-coli tRNA	Sigma Chemical Co.
EDTA	J.T. Baker Chemical Co.
Ethidium bromide	Sigma Chemical Co.
Gelatin	Fischer Scientific Co.
Hydroxyapatite	Bio-Rad Laboratories
Carrier-free sodium ^{125}I (~500mCi/ml)	Amersham Searle Co.
2-mercaptoethanol	Bio-Rad Laboratories
Sodium lauryl sulphate (SDS)	BDH Chemicals
Sucrose	J.T. Baker
Tetracycline	Sigma Chemical Co.

Description	Source
Thallium chloride	ICN. K & K Labs., INC.
Thiamine	Calbiochem
Thymidine	Worthington Biochemical Corp.
Toluidine blue O	Baker Chemical Co.
Triethanolamine	Fischer Scientific Co.
Triton X-100	Sigma Chemical Co.
Tryptophan	Fischer Scientific Co.
Uridine	Sigma Chemical Co.

II. Enzymes

Bacterial alkaline phosphatase-F	Worthington Biochemical Corp.
DNase I	Worthington Biochemical Corp.
Lysozyme (egg white)	Sigma Chemical Co.
Pronase	Calbiochem
Proteinase K (fungal)	BDH Chemicals
RNase-A (Bovine pancreas)	Sigma Chemical Co.
RNA Polymerase I (E-coli)	P-L Biochemicals Inc.
Restriction endonuclease HindIII	New England Biolabs
T_4 polynucleotide ligase	Provided by Dr. R.C. Miller

III. Solutions

1. For preparation of plasmid DNA

i)	Tris-HCl	0.05M	pH8.0
	Sucrose	25%	

ii) Triton X-100 10%

Tris-HCl 0.05M pH8.0

EDTA 0.0625M

iii) Dialysis buffer (TEN)

Sodium chloride 0.02M

Tris-HCl 0.02M pH8.0

EDTA 0.001M

2. Restriction Endonuclease buffers

i) HindIII buffer

Sodium chloride 60mM

Magnesium chloride 7mM

Tris-HCl 10mM pH7.4

Gelatin 100 ug/ml

3. Agarose gel electrophoresis

i) Tris-Phosphate buffer

Tris 0.04M pH8.0

Sodium phosphate (NaH_2PO_4) 0.02M

EDTA 0.001M

ii) Loading mix

Sucrose 40%

EDTA 0.025M

Bromophenol blue 0.02%

4. Southern transfer and Hybridization

i) Denaturing solution

Sodium hydroxide 0.2M

Sodium chloride 0.6M

Thymol blue 20 mg/l

ii) Neutralizing buffer

Sodium chloride	1.5M	
Tris-HCl	0.5M	pH7.4

iii) 10X SSC

Sodium chloride	1.5M
Sodium citrate	0.15M

iv) 2XSSC + 0.5% SDS

Sodium chloride	0.3M
Sodium citrate	0.03M
sodium dodecyl sulphate	0.5%

5. Recloning procedure and Hogness hybridization

i) Ligation buffer

Tris-HCl	50mM	pH7.5
Magnesium chloride	10mM	
DDT	1mM	
ATP	1mM	

ii)

Sodium chloride	1.5mM	
Tris-HCl	0.5M	pH7.4

6. In situ hybridization

i) Beadle and Ephrussi's Ringers Solution

Sodium chloride	7.50 g
Potassium chloride	0.35 g
Calcium chloride	0.31 g

Make up the volume to a liter

ii) Formamide buffer

Potassium phosphate (KH_2PO_4)	0.06M	
Potassium phosphate (K_2HPO_4)	0.06M	
EDTA	0.01M	
Potassium hydroxide	0.027M	
Potassium chloride	0.5M	
Formamide	70%	pH 7.0

IV. Bacterial and Drosophila strains

i) E. coli - SF8 was obtained from M. Olson (Olson et al., 1977)

ii) pBR322 - Bacterial strain containing the plasmid pBR322 (Bolivar et al., 1977) was provided by H. Boyer.

iii) Mutant, giant, of Drosophila melanogaster (Kaufman, 1971)

V. Growth Media

i) Luria Broth

Tryptone	10g
Yeast extract	5g
Sodium chloride	5g
Distilled water	1l (volumetrically) pH7.2

Plates of LB were made with 10 g/l agar

LB agar - ampicillin plates contained 50 ug/ml ampicillin

LB agar - tetracycline plates contained 20 ug/ml tetracycline

ii) M9S medium

M9 medium (Champe and Benzer, 1962) plus

Casamino acids 0.2%

iii) M9S medium plus

Uridine	200 ug/ml
Thiamine	0.5 ug/ml
Tryptophan	50 ug/ml

iv) Drosophila growth medium (Lewis, 1960)

METHODS

I. Isolation of recombinant plasmids carrying Drosophila melanogaster tRNA genes

Recombinant plasmids carrying Drosophila melanogaster tRNA genes were constructed by the "shot gun" technique where HindIII cleaved Drosophila DNA was ligated to HindIII cut pBR322 DNA. This work was done by Dunn et al. (1979b). The plasmids were numbered sequentially as they were isolated and identified as plasmid Drosophila tRNA N, pDtN.

II. Preparation of Plasmid DNA

i) pDt16, pDt27RC, pDt92 and pDt120 plasmid DNAs were prepared as described by Dunn et al. (1979b).

ii) pDt17RC DNA was prepared by a slight modification of the procedure by Dunn et al. (1979b). Norgard et al. (1979) reported about three times greater yield of plasmid DNA when plasmid carrying bacteria was grown in presence of uridine and later treated with chloramphenicol, than would normally be obtained by routine plasmid amplification procedures. Therefore, E-coli containing pDt17RC was grown to about 6.10^8 bacteria/ml at 30^oC in M9S medium plus uridine, thiamine and tryptophan. Chloramphenicol (80 mg/ml in 95% ethanol) was added to a final concentration of 200 ug/ml and the culture was incubated overnight. One liter of the cells was collected by centrifugation (8000 RPM - 15 min.) and the cells were resuspended in 5ml 25% sucrose, 0.05M Tris (pH8.0). 2.5 ml 0.5M EDTA was added and mixed. 1ml lysozyme (5mg/ml) then was

added and the mixture was incubated at 4°C for 10 min. Finally 7ml Triton (2%) was pipetted rapidly into the solution and the lysate was centrifuged at 4°C for 60 min. at 25,000 RPM. The supernatant (cleared lysate) was collected and centrifuged to equilibrium in CsCl and ethidium bromide according to Bazaral and Helinski (1968). The plasmid DNA was collected and centrifuged to equilibrium in CsCl and ethidium bromide once again. The plasmid DNA was collected, the ethidium bromide was removed with butanol, and the solution was dialyzed against TEN buffer overnight at 4°C. The solution of plasmid DNA was phenol extracted, ether washed and stored at -20°C.

III. Digestion of plasmid DNA with restriction endonuclease

i) HindIII $\begin{pmatrix} 5' \downarrow A\,AGCTT\, 3' \\ 3' TTCGA\,\uparrow A\, 5' \end{pmatrix}$

Plasmid DNA was cut with HindIII in HindIII buffer at the enzyme concentration of 1 unit/ug DNA in the reaction volume of 20-50 ul. The digestion was done at 37°C for 2 hours.

IV. Agarose gel electrophoresis

0.5%, 1% or 2% agarose gels were run in Tris-phosphate (pH8.0) buffer with 1 ug/ml ethidium bromide, in a Studier gel apparatus (McDonell et al., 1977). DNA samples were mixed with 0.2 volume of loading mix before loading the gel. The electrophoresis was carried out at 4°C for 1.5-3.5 hours at 85V (6.5V/cm). The gel was exposed to U.V. light and photographed through an orange filter using Type 57 Polaroid film.

V. Size determination of DNA fragments

The size of DNA fragments resulting from HindIII digestion of recombinant plasmids was determined by linear regression analysis, using HindIII cut lambda DNA as a reference (Murray and Murray, 1975). Linear regression analysis was performed using a HP9810 A calculator made by Hewlett-Packard.

VI. Southern transfer

The DNA in the agarose gel was transfered to nitrocellulose paper by the procedure of Southern (1975).

VII. Isolation and iodination of purified Drosophila tRNAs

tRNA from adult Drosophila melanogaster was isolated and purified using a number of chromatographic procedures (Dunn et al., 1979b). First it was separated on BD-cellulose column (White and Tener, 1973), then on Sepharose 6B in the presence of ammonium sulphate (Holmes et al., 1975). Fractions were further purified on RPC-5 column (Pearson et al., 1971). Purification by similar methods of three species of $tRNA^{Val}$ has been described by Dunn et al. (1978). The system of numbering the isoacceptors of the various tRNAs was taken from White et al. (1973a). Amino acids acceptance studies and other criteria of purity suggested that the purified tRNAs were not less than 95% pure with no single contaminant representing more than 2% of the total material. The purified tRNAs were isolated by and obtained from Dr. Ian Gillam.

The purified tRNAs were iodinated with $[^{125}I]$ by the procedure of Commerford (1971).

VIII. Hybridization of DNA on nitrocellulose filter to [^{125}I] tRNA

The filter with DNA was saturated with 2XSSC containing approximately 5.10^5-1.10^6 cpm of [^{125}I] tRNA. The filter was placed between mylar sheets and glass plates, wrapped in Saran wrap and incubated at 65°C for 6 hours. After incubation the filter was washed three times with 2XSSC, incubated for 30 min. at 37°C in 10 ug/ml RNase A, washed three times in 2XSSC, 0.1% SDS and finally washed three more times in 2XSCC. The filter was dried and autoradiographed for 48 hours using preflashed X-ray screen film and Dupont screen in a cassette at -70°C.

IX. Recloning procedure

i) Ligation

10 ug of pBR322 DNA cut with HindIII was incubated with 1.4 units of bacterial alkaline phosphatase in 50 ul containing 20 ul of TEN buffer and 5 ul of 1M Tris pH8.0. This mixture was incubated at 60°C for 60 min. 4 ug of BAP-treated pBR322 was coprecipitated with 1 ug of HindIII cut pDt92 or pDt120 in 0.25M sodium acetate (pH4.5) and four volumes of 95% ethanol at -20°C overnight. The precipitate was resuspended in 50 ul of ligation buffer. 20 units of T_4 polynucleotide ligase were added and the mixture was incubated at 12°C for 4 hours.

ii) Transformation

0.2 ul of $CaCl_2$-treated SF8 cells (Dunn et al., 1979b) were mixed with 30ul of 0.1M $CaCl_2$ and 50ul of ligated pDt92 or pDt120 in ligation buffer. The transformation mixture was incubated

at 4°C for 60 min. after which it was heat-pulsed at 42°C for 10 min. The culture then was diluted into 10ml of LB and grown for 3 hours at 30°C. The bacteria were plated on LB-ampicillin or LB-tetracycline plates and scored for drug resistance.

iii) Hogness procedure

Bacteria transformed with pDt92 or pDt120 were plated on large (140mm diameter) LB-ampicillin plates to yield approximately 300 colonies per plate. The colonies were replica plated onto nitrocellulose filters on LB-ampicillin plates. The filters were treated as described by Grunstein and Hogness (1976). The actual procedure followed was as described by Dunn et al. (1979b). The filters then were hybridized to $[^{125}I]$ $tRNA_4^{Val}$ as described earlier.

iv) Single colony isolation

Colonies containing plasmids carrying tRNA genes were located on master plates and streaked for single colonies on LB agar without any antibiotic. 9-10 isolated colonies were picked from each plate and transferred simultaneously to an LB-ampicillin plate and onto a nitrocellulose filter on an LB-ampicillin plate. The colonies were lysed and hybridized to $[^{125}I]$ $tRNA_4^{Val}$ as described above.

Streaking for single colonies on non-selective medium (LB agar) was done to isolate non-transformed colonies (these are ampicillin sensitive and appear as tiny satellite colonies around transformed, ampicillin resistant colonies on selective LB-ampicillin medium) from the transformed ones.

v) Checking clones for single insert carrying tRNA gene

Positive colonies containing tRNA genes after second hybridization were grown up in M9S medium; plasmid DNA was isolated, cut with HindIII, run on an agarose gel, transferred to a nitrocellulose filter, (Southern transfer) and hybridized to $[^{125}I]$ $tRNA_4^{Val}$.

X. In situ hybridization of plasmid DNA to polytene chromosomes

i) Preparation of salivary gland squashes

Polytene chromosomes were prepared from salivary glands of late third instar larvae of the mutant, giant, of Drosophila melanogaster (Kaufman, 1971) according to the method of Atherton and Gall (1972).

ii) Preparation of slides

Slides with squashes were prepared for in situ hybridization according to the procedure of Gall and Pardue (1971) with the additional incubation of the preparation in 2XSSC for 30 min. at 70°C (Bonner and Pardue, 1976) prior to initial RNase treatment and the acetylation of squashes (Hayashi et al., 1978) to reduce background on autoradiography.

iii) Iodination of plasmid DNA (pDt16, pDt27RC, pDt92RC, pDt120RC

5 ug of plasmid DNA (dried) was denatured in 20 ul of 0.3M sodium hydroxide. Enough 1M acetic acid was added to bring the pH up to 4.5-4.7 followed by addition of water to bring the total volume up to 40 ul. The denatured DNA then was iodinated with $[^{125}I]$ by a procedure adapted from the methods of Commerford (1971). 10 ul of 5mM thallium chloride was added to the denatured

sample, and the mixture was incubated at 70°C for 10 min. Approximately 1mCi of carrier-free Na [^{125}I] was mixed with 3 ul of 0.4mM NaI before addition to the DNA sample. Iodination was allowed to proceed at 70°C for 30 min. After iodination, 400 ul of 0.03M sodium phosphate and 10 ul of 0.1M sodium sulphite were added and mixed thoroughly. Finally, 135 ug of E-coli tRNA was added as carrier, and the mixture was loaded onto a hydroxylapatite column (0.5X1cm). The column was washed with 25ml of 0.03M sodium phosphate buffer, pH7.0 and the DNA was eluted with 0.48M sodium phosphate pH7.0. The ^{125}I-labelled plasmid DNA was heated at 70°C for 10 min. and then chromatographed on a Sephadex G-25 column (0.9X 40cm) in 70% formamide buffer, pH7.0. The specific activity obtained was usually around 1-2.10^{7} cpm/ug DNA.

iv) Synthesis and iodination of cRNA (pDt17RC)

[^{125}I] CTP was provided by Dr. Shizu Hayashi.

^{125}I-cRNA was synthesized from pDt17RC DNA by the procedure described by Wensink et al. (1974) with a few modifications. 5 ug of pDt17RC DNA was preincubated with 15 ul of 0.08M Tris-HCl pH8.0, 3 ul of 0.1M $MgCl_2$, 3 ul of 0.1M 2-ME, 1 unit of E-coli RNA polymerase and 6 ul of water at 37°C for 10 min. 0.6 ul each of 10^{-3}M ATP, GTP and UTP was added and this mixture then was added to about 35 uCi of dessicated [^{125}I] CTP and incubated at 37°C for 2 hours. Following this reaction, the DNA template was destroyed by digestion with DNase. 200 ul of 0.08M Tris-HCl pH7.8, 47.1 ul E-coli tRNA (100 ug), 152.9 ul water and 1 ul of 10 ug/ml DNase I was added, and the mixture was incubated at 25°C for 20 min. This

was followed by phenol (3X) and ether (3X) extractions. cRNA was separated from nucleoside triphosphates by Sephadex G-25 chromatography in 0.3M sodium acetate pH6.5. The labelled cRNA was ethanol precipitated, and the precipitate was resuspended in 70% formamide buffer.

v) In situ hybridization

50-55 ul of $[^{125}I]$ plasmid DNA or 25 ul of $[^{125}I]$ cRNA in 70% formamide buffer was used to hybridize to chromosome squashes at 35°C and 45°C respectively for varying periods of time. After hybridization, unbound plasmid DNA or cRNA was removed as described by Gall and Pardue (1971).

vi) Autoradiography

The slides were prepared for autoradiography according to Grigliatti et al. (1974). After developing the emulsion, chromosomes were stained with 0.4% toluidine blue O in 2XSSC.

vii) Analysis of Autoradiograms

The labelled bands were identified with the aid of photographic representations of the polytene banding pattern of D. melanogaster by Lefevre (1976).

XI. Containment

Bacteria containing recombinant plasmids were handled under B-M containment conditions, as specified by the regulations of the Medical Research and National Science and Engineering Research Councils of Canada.

RESULTS

I. Characterization of pDt92 and pDt120

pDt92 and pDt120 were characterized initially to determine the number of inserts (i.e. *Drosophila* DNA fragments) they contained, the size of the inserts and the insert which carried the tRNA gene.

Both, pDt92 and pDt120 when cut with HindIII and run on agarose gels showed 3 bands each (Figs. 1 and 2 respectively). The sizes of the 3 bands of pDt92 were 0.5kb, 1.7kb and 4.4kb, whereas the sizes of the 3 bands of pDt120 were 2.0kb, 4.4kb and 5.4kb. The 4.4kb band in each case corresponded to pBR322, therefore pDt92 and pDt120 contained two inserts each.

Southern transfer to nitrocellulose filter and hybridization to $[^{125}I]$ $tRNA_4^{Val}$ showed that the 0.5kb fragment of pDt92 (Fig. 1) and 2.0kb fragment of pDt120 (Fig. 2) contained the $tRNA_4^{Val}$ genes.

II. Recloning of pDt92 and pDt120

pDt92 and pDt120 were recloned to contain only the inserts which carried the $tRNA_4^{Val}$ genes i.e. the 0.5kb and the 2.0kb fragment respectively.

Fig. 3 is a photograph of an autoradiogram of a hybridization of $[^{125}I]$ $tRNA_4^{Val}$ to bacterial colonies after the first Hogness procedure (METHODS). 6 clones out of a total of 300 colonies on that filter hybridized, a frequency of about 2%. This autoradiogram represents the result obtained with pDt120; a similar result

Fig. 1. Agarose gel analysis and hybridization of HindIII digested pDt92 with $[^{125}I]$ $tRNA_4^{Val}$.

The plasmid pDt92 and control DNAs were cleaved with HindIII and were electrophoresed on 1% agarose gel in Tris-phosphate buffer (pH8.0) for 2.5 hours at 85 volts. The fragments were visualized by staining with ethidium bromide and exposure to U.V. light. Lane 1, λ DNA; lane 2, pDt92; lane 3, pBR322.

The agarose gel was treated by the procedure of Southern (1975). The transfered DNA was incubated with $[^{125}I]$ $tRNA_4^{Val}$ followed by autoradiography. The unmarked lane on the right corresponds to lane 2, i.e. pDt92.

Fig. 2. Agarose gel analysis and hybridization of HindIII digested pDt120 with $[^{125}I]$ $tRNA_4^{Val}$.

The plasmid pDt120 and control DNAs were cleaved with HindIII and were electrophoresed on 2% agarose gel in Tris-phosphate buffer (pH8.0) for 2.75 hours at 85 volts. The gel was stained with ethidium bromide and photographed under U.V. light. Lane 1, λ DNA; lane 2, pBR322; lane 3, pDt120.

The agarose gel was treated by the procedure of Southern (1975) and incubated with $[^{125}I]$ $tRNA_4^{Val}$. The unmarked lane on the right corresponds to lane 3, i.e. pDt120.

Fig. 1.

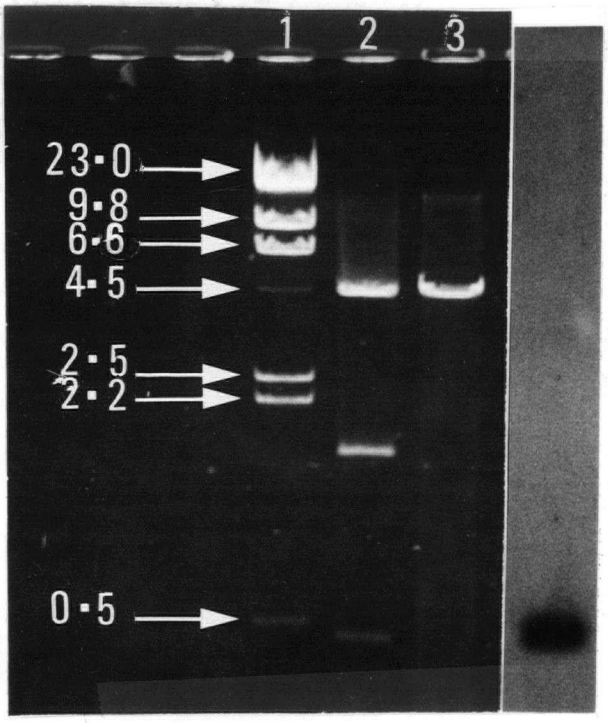
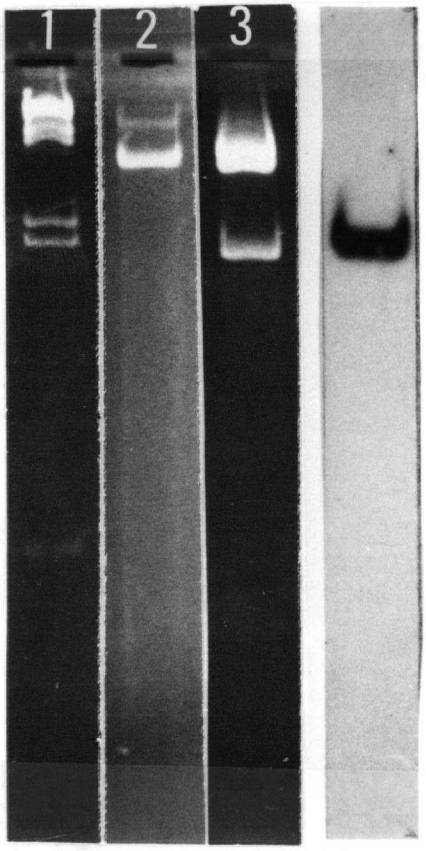


Fig. 2.



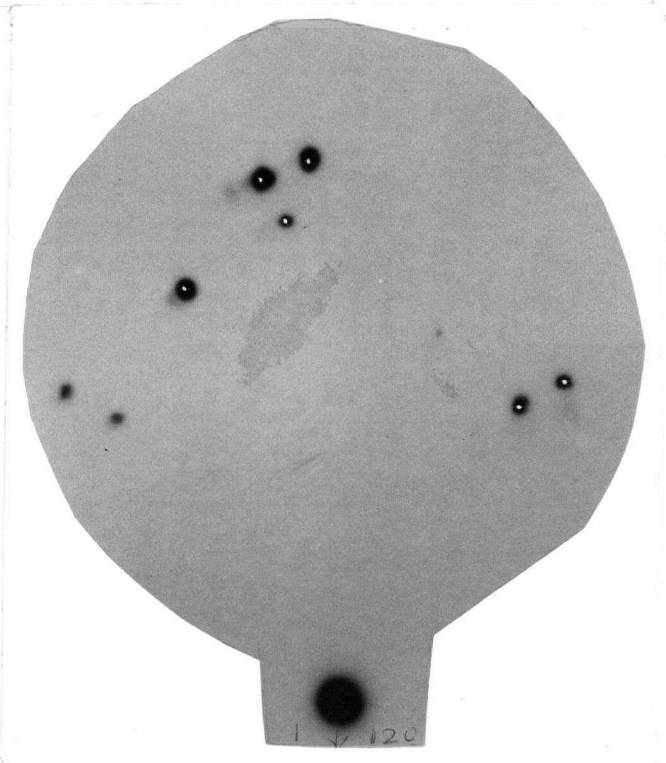


Fig. 3. Isolation of reclones containing recombinant plasmids carrying $tRNA_4^{Val}$ genes.

HindIII cut and bacterial alkaline phosphatase treated pBR322 was ligated to HindIII cut pDt120. The ligated DNA was used to transform E-coli. Bacteria grown on the nitrocellulose filters were treated according to the procedures of Grunstein and Hogness (1976) and incubated with $[^{125}I]$ $tRNA_4^{Val}$. The filters then were washed and developed for autoradiography. This photograph represents a typical autoradiogram after such a procedure.

was obtained with pDt92, though the frequency was only about 1%.

Fig. 4 represents the autoradiogram of the hybridization with bacterial colonies after the second Hogness procedure (METHODS) i.e. after the positive colonies were streaked for isolation on non-selective medium to eliminate contaminating non-transformed cells. Not all the colonies transfered to the filter contained a plasmid at this point, indicating that these colonies were not transformed, thus confirming the need for streaking on non-selective medium.

6 clones of pDt120 and 1 clone of pDt92 giving hybridization with $[^{125}I]$ $tRNA_4^{Val}$ after the second Hogness were tested for single inserts.

Fig. 5 shows that one clone of pDt92 and 3 out of 6 clones of pDt120 contained single inserts, and Fig. 6 shows that these single inserts carried the $tRNA_4^{Val}$ genes.

III. In situ hybridization

Five recombinant plasmids, pDt16, pDt17RC, pDt27RC, pDt92RC and pDt120RC were characterized further by in situ hybridization.

pDt16, pDt17RC and pDt27RC have inserts of sizes 6.7kb, 3.3kb and 6.1kb respectively (Fig. 7) and they all carry $tRNA_{4,7}^{Ser}$ genes (Fig. 8).

The plasmid DNAs of pDt16, pDt27RC, pDt92RC and pDt120RC were labelled with $[^{125}I]$ and hybridized to squashes of polytene chromosomes. All four of these plasmids hybridized to only one site on the Drosophila chromosome. pDt92RC (Fig. 9) and pDt120RC (Fig. 10) hybridized to the 90BC site on the right arm of the third chromosome; pDt16 (Fig. 11) and pDt27RC (Fig. 12) hybridized to the 12DE site on the X chromosome.

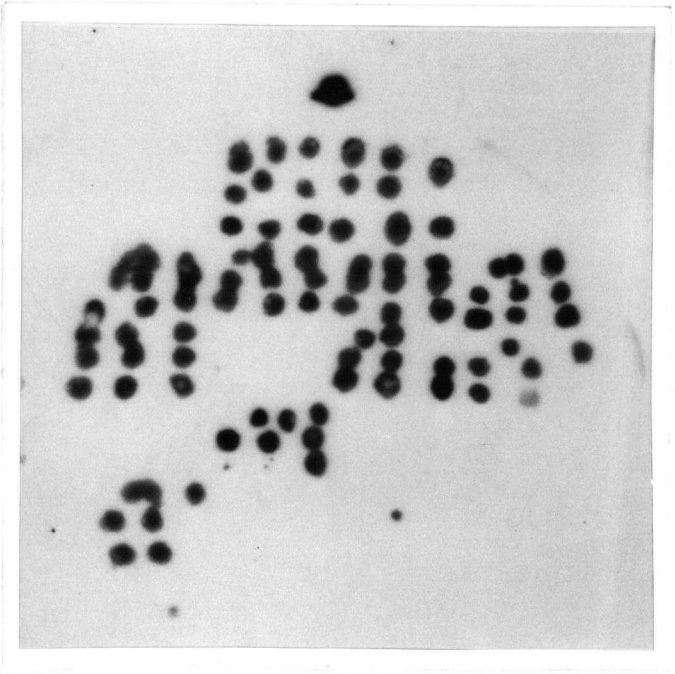


Fig. 4. Single colony isolation of bacteria containing recombinant plasmids carrying $tRNA_4^{Val}$ genes.

Positive colonies containing plasmids carrying $tRNA_4^{Val}$ genes were picked from the master plates and were streaked for isolation on LB-agar in the absence of any antibiotic. About 9-10 isolated colonies from the non-selective LB-agar plates were transfered to secondary master plates (LB-ampicillin), replicated to nitrocellulose filters on LB-ampicillin agar and treated as described in Fig. 3 and METHODS. This photograph is an autoradiogram after such a procedure.

Fig. 5. Checking reclones of pDt92 and pDt120 for single inserts.

Plasmid DNA was prepared from several positive clones from the secondary master plates. The recloned plasmid DNAs and control DNAs were cleaved with HindIII and were electrophoresed on 1% agarose gel in Tris-phosphate buffer (pH8.0) for 2.75 hours at 85 volts. The gel was stained with ethidium bromide and photographed under U.V. light. Lanes 1-6, six different reclones of pDt120; lane 7, pBR322; lane 8, λ DNA; lane 9, a reclone of pDt92; lane 10, unrecloned pDt92. Note, that three reclones of pDt120 in lanes 2-4 and one reclone of pDt92 in lane 9 have single inserts.

Fig. 6. Hybridization of $[^{125}I]$ $tRNA_4^{Val}$ with HindIII digested DNAs from reclones of pDt92 and pDt120.

The agarose gel photographed in Fig. 5 was treated by the procedure of Southern (1975) and incubated with $[^{125}I]$ $tRNA_4^{Val}$. Lanes 1-10 correspond to lanes 1-10 of Fig. 5; i.e. lanes 1-6, reclones of pDt120; lane 7, pBR322; lane 8, λ DNA; lane 9, reclone of pDt92; lane 10, unrecloned pDt92.

Fig. 5.

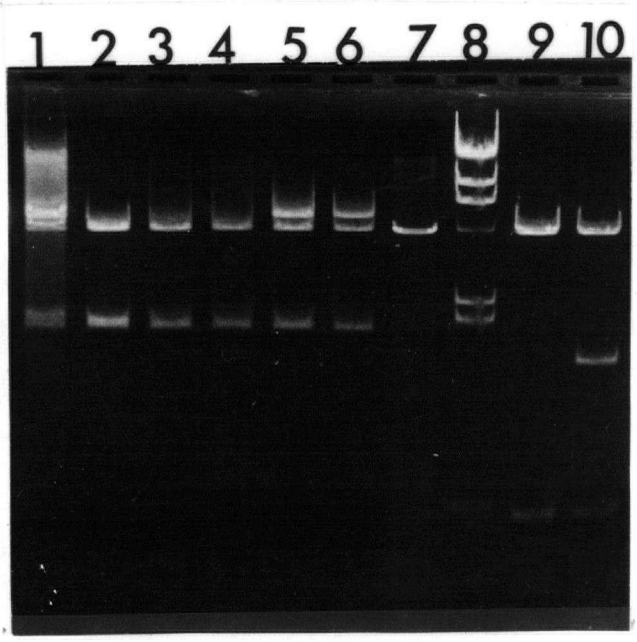
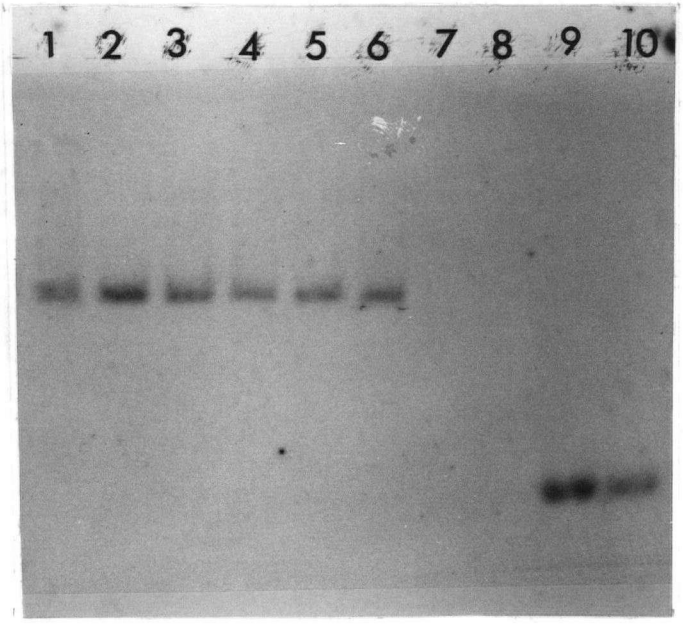


Fig. 6.



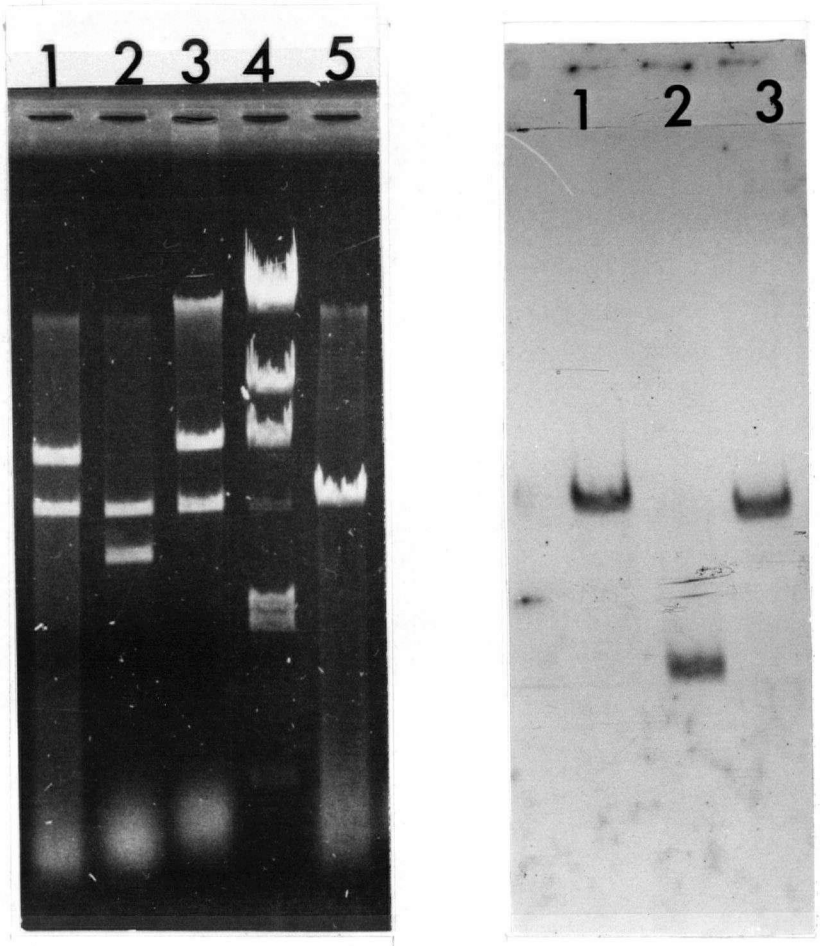


Fig. 7. Agarose gel analysis of HindIII digested pDt16, pDt17RC and pDt27RC.

The plasmids pDt16, pDt17RC, pDt27RC and control DNAs were cleaved with HindIII and were electrophoresed on 0.5% agarose gel in Tris-phosphate buffer (pH8.0) for 2.5 hours at 85 volts. The gel was photographed under U.V. light after staining with ethidium bromide. Lane 1, pDt27RC; lane 2, pDt17RC; lane 3, pDt16; lane 4, λ DNA; lane 5, pBR322.

Fig. 8. Hybridization of HindIII digested pDt16, pDt17RC and pDt27RC with $[^{125}I]$ $tRNA_7^{Ser}$.

The agarose gel photographed in Fig. 7 was treated by the procedure of Southern (1975). The transfered DNA was incubated with $[^{125}I]$ $tRNA_7^{Ser}$ followed by autoradiography. Lane 1, pDt16; lane 2, pDt17RC; lane 3, pDt27RC.

Fig. 9. *In situ* hybridization of pDt92RC.

pDt92RC was labelled with [^{125}I] to a specific activity of approximately $3.5 \cdot 10^7$ cpm/ug DNA and then hybridized to *Drosophila* salivary gland chromosomes as described in METHODS. The photographic emulsion was exposed for 9.5 weeks and on development showed grains over the region 90BC (arrow).

Fig. 10. *In situ* hybridization of pDt120RC.

[^{125}I] pDt120RC ($2.9 \cdot 10^7$ cpm/ug) was hybridized as outlined in the legend to Fig. 9. Region 90BC (arrow) was labelled after 4 weeks.

Fig. 9.

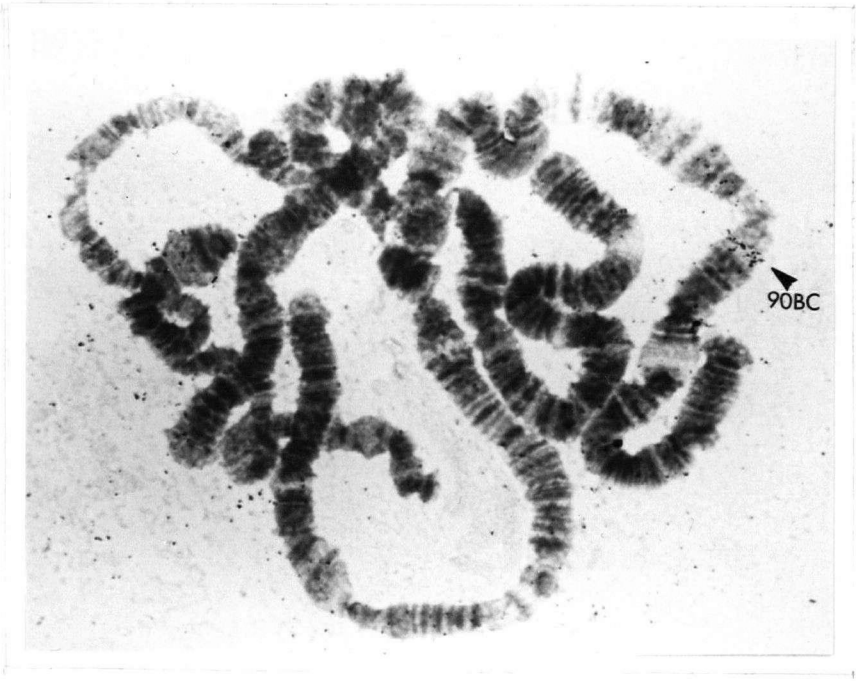


Fig. 10.

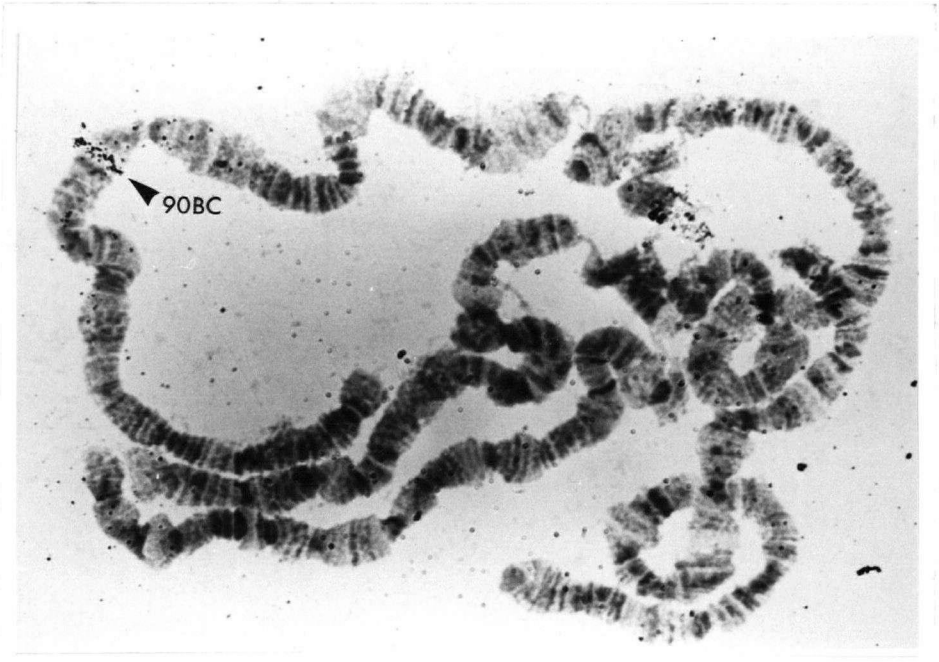


Fig. 11. In situ hybridization of pDt16.

[^{125}I] pDt16 ($1.1 \cdot 10^7$ cpm/ug) was hybridized as described in the legend to Fig. 9. Site 12DE (arrow) was labelled after 16 days.

Fig. 12. In situ hybridization of pDt27RC.

[^{125}I] pDt27RC ($2.5 \cdot 10^7$ cpm/ug) was hybridized as outlined in the legend to Fig. 9. Site 12DE (arrow) was labelled after 27 days.

Fig. 11.

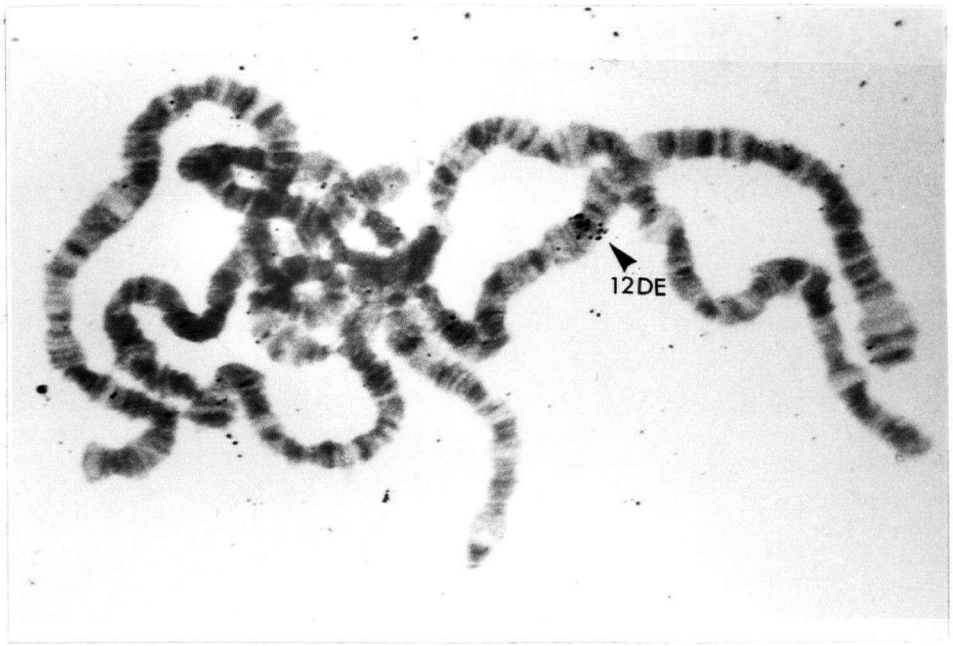
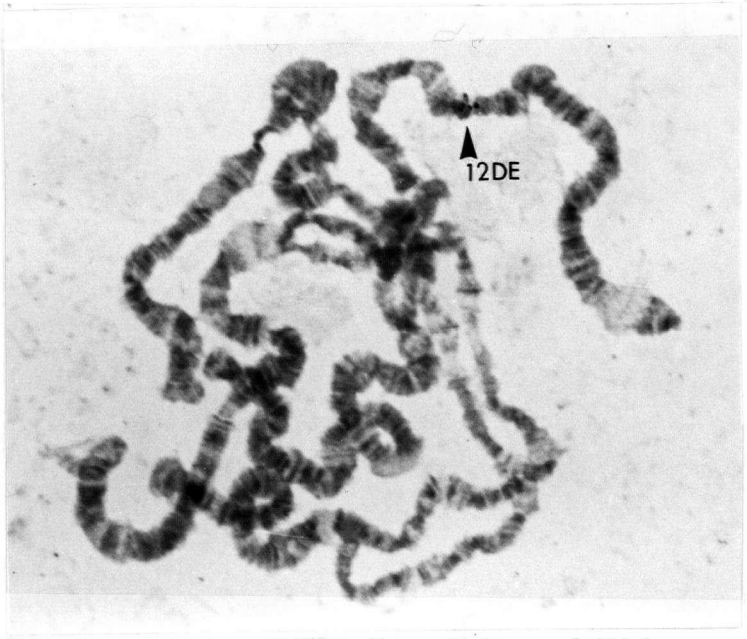


Fig. 12.



In the case of pDt17RC, ^{125}I-labelled cRNA was synthesized first using the plasmid DNA as template. The cRNA then was used to hybridize to squashes of polytene chromosomes. Several advantages were observed using this method over the DNA:DNA hybridization method described earlier:

i) The specific activity achieved by this method (about $2\text{-}3.10^9$ cpm/ug RNA) was much higher than that obtained by the other method ($1\text{-}2.10^7$ cpm/ug DNA), allowing grains to be seen in a matter of few days to a week, as opposed to 3-10 weeks for the DNA-DNA hybridization method.

ii) The labelling of cRNA with $[^{125}I]$-CTP is more specific, being restricted to the transcript being made. Commerford's method of labelling plasmid DNA with $[^{125}I]$ is not specific because RNA and proteins if present in the plasmid preparation also get labelled, contributing to higher background.

Unlike the results obtained with the other four plasmids, pDt17RC-cRNA hybridized to more than one site on the Drosophila chromosomes (Fig. 13). The highest number of grains was seen at the 12DE site; fewer, but with consistency, grains were also seen at 23E (2L), 56D (2R), 62D (3L) and 64D (3L).

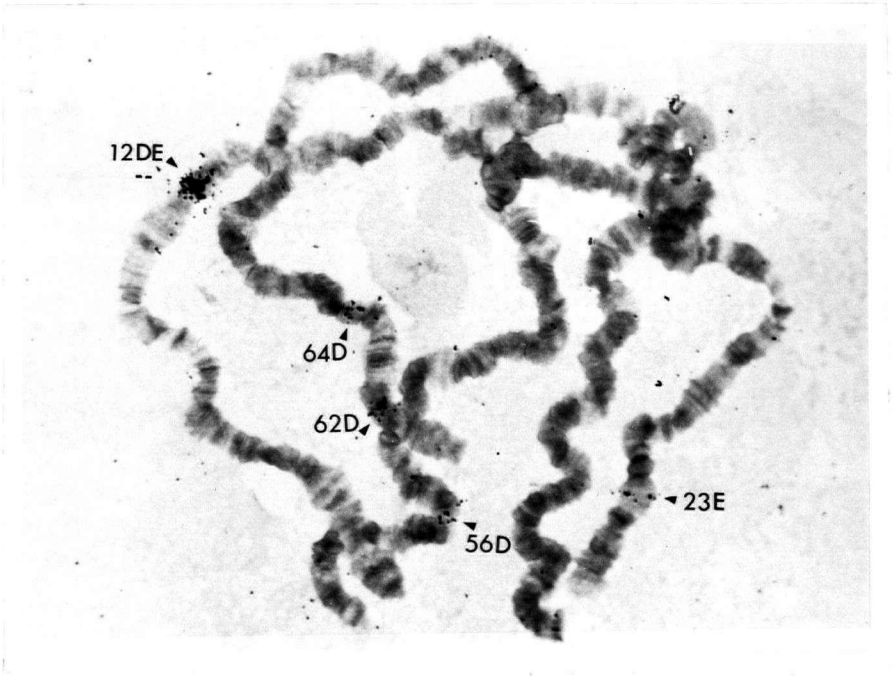


Fig. 13. In situ hybridization of pDt17RC.

pDt17RC was used as a template to synthesize cRNA which was labelled with $[^{125}I]$-CTP to a specific activity of approximately 10^9 cpm/ug RNA and then hybridized to Drosophila salivary gland chromosomes as described in METHODS. The photographic emulsion was exposed for 8 days and on development showed grains over 12DE, 23E, 56D, 62D and 64D (arrows).

DISCUSSION

When all the $tRNA_4^{Val}$ gene containing plasmids (Dunn et al., 1979b) were studied together as a group, it was found that they fell into four different sized groups (Table I) with pDt23, pDt55, pDt92RC and pDt120RC with insert sizes of 12.0kb, 8.0kb, 0.5kb and 2.0kb respectively being representatives of each of these groups.

Similarly, the plasmids containing $tRNA_{4,7}^{Ser}$ genes (Dunn et al., 1979b) fell into five different sized groups (Table I) of insert sizes 3.3kb, 4.4kb, 4.7kb, 6.1kb and 6.7kb. These plasmids are classified as $tRNA_{4,7}^{Ser}$ because both, highly purified $tRNA_4^{Ser}$ and $tRNA_7^{Ser}$, hybridize to all these plasmids. $tRNA_4^{Ser}$ and $tRNA_7^{Ser}$ are also known to hybridize to identical sites on the Drosophila chromosomes (Hayashi et al., 1980). It has been shown that $tRNA_4^{Ser}$ and $tRNA_7^{Ser}$ have different anticodons (White et al., 1975) but sequencing data indicates that the two tRNAs differ by only a few nucleotides (D. Cribbs, unpublished). Thus these results may be explained most easily by cross-hybridization.

Plasmids carrying more than one insert were recloned to contain only the inserts with the tRNA genes in order to assign them unambiguous location on the Drosophila chromosomes by in situ hybridization.

In situ hybridization of twelve tRNAs by Hayashi et al. (1980) showed that essentially all the purified tRNAs hybridized to more than one site on the polytene chromosomes of the salivary glands of D. melanogaster (Fig. 14). Sites which were heavily labelled were termed 'major' sites, and lightly labelled sites were called

Table I. HindIII Fragments and *In Situ* Hybridization Results of Recombinant Plasmids Carrying Genes for $tRNA_4^{Val}$, $tRNA_{3b}^{Val}$ and $tRNA_{4,7}^{Ser}$

Plasmid	tRNA Gene	HindIII fragment (Size, kb)	Hybridization Site[1]	tRNA hybridization at this site[2]
pDt23	Val-4	12.0	89B	minor
pDt55	Val-4	8.0	70BC	major
pDt92RC	Val-4	0.5	90BC	minor
pDt120RC	Val-4	2.0	90BC	minor
pDt41RC	Val-3b	2.0	90BC	minor
pDt48	Val-3b	2.4	90BC	minor
pDt78RC	Val-3b	5.2	84D	major
pDt5	Ser-4,7	4.4	23E	major
pDt16	Ser-4,7	6.7	12DE	major
pDt17RC	Ser-4,7	3.3	12DE, 23E, 56D 62D, 64D	major, minor
pDt27RC	Ser-4,7	6.1	12DE	major
pDt73	Ser-4,7	4.7	12DE	major

[1]Plasmid DNA was used for hybridization in each of these cases except pDt17RC, where cRNA synthesized from pDt17RC was used.

[2]tRNA *in situ* hybridizations were done by Hayashi et al. (1980).

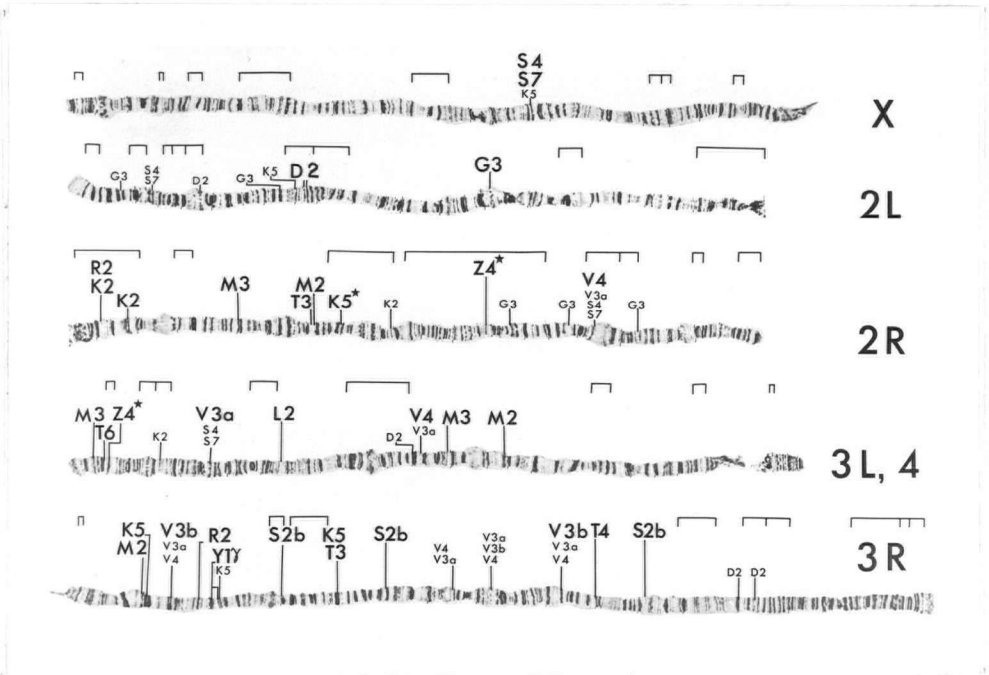


Fig. 14. Localization of tRNA genes on the polytene chromosomes of Drosophila (S. Hayashi).

In situ hybridization of about twelve purified, [^{125}I] tRNAs was performed by Hayashi et al. (1980). These results are plotted on the composite photographs of polytene chromosomes prepared by Lefevre (1976). Standard amino acid abbreviations are used to designate sites for the corresponding tRNA genes. The relevant ones are: V3b, $tRNA^{Val}_{3b}$; V4, $tRNA^{Val}_{4}$; S4, S7, $tRNA^{Ser}_{4,7}$. The heavier, larger type designates 'major' sites and the lighter, smaller print designates 'minor' sites. The lines above the chromosomes cover regions where Minute mutants map.

'minor' sites. $tRNA^{Val}_{3b}$ hybridized to two major sites, 84D and 92B and one minor site 90BC, all these sites being present on the right arm of the third chromosome (3R). On the other hand, $tRNA^{Val}_{4}$ hybridized to 56D (2R), 70BC (3L), the two major sites and 84D (3R), 89B (3R), 90BC (3R) and 92B (3R), the many minor sites; all these sites being distributed on different arms and different chromosomes. Similarly the major sites for $tRNA^{Ser}_{4,7}$, 12DE (X chromosome), 23E (2L) and the minor sites, 56D (2R), 64D (3L) lie on different chromosomes.

The confidence that can be placed in the results of the tRNA in situ hybridization depends critically on the specificity of binding of the tRNA to the corresponding gene and on the purity of the labelled tRNA probe. For these reasons, the tRNA in situ data reviewed above was not completely convincing. First, it was possible that some of the tRNA hybridization sites, particularly the minor sites, did not represent the presence of a tRNA gene. There are several possibilities as to what the minor sites could represent:

i) they could represent unrelated genes which possibly occur as multiple copies and correspond to contaminants in the tRNA probes used for hybridization,

ii) they could represent genes of related sequences which have some homology to the tRNAs used as probes, or

iii) they could indeed represent single or a few copies of the tRNA gene which is present in a greater number at the respective major sites and which is truly homologous to the tRNA probe used for hybridization.

All these possibilities would cause formation of fewer grains at the minor sites compared to the major sites.

Second, the sharing of the major and the minor sites of hybridization by the $tRNA^{Val}$ isoacceptors was ambiguous. Again, several explanations are possible:

i) there could have been contamination of one valine isoacceptor with another,

ii) there possibly exists some sequence homology between $tRNA_{3b}^{Val}$ and $tRNA_{4}^{Val}$, or

iii) genes for both these isoacceptors exist at the shared site.

There is some evidence for sequence homology between $tRNA_{3b}^{Val}$ and $tRNA_{4}^{Val}$ (Figs. 15-17); plasmids containing $tRNA_{3b}^{Val}$ genes hybridize strongly to $[^{125}I]$ $tRNA_{3b}^{Val}$ on Southern transfer and weakly to $[^{125}I]$ $tRNA_{4}^{Val}$. Also, plasmids with $tRNA_{4}^{Val}$ genes hybridize strongly to $[^{125}I]$ $tRNA_{4}^{Val}$ and weakly to $[^{125}I]$ $tRNA_{3b}^{Val}$.

<u>In situ</u> hybridization with the plasmid DNAs was undertaken in order to discriminate among the various possibilities stated above and to confirm the sites determined by tRNA <u>in situ</u> hybridization. Table I summarizes the hybridization sites for various plasmids carrying the $tRNA_{4}^{Val}$ genes, $tRNA_{3b}^{Val}$ genes and the $tRNA_{4,7}^{Ser}$ genes. The data represented in this table is collective data from work reported here and that reported by Dunn et al. (1979b). It was observed in general, that a plasmid carrying a single insert hybridized preferentially to only one of the tRNA-sites, determined by Hayashi et al. (1980), for that respective tRNA. Other tRNA-

Fig. 15. Agarose gel analysis of HindIII digested plasmids carrying genes for $tRNA_{3b}^{Val}$ or $tRNA_{4}^{Val}$.

Five valine-4 plasmids, pDt23, pDt55, pDt92RC, pDt120RC and pDt110, three valine-3b plasmids, pDt41RC, pDt48 and pDt78RC and control DNAs were cleaved with HindIII and were electrophoresed on 1% agarose gel in Tris-phosphate buffer (pH8.0) for 2.75 hours at 85 volts. The gel was stained with ethidium bromide and photographed under U.V. light. Lane 1, pDt110; lane 2, pDt120RC; lane 3, pDt92RC; lane 4, pDt55; lane 5, pDt23; lane 6, pBR322; lane 7, λ DNA; lane 8, pDt78RC; lane 9, pDt48; lane 10, pDt41RC.

Fig. 16. Hybridization of $[^{125}I]$ $tRNA_{3b}^{Val}$ with Hind III digested plasmids carrying genes for $tRNA_{3b}^{Val}$ or $tRNA_{4}^{Val}$.

The agarose gel photographed in Fig. 15 was treated by the procedure of Southern (1975). The transfered DNA was incubated with $[^{125}I]$ $tRNA_{3b}^{Val}$ followed by autoradiography. Lanes 1-10 correspond to lanes 1-10 of Fig. 15; i.e. lane 1, pDt110; lane 2, pDt120RC; lane 3, pDt92RC; lane 4, pDt55, lane 5, pDt23; lane 6, pBR322; lane 7, λ DNA; lane 8, pDt78RC; lane 9, pDt48; lane 10, pDt41RC.

Fig. 15.

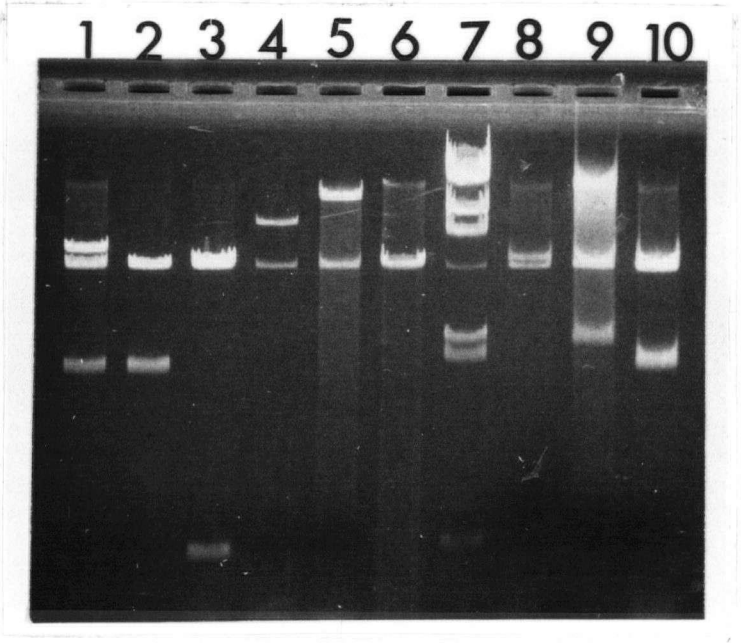
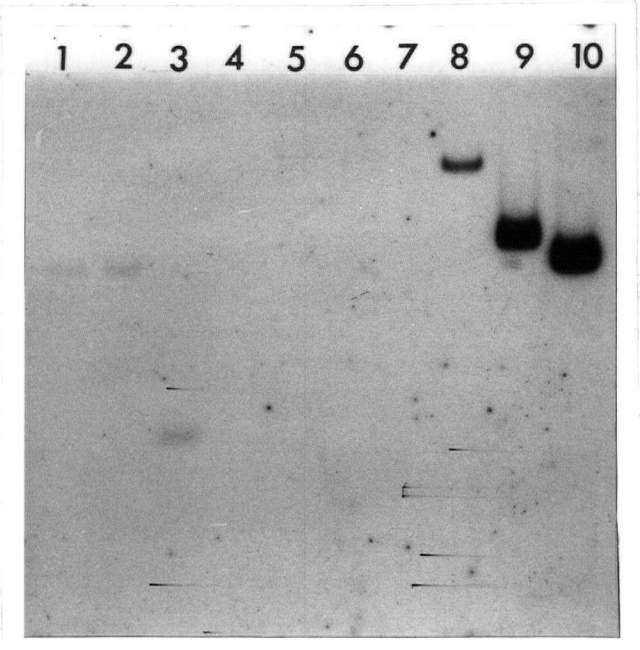


Fig. 16.



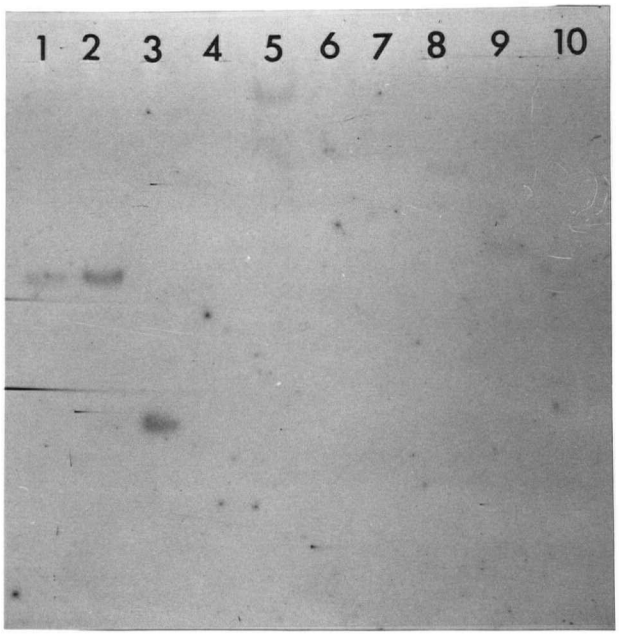


Fig. 17. Hybridization of $[^{125}I]$ $tRNA_4^{Val}$ with HindIII digested plasmids carrying genes for $tRNA_{3b}^{Val}$ or $tRNA_4^{Val}$.

An agarose gel identical to that photographed in Fig. 15 was treated by the procedure of Southern (1975). The transfered DNA was incubated with $[^{125}I]$ $tRNA_4^{Val}$ followed by autoradiography. Lane 1, pDt110; lane 2, pDt120RC; lane 3, pDt92RC; lane 4, pDt55; lane 5, pDt23; lane 6, pBR322; lane 7, λ DNA; lane 8, pDt78RC; lane 9, pDt48; lane 10, pDt41RC.

sites might have been labelled also, but under the conditions used, grains at those sites could not be distinguished above background. If the hybridization at the preferential site was due to additional homology offered by the flanking sequences (i.e. in addition to the homology due to the gene), then the hybridization site denotes the site on the Drosophila chromosome from which the insert originated. Hybridization at a given site would therefore confirm the presence of the tRNA gene at that site. This pattern of hybridization also made it possible to see if plasmids with different sized inserts but carrying the same tRNA gene hybridized to the same site or to a different site. Such information would throw some light on the organization of tRNA genes in Drosophila.

In situ hybridization data on the five serine plasmids (Table I) confirmed the presence of $tRNA^{Ser}_{4,7}$ genes at 12DE and 23E, the two major $tRNA^{Ser}_{4,7}$ sites according to Hayashi et al. (1980). The four plasmids which hybridized to 12DE had different sized inserts, suggesting the presence of multiple copies of $tRNA^{Ser}_{4,7}$ gene at this site. In fact, Elder et al. (1980) have estimated 5 copies of a purified 4S RNA gene at 12E and 2 copies of the same gene at 23EF.

The hybridization pattern of pDt17RC was different from other plasmids in that it hybridized to more than one site. It should be noted that cRNA was used for hybridization in this case instead of plasmid DNA (METHODS), but this difference may not be the reason for hybridization at multiple sites because such a trend was not observed for several other plasmids where cRNA was used for hybridization (S. Hayashi, personal communication). The other point to

note is that pDt17RC-cRNA not only hybridized to all the $tRNA^{Ser}_{4,7}$ sites (12DE, 23EF, 56D, 64D) but also to 62D, a site which has not been previously characterized as a $tRNA^{Ser}_{4,7}$ site. It is conceivable that there was fragmentation of the initial transcript and fragments containing the gene and/or flanking sequences hybridized to the various sites. 12DE and 23E have already been established as $tRNA^{Ser}_{4,7}$ gene sites (above). 56D and 64D are minor $tRNA^{Ser}_{4,7}$ sites according to Hayashi et al. (1980) and the fact that grains were seen at these sites by two independent methods lends credibility to the presence of $tRNA^{Ser}_{4,7}$ genes at these sites. Additional support for this observation is provided by Elder et al. (1980) who reported weak hybridization at 56D and 64DE with 4S RNA. Hybridization at 62D site is a little more difficult to explain. HindIII digestion and agarose gel analysis (Fig. 7) confirms that pDt17RC has only one insert, so the hybridization at 62D site is not due to the presence of an extra fragment. It is possible that fragments containing the flanking sequences hybridized to 62D site, which must mean that the flanking region represents some sequence which is present at multiple sites on the genome of Drosophila. It could be another tRNA gene which was not detected by the probe of 12 tRNAs used by Dunn et al. (1979b) or it could be some other small RNA species (Elder et al., 1980).

In situ hybridization results of Valine-4 and Valine-3b plasmids (Table I) confirmed the presence of $tRNA^{Val}_{4}$ genes at the 70BC and 89B sites and $tRNA^{Val}_{3b}$ gene at the 84D site. Additional evidence for the presence of $tRNA^{Val}_{3b}$ genes at the 84D site is provided

by the studies of Dunn et al. (1979a) on the effect of duplication and deletion in the 84D region on the level of $tRNA_{3b}^{Val}$ in flies. 90BC is an interesting site in that two plasmids from each group hybridized to this site. There was some evidence given earlier (Figs. 15-17) that this sharing of sites may be due to sequence homology between $tRNA_{3b}^{Val}$ and $tRNA_{4}^{Val}$. But if the assumption, that the additional sequence homology provided by the flanking regions causes the plasmid DNA to preferentially hybridize to one site on the Drosophila chromosome, is correct, only the fragment which originated from the site should be able to hybridize there. A fragment which had originated from another site but with sequence homology (either complete or partial) only in the coding region would not be able to hybridize to the site in question because it would not have the proper flanking sequences. Therefore hybridization of fragments containing either $tRNA_{3b}^{Val}$ or $tRNA_{4}^{Val}$ gene to the 90BC site must represent presence of both of these genes at the 90BC site. Indeed DNA sequence data on pDt92RC (Carolyn Astell, unpublished) and pDt120RC (author, unpublished), the two valine-4 plasmids which hybridize to the 90BC site, shows the presence of a tRNA gene whose sequence is very similar to that of Drosophila $tRNA_{4}^{Val}$ (Bill Addison, unpublished). No such sequence data is yet available on the two valine-3b plasmids which hybridize to the 90BC site nor is the sequence of Drosophila $tRNA_{3b}^{Val}$ yet known. Elder et al. (1980) have estimated that about 9 copies of 4S RNA gene occur at the 90C site, a large enough number to accomodate genes for two species of tRNA.

Another feature of importance to note is that of the seven valine plasmids tested, five hybridized to their respective minor sites (Table I) as determined by Hayashi et al. (1980). This proves that the minor sites do represent the presence of tRNA genes at those loci. This observation is substantiated by Elder et al. (1980) who estimated about 1-3 copies of a tRNA gene at such weakly labelled sites.

Conclusion

The *in situ* hybridization study presented here confirmed the presence of $tRNA^{Ser}_{4,7}$ genes at 12DE, 23E and possibly at 56D and 64D and $tRNA^{Val}_{4}$ genes at 70BC, 89B and 90BC sites. *In situ* hybridization with plasmid DNAs also confirmed that minor sites of hybridization seen with tRNA - *in situ* hybridization represent the presence of a few copies of tRNA genes at those loci.

These results along with others reported elsewhere (Kubli and Schmidt, 1978; T. Schmidt et al., 1978; Elder, 1978; Hayashi et al., 1980) show that genes for many individual tRNAs occur as multiple copies in more than one cluster at sites widely scattered on the chromosomes. A cluster at a site may contain genes for a single tRNA species or it may contain genes for more than one tRNA species (Yen et al., 1977; O. Schmidt et al., 1978; Hovemann et al., 1980).

The arrangement of tRNA genes in *Drosophila* does not seem to follow any obvious pattern and thus appears to be markedly different from that described in yeast (Feldman, 1976; Olson et al, 1977)

and Xenopus laevis (Clarkson et al., 1973; Clarkson and Kurer, 1976; Clarkson et al., 1978). The biological significance of such an arrangement of genes and the regulatory mechanism by which the cell maintains the fidelity of sequence for genes so widely scattered on the chromosomes are not known yet.

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