

AGING AND PROTEIN SYNTHESIS;
SERINE AND LEUCINE TRANSFER RNA GENES IN DROSOPHILA MELANOGASTER

by

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B.Sc., University of British Columbia, 1983

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
UNIVERSITY OF BRITISH COLUMBIA

We accept this thesis as conforming
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December, 1988

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ABSTRACT

This thesis consists of two distinct parts. Part I describes preliminary studies undertaken to investigate whether defective tRNA molecules may be at least partially responsible for the general decrease in protein synthesis ability seen with advancing age. Part II describes the cloning of three recombinant plasmids, each containing at least one putative gene for one of Drosophila melanogaster tRNA^{Ser}_{2b}, tRNA^{Ser}_{UCA}, or tRNA^{Leu}_{CUG}.

To approach the first problem from an in vitro perspective, unfractionated Drosophila melanogaster tRNA was either degraded by approximately four nucleotides or elongated by one nucleoside 3',5'-diphosphate at the 3'-terminus, and these defective tRNAs were added to a rabbit reticulocyte lysate protein synthesis system in varying amounts. It was found that these molecules did not produce appreciable inhibition of protein synthesis in this cell-free system until they were present in quantities similar to the estimated amount of endogenous tRNA present. This finding suggests that such defective molecules would not play an appreciable role in the age-related protein synthesis decrease seen in vivo, as it is highly improbable that defective tRNA molecules would accumulate at levels approximating the levels of active tRNA in the cell.

A search was conducted for partially 3'-degraded tRNA molecules among the entire tRNA population isolated from aging Drosophila, and among both ribosome-associated and non ribosome-associated tRNAs from such organisms. These tRNA samples were treated with alkaline

phosphatase and 5'-labelled with [γ - 32 P]ATP and polynucleotide kinase. Differential labelling of tRNA bands from the aged population could indicate the presence of partially 3'-degraded tRNA in this population; this was not observed, however, other than for one band which appeared to label somewhat more darkly in the older population in two independent experiments. The significance of this band is not clear, but it does not appear abundant enough to affect protein synthesis, on the basis of the in vitro results described.

Analysis of whole tRNA isolates from both young and aged Drosophila by two-dimensional polyacrylamide gel electrophoresis did not reveal consistent differences between these two tRNA populations.

In Part II, two oligonucleotides complementary to the known tRNA^{Ser}_{2b} sequence were used to screen pUC 13 recombinant DNA libraries containing inserts of Drosophila genomic DNA purified by size. One of these oligonucleotides, GT8, hybridized to a 5.1 kb HindIII restriction fragment containing tRNA^{Ser}_{2b}, a 3.6 kb EcoRI fragment containing an apparent tRNA^{Ser}_{UCA} gene, and a 3.6 kb EcoRI fragment containing a tRNA^{Leu}_{CUG} gene.

The entire structural genes for both tRNA^{Ser}_{2b} and tRNA^{Leu}_{CUG} were sequenced, as well as the 56 3'-nucleotides of the putative tRNA^{Ser}_{UCA} structural gene. The sequence data suggests that examples of genetic microheterogeneity are seen here for both tRNA^{Ser}_{2b} and tRNA^{Ser}_{UCA}. The sequence of the tRNA^{Leu}_{CUG} gene corresponded exactly to that of a previously cloned Drosophila tRNA^{Leu}_{CUG} gene (Glew et al. (1986) Gene 44, 307-314), but the flanking sequences of these two clones were different. Thus, a second copy of this tRNA^{Leu}_{CUG} gene was obtained in this work.

In situ hybridization studies showed the three fragments to derive from chromosomal sites 88A (tRNA^{Ser}_{2b}), 58AB (tRNA^{Ser}_{UCA}), and 66B (tRNA^{Leu}_{CUG}).

TABLE OF CONTENTS

	<u>Page</u>
Abstract	ii
Table of Contents	v
List of Figures	viii
Abbreviations	ix
Acknowledgements	xi
Dedication	xii

PART I

I. Introduction	2
II. Materials and Methods	12
i. Isolation of tRNA from <u>Drosophila</u> - Method I	12
ii. Isolation of tRNA from <u>Drosophila</u> - Method II	13
iii. Sephadex® G-100 Gel Filtration of RNA	13
iv. Isolation of tRNA from Post-Ribosomal Supernatant and from Salt-Washed Ribosomes	14
v. Partial Enzymatic 3'-Degradation of tRNA	15
vi. 3'-Elongation of tRNA	16
vii. Reticulocyte Lysate Protein Synthesis Assays	16
viii. Determination of Reticulocyte Lysate Endogenous tRNA	18
ix. One-Dimensional Polyacrylamide Gel Electrophoresis	18
x. Two-Dimensional Polyacrylamide Gel Electrophoresis	20
xi. 5'-Labelling of tRNA with ^{32}P	20
III. Results and Discussion	22
i. Partial Enzymatic 3'-Degradation of tRNA	22
ii. 3'-Elongation of tRNA	26

	<u>Page</u>
iii. Inhibition of Protein Synthesis by Defective tRNA . . .	29
iv. Two-Dimensional Electrophoresis of tRNA from Young and Aged <u>Drosophila</u>	35
v. 5'-Kinasing Studies of Young and Aged tRNA Populations	39
a) Whole tRNA Extracts	41
b) Free and Ribosome-Associated tRNA	46

PART II

I. Introduction	49
II. Materials and Methods	55
i. Isolation of Genomic DNA	55
ii. Agarose Gel Electrophoresis	56
iii. Restriction Endonuclease Digestion	56
iv. Genomic Southern and Filter Hybridizations	57
v. Purification of DNA Fragments of Selected Size	58
vi. Oligonucleotide Preparation and Radioactive Labelling	58
vii. DNA Ligation, Bacterial Transformation, and Colony Screening	59
viii. Plasmid Isolation and Purification for Sequencing . . .	61
ix. DNA Sequencing	62
III. Results and Discussion	65
i. tRNA ^{Ser} _{2b} Gene Localization	65
ii. Cloning of tRNA ^{Ser} _{2b} , tRNA ^{Ser} _{UCA} , and tRNA ^{Leu} _{CUG}	68
iii. Sequencing and <u>in situ</u> Hybridization of tRNA ^{Ser} _{2b} , tRNA ^{Ser} _{UCA} , and tRNA ^{Leu} _{CUG} Clones	73

	<u>Page</u>
a) tRNA ^{Ser} _{2b}	74
b) tRNA ^{Ser} _{UCA}	76
c) tRNA ^{Leu} _{CUG}	84
References	90
Appendix	95

LIST OF FIGURESPagePART I

Figure 1.	Comparison of unaltered and partially 3'-degraded tRNA	24
Figure 2.	Comparison of unaltered and 3'-elongated tRNA	27
Figure 3.	Effect of high levels of whole and partially degraded tRNA on protein synthesis	31
Figure 4.	Effect of low levels of partially degraded tRNA on protein synthesis	32
Figure 5.	Effect of high levels of elongated tRNA-pCp on protein synthesis	34
Figure 6.	Two-dimensional electrophoretic comparison of tRNA populations from newly-hatched and 32 day old <u>Drosophila</u>	37
Figure 7.	Comparison of 5'-kinasing susceptibility of tRNA from newly-hatched and 30 day old <u>Drosophila</u>	42
Figure 8.	Comparison of 5'-kinasing susceptibility of ribosome-associated vs. free tRNA from newly-hatched and 25 day old <u>Drosophila</u>	44

PART II

Figure 9.	Cloverleaf structure of tRNA ^{Ser} _{2b}	53
Figure 10.	Genomic Southern analysis of tRNA ^{Ser} _{2b} genes	66
Figure 11.	Comparison of tRNA gene sequences	70
Figure 12.	<u>In situ</u> hybridization of the tRNA ^{Ser} _{2b} gene	77
Figure 13.	Cloverleaf structures of cloned tRNA genes as predicted from the DNA sequence data	79
Figure 14.	<u>In situ</u> hybridization of the tRNA ^{Ser} _{UCA} gene	82
Figure 15.	<u>In situ</u> hybridization of the tRNA ^{Leu} _{CUG} gene	86

ABBREVIATIONS

A ₂₆₀	- absorbance at 260 nm
Bis-MSB	- p-bis-(O-methylstyryl) benzene
bp	- basepairs
BSA	- bovine serum albumin (Fraction V)
ddNTP	- dideoxynucleoside triphosphate
DMSO	- dimethylsulfoxide
dN	- deoxynucleoside
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleoside triphosphate
DTT	- dithiothreitol
EDTA	- ethylenediamine tetraacetate (disodium salt)
EGTA	- ethylene glycol bis-(β -aminoethyl ether)-N,N'-tetraacetic acid
EtBr	- ethidium bromide
HEPES	- N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
HSP	- high speed pellet
HSS	- high speed supernatant
kb	- kilobasepairs
mRNA	- messenger ribonucleic acid
OAc	- acetate
PAGE	- polyacrylamide gel electrophoresis
pCp	- cytidine 3',5'-diphosphate
PPO	- 2,5-diphenyloxazole
Ψ	- pseudouridine
RNA	- ribonucleic acid
rRNA	- ribosomal ribonucleic acid

SDS - sodium dodecyl sulphate
TEMED - N,N,N',N'-tetramethylethylenediamine
tris - tris(hydroxymethyl)aminomethane
tRNA - transfer ribonucleic acid

1 x Denhardt's solution = 0.02% each of Ficoll®, polyvinylpyrrolidone,
and BSA (Fraction V)

1 x SSC = 0.15 M NaCl / 0.015 M Na₃citrate

1 x TBE = 80 mM tris / 80 mM boric acid / 1 mM EDTA

1 x YT = 1% Difco bactotryptone / 0.5% Difco yeast extract / 0.5% NaCl

ACKNOWLEDGEMENTS

I would sincerely like to thank my supervisor, Gordon Tener; without his enduring patience and support this work could not have been carried out. The members of his laboratory have also been extremely helpful throughout: Shizu Hayashi, who most generously contributed the in situ hybridization studies and greatly helped me with the intricacies of the UBC computer, Don Sinclair, who always had a smile and an uplifting word when the gremlin would strike, Ian Gillam, Jeff Leung, Craig Newton, and Nina Seto, all of whom contributed protocols, discussion, and much encouragement. My sincere gratitude goes to each of you. I am also thankful to my parents for all their encouragement, and to my mother for most kindly typing this manuscript. Finally, I gratefully acknowledge receipt of a Medical Research Council Studentship.

To Heather

Part I

I. INTRODUCTION

The quest for immortality, the "fountain of youth", has probably dwelt in the human race since the dawn of our ability to reason. In this century, the quest has been continued in the scientific investigations of many researchers. These researchers have amassed a great deal of information, sometimes conflicting, on age-related changes at the biochemical, cellular, and higher levels of organization, in a large number of tissues and organisms. It is unclear, but probable that most changes are a result of, rather than a cause of aging. To find the primary "trigger" of the aging process, assuming there is one, or a few, is an important research goal. However, it is also important to use any accumulated information to ameliorate the effects of aging as far as possible by delaying the occurrence or lessening the severity of the degenerative diseases common in old age.

Many diverse theories on the basis of aging have been constructed from this array of information. These theories can be roughly divided into two broad categories: Theories regarding aging as a genetically programmed developmental stage, and those concerning the deleterious effects of accumulation of random sub-lethal damage over time. Proposals of the first type have tended to suffer from a scarcity of evidence for specific mechanisms by which they may operate, and also from a lack of convincing theoretical arguments supporting the evolution of such a genetic program.

One model, based on a study of cell senescence in vitro, has

involved the eventual production of an inhibitor of DNA replication (Smith, 1984). Intriguing research supporting the programmed release of a "death hormone" from the pituitary has also been carried out (Bolla, 1982). It was shown that in rats hypophysectomized early in life (at 3-6 months), and maintained on minimal hormone replacement therapy, a number of parameters which normally decrease with age remained at "young" levels, even in 18-24 month old rats. These parameters include total RNA synthesis, initiation of RNA synthesis, liver aldolase synthesis, and a number of thyroid dependent physiological parameters.

On the theoretical side, it is difficult to rationalize the existence of "genetically programmed" death as a way of preventing species overcrowding in light of the fact that most organisms, including humans until recent times, die of environmental causes before they live long enough to senesce. This would eliminate any selection pressure toward programmed aging (Kirkwood, 1984). Perhaps the strongest theory in this direction is the "disposable soma" theory (Kirkwood and Holliday, 1979) which suggests that energy is conserved and allocated toward growth, reproduction, and repair in such a way that somatic cell processes are not maintained at the level of accuracy they would require for extended survival. Thus, reproduction and continuation of the species is promoted at the expense of somatic immortality.

The second category of aging theories, those concerning accumulations of unrepaired sub-lethal damage, have a much wider base of experimental data to support them. These theories address damage which occurs over time to the integral components of the cell such

as DNA (Burnet, 1974) and protein (Orgel, 1963).

One example is the error catastrophe theory put forward by Orgel (1963). He postulated that errors could accumulate in the proteins of the protein synthetic machinery over time, thus making it more prone to synthesizing defective proteins. Some of these, in turn, form part of the new synthetic machinery and thus give rise to even more unfaithful translations. Eventually protein synthesis becomes so error-prone that a "catastrophe" ensues causing, presumably, cell death. Although it is now generally believed that such a dramatic escalation of errors does not take place during aging, age-related changes to such cellular components as chromatin and protein are observed by some investigators (Makrides, 1983; Thakur, 1984). Orgel later amended his proposal to allow for a steady-state error frequency during aging, as opposed to a catastrophic escalation (Orgel, 1970). Experiments designed to support or refute the error theories of aging have been carefully reviewed (Laughrea, 1982) and the conclusion drawn that a modest increase in error frequency does accompany advancing age. It is not clear, however, whether such damage is a major or significant cause of aging in organisms, or of cell senescence in model fibroblast systems.

A major type of damage to cellular components is thought to be caused by free radicals. Free radicals arise from exposure to ionizing radiation, and from both enzymatic (during reduction of O_2 to water) and nonenzymatic (O_2 with organic compounds) reactions. The theory proposes that these could negatively affect cells in a variety of ways. These effects include oxidative damage (i.e., cross-linking) to long-lived molecules such as collagen, elastin, and chroma-

tin; oxidative degradation of mucopolysaccharides; production of lipofuscin (age pigment) by oxidative polymerization of lipids and proteins; lipid peroxidation of organelle membranes; post-injury blood vessel damage by peroxidation of serum and vessel wall components (Harman, 1981).

Living organisms have developed a number of defense mechanisms against oxygen free radicals, including antioxidants (i.e., tocopherols and carotenes), heme-containing peroxidases (i.e., catalase), glutathione peroxidase, superoxide dismutase, and DNA repair enzymes (Harman, 1981). The question might be asked whether individuals or species with relatively higher amounts of these protective agents in their tissues would age more slowly.

One very interesting result has been found regarding superoxide dismutase. A variety of mammalian species (both rodents and primates, including humans) were tested for superoxide dismutase levels in both liver and brain. An excellent linear relationship was found between the quotient of superoxide dismutase/specific metabolic rate vs. lifespan potential for each species (Cutler, 1983). Similar relationships between superoxide dismutase and lifespan have also been observed for the housefly, Musca domestica (Sohal et al., 1986 and 1987). This proportionality between potential lifespan and superoxide dismutase content gives considerable experimental support to the free radical theory of aging, as the only known function of superoxide dismutase is to inactivate the highly toxic superoxide free radical.

The theory also suggests that dietary ingestion of antioxidants could potentially retard the aging process. This has, in fact, been

found to be the case in a number of species including fruit flies, nematodes, mice, and rats (Harman, 1981). Antioxidants were able to increase the average, but not the maximum lifespan of various species studied. The lifespan benefits of excessive antioxidant consumption by humans have yet to be shown.

A dietary manipulation that has yielded dramatic results is food restriction (reviewed by Masoro, 1986). It has been possible to extend the maximum lifespan of rats 50% by reducing their caloric intake to 60% of the normal ad libitum level from the time of weaning (Yu et al., 1982). Food restricted rats show markedly reduced lipid peroxidation of liver subcellular organelles compared to control rats, at ages 6, 12, and 24 months (Laganriere and Yu, 1987). Restricted mice show increased liver catalase activity of approximately 50% at 12 and 24 months, and less lipid peroxidation at both ages, although the 13% difference at 24 months was not statistically significant (Koizumi et al., 1987). Furthermore, mice show less lipofuscin deposition in the hippocampus and frontal cortex of the brain (sites commonly affected in Alzheimer's disease), and better learning and performance in a maze test than control mice after 12 months of a restricted diet (Idrobo et al., 1987). Results like these seem to suggest that restricting caloric intake while ensuring full consumption of vitamins and minerals reduces the cumulative amount of oxidative damage to tissues, dramatically slowing the aging process and extending not only the average but also the maximum lifespan. This gives support to the theories on oxidative damage and aging, and also suggests possible strategies for slowing the aging process in humans.

It is well established that the rate and amount of protein synthe-

sis decreases during aging, in a wide variety of organisms (Gabijs et al., 1982; Makrides, 1983). The reason or reasons for this decrease may hold an important key to the mystery of aging, as proteins are so integral to the structure and function of cells and their organelles.

In vitro protein synthesis systems derived from aged organisms also show decreases in synthesis ability (Webster and Webster, 1979; Gabijs et al., 1983; Khasigov and Nikolaev, 1987). Such a system from Drosophila melanogaster exhibited a 70% drop in rate of synthesis by just 14 days after eclosure (Webster and Webster, 1979). This system has been studied intensively to determine the step or steps which show the greatest decrease (Webster and Webster, 1979, 1981, and 1982; Webster et al., 1981). It was seen (Webster and Webster, 1982) that the elongation stage of protein synthesis, and more specifically, binding of the aminoacyl tRNA to ribosomes, showed the greatest rate decrease with age: 60-70% after 20 days. This same step of the elongation process also showed the greatest rate decline with age (30-40%) in an analysis of cell-free protein synthesis from rat liver and kidney (Gabijs et al., 1983). Further, elongation also showed the greatest rate decrease in cell-free systems from mouse brain, liver, kidney, and skeletal muscle (Blazejowski and Webster, 1984), and rat liver and brain cortex (Khasigov and Nikolaev, 1987).

In Drosophila, at least part of this decline may be the result of a precipitous drop in the level of EF-1 mRNA available for translation (Webster and Webster, 1983 and 1984), which happens about five days previously to the decline. Experiments on a cell-free system from rat liver also show EF-1 from aged rats 30-40% less active than EF-1 from young rats (Moldave et al., 1979), however this has not

been consistently observed (Sojar and Rothstein, 1986).

A soluble factor from high-salt washes of young rat liver ribosomes has been found to significantly stimulate old ribosome preparations; the active factor does not appear to be present in high-salt washes from old ribosomes (Sojar and Rothstein, 1986). A similar situation has been found in the study of ribosomal material from young and old rabbit reticulocytes (Rowley et al., 1971). However, a proportion of the ribosomes of senescent nematodes are inactive and do not show stimulation by material obtained from salt washes of young ribosomes (Egilmez and Rothstein, 1985). The binding of the ternary complex EF-1 • GTP • aminoacyl tRNA is found to be reduced for these inactive ribosomes, but not for the aged rat liver system described above. It is seen, therefore, that the nature of the decline in protein synthesis is still somewhat obscure. It appears that ribosomes can become inactivated in aged organisms, but that soluble factors such as EF-1 can also play a role in the decreased ability to synthesize protein.

There has been some evidence suggesting the buildup during aging of defective material which co-purifies with tRNA, possibly being an inactive form of this vital adaptor molecule. Indirect evidence for this is the significantly decreased ability of nine different tRNAs from 35 day old Drosophila to be aminoacylated by aminoacyl-tRNA synthetases from either young or old flies (Hosbach and Kubli, 1979). The ability of eight tRNAs was reduced 10-25%, even using young synthetases, and for tRNA^{Leu} a 50% reduction was seen. Further, tRNAs from aged rat liver were found to be somewhat less efficient than young tRNAs from the same source in a Krebs ascites cell tRNA-dependent

cell-free protein synthesis system (Mays-Hoopers et al., 1983).

Possibly even more informative is the following data on aminoacylation of young and old rat liver tRNA: On comparison of amino acid acceptance of young and old whole tRNA extracts, testing for 17 amino acids, old tRNA isolated from whole tissue showed an average 64% decreased acceptance, whereas old tRNA isolated from the post-ribosomal supernatant (HSS) only showed an average decrease of 7% (Mays et al., 1979; Lawrence et al., 1979). This suggests the presence of defective or inhibitory material, which co-purifies with tRNA, bound to the aged ribosomes. Possible candidates could be partially degraded or otherwise damaged rRNA and/or tRNA molecules. On analysis of the material bound to young and old ribosomes (HSP), it was found that material derived from old ribosomes had only 64% of the acceptance capacity of young ribosome-derived material.

During purification of Drosophila melanogaster tRNA isoacceptors for sequencing in our laboratory, a contaminant was found co-purifying with the tRNA₅^{Lys} fraction. On sequencing, this contaminant was found to be a tRNA^{Arg} molecule which had lost five nucleotides from the 3'-end (Cribbs, 1982). Even though the molecule was only present in very low amounts, it labelled at the 5'-end with [γ -³²P]ATP and polynucleotide kinase to give a significantly darker band than the much more abundant intact tRNA₅^{Lys} also present, presumably due to the absence of steric hinderance from the missing 3'-nucleotides, creating a one-nucleotide 5'-overhang. It is not clear whether this partially degraded species is an artifact of the tRNA purification process or whether it is actually present in live Drosophila, but this points to the possibility of such defective but largely intact

tRNA molecules being present in vivo, possibly due to lowered activity of degradative enzymes. These molecules could form part of the material which causes lowered amino acid acceptance of tRNA from aged organisms. If such material tended to bind tightly to ribosomes, it would be largely removed from the post-ribosomal supernatant, accounting for the increased acceptance of tRNA from this fraction mentioned earlier.

Finally, it has been shown that unprocessed tRNA^{Met} and tRNA^{Leu} precursors are stably present in HeLa cells, with four extra 5'-nucleotides and nine extra 3'-nucleotides (Harada et al., 1984). Similar molecules may also become candidates for defective ribosome-bound RNA during aging, if the activity of processing enzymes was decreased, causing a significant increase in their abundance.

The research contained in this thesis is a preliminary investigation of the hypothesis that tRNA partially degraded or elongated at the 3'-end could bind to ribosomes and interfere with protein synthesis. tRNA has been partially 3'-degraded by removing the single stranded nucleotides using snake venom phosphodiesterase, and 3'-elongated by one nucleotide using pCp and RNA ligase. These modified molecules were added to a rabbit reticulocyte lysate protein synthesis system, but inhibition was not seen until the ratio of defective : whole tRNA was almost 1:1.

Further, 5'-labelling studies using [γ -³²P]ATP, alkaline phosphatase, and polynucleotide kinase, designed to detect 3'-degraded tRNA in whole extracts from young and old Drosophila melanogaster, showed no marked differences between the two tRNA populations, using this assay. Further, young and old tRNA from both post-ribosomal supernatant (HSS) and ribosomal bound fractions (HSP) showed a similar labelling

profile in the tRNA range. Only one minor band possibly showed differential labelling in two independent experiments. The conclusion is drawn, from these preliminary studies, that partially 3'-degraded or elongated tRNA does not have the potency or in vivo abundance to be a significant inhibitor of protein synthesis. The synthesis machinery appears able to discriminate efficiently between whole and defective tRNA in these in vitro assays, only becoming inhibited with very high levels of defective tRNA.

II. MATERIALS AND METHODS

All enzymes and lysate were obtained commercially, and the suppliers given were used exclusively. All chemicals used were reagent grade. Drosophila melanogaster, strain Oregon R, were grown by Dr. G.M. Tener, and were a gift from him.

i. Isolation of tRNA from Drosophila - Method I

For isolation of tRNA, freshly killed Drosophila melanogaster were preferable, but the tRNA of such flies frozen at -70°C for a few days or weeks also appeared intact by PAGE. The method was scaled down or up to cover weights of flies between a fraction of a gram and 100 gm.

In a typical experiment by this method, derived from the method of Roe (1975), 1 gm of flies was ground in a 15 ml Corex® tube for 3 minutes with a Polytron® apparatus at setting "4" in a 1:1 mixture of 0.14 M NaOAc pH 4.5 (HOAc) : 88% phenol (total volume = 2.8 ml). This was centrifuged at $1,000 \times g_{\text{max}}$ for 15 minutes. The aqueous layer was further extracted twice with phenol and applied to a 3 ml DEAE-cellulose column equilibrated with 0.14 M NaOAc pH 4.5 (HOAc) (= buffer A). The column was washed with buffer A until the eluant $A_{260} = 0.4$ (50 ml); the column buffer was then changed to buffer A / 0.3 M NaCl and the column washed until the eluant $A_{260} = 0.03$ (20 ml); finally, the tRNA was eluted with 8 ml buffer A / 1.1 M NaCl, and ethanol precipitated. tRNA isolated in this manner contained considerable 5S rRNA, as well as larger rRNAs. Where necessary, a Sephadex® G-100 column was used to remove these, as described below.

For isolation of tRNA from 100-200 flies, the method was scaled down as follows: The flies were ground in 600 μ l of 1:1 buffer A : phenol in a v-shaped tube 1.3 cm wide, with four passes of a Potter-Elvehjem homogenizer pestle at setting "4". This was centrifuged at $77 \times g_{\max}$ for 5 minutes. After two more phenol extractions, the aqueous phase was applied to a 1 ml DEAE-cellulose column as above and eluted step-wise with 30 ml buffer A, 24 ml buffer A / 0.3 M NaCl, and 2 ml buffer A / 1.1 M NaCl. The latter eluate, which contains the tRNA, was desalted using an Amicon® centricon 10 microconcentrator unit and dessicated.

ii. Isolation of tRNA from *Drosophila* - Method II

To isolate tRNA from 1 gm of *Drosophila* by this method, the flies were ground for 1-2 minutes with a Polytron® apparatus at setting "4", in 2.5 ml of 2% SDS / 0.14 M NaOAc pH 4.5 (HOAc) / 1.5 M NaCl, plus 2.5 ml of 88% phenol. This was centrifuged at $1,000 \times g_{\max}$ for 5 minutes. The aqueous phase was further extracted three times with phenol, back extracting the phenol phase with H₂O each time, and pooling the resulting aqueous layers. The final aqueous phase was ethanol precipitated, taken up in 3 ml buffer B (see below) / 10% sucrose, and chromatographed directly on the Sephadex® G-100 column described below.

iii. Sephadex® G-100 Gel Filtration of RNA

The tRNA isolated from *Drosophila* was contaminated with 5S RNA as well as significant amounts of larger rRNAs. To remove these contaminants where relatively pure tRNA was needed, gel filtration

using Sephadex® G-100 proved to be effective.

A Pharmacia 1.6 cm x 100 cm column was used. This was packed with Sephadex® G-100 (fine grade) at 65 cm pressure to a height of 97 cm. The column buffer was 0.75 M NaCl / 0.01 M HOAc pH 5.0 / 1% methanol / 0.02% NaN₃ (= buffer B). The sample was loaded in 3 ml of buffer B with 10% sucrose added; this was followed by a 4 ml "cushion" of 15% sucrose / buffer B. Both solutions were filtered through a 0.45 µ Millipore® filter before loading. The column was run against gravity at a flow rate of 16 ml/hr, and 1.4 ml fractions were collected.

Totally excluded material, such as large rRNAs, eluted in the fractions from 63 ml to 91 ml; 5S RNA eluted from 91 ml to 122 ml; degraded, elongated, and whole tRNA eluted from 122 ml to 160 ml; totally included molecules eluted from 189 ml to 245 ml. The desired fractions were pooled and ethanol precipitated.

iv. Isolation of tRNA from Post-Ribosomal Supernatant and from Salt-Washed Ribosomes

This method is modified from two previous methods (Pelly and Stafford, 1970; Webster et al., 1981). D. melanogaster (1 gm) was ground for 25 seconds with a Polytron® apparatus in a 30 ml Corex® tube, in 20 ml ribosome homogenization buffer: 0.25 M sucrose, 50 mM tris pH 7.2 (HCl), 100 mM KCl, 6 mM Mg(OAc)₂, 1 mM EDTA, 7 mM β-mercaptoethanol, and 10 µg/ml polyvinyl sulfate. The mixture was centrifuged at 4°C for 10 minutes at 3,500 x g_{max}; this pellets nuclei and mitochondria (material > 40,000 S).

The supernatant was centrifuged at 4°C for 90 minutes at 106,000

$\times g_{\max}$. The resulting "high speed supernatant" (HSS) and "high speed pellet" (HSP) were treated as follows: The HSS was phenol extracted three times, ethanol precipitated, and chromatographed on a Sephadex® G-100 column as described. The HSP was resuspended in 10 ml buffer A / 1.1 M NaCl, phenol extracted, ethanol precipitated, and chromatographed as for the HSS.

v. Partial Enzymatic 3'-Degradation of tRNA

Drosophila tRNA was treated with snake venom phosphodiesterase (phosphodiesterase I) to selectively remove the four single-stranded nucleotides at the 3'-terminus of the molecule (Zubay and Takanami, 1964; von der Haar et al., 1971; Sprinzl et al., 1972; Addison, 1982). The enzyme was purchased from P-L Biochemicals as a 20 U/mg lyophilized powder. It was stored at -20°C in a 10 mM $\text{Mg}(\text{OAc})_2$ / 10% glycerol solution at 1 mg/ml. The enzyme is stable for four months under these conditions (Miller et al., 1970).

The reaction contained 1.0 $\mu\text{g}/\mu\text{l}$ D. melanogaster tRNA, 0.1 $\mu\text{g}/\mu\text{l}$ snake venom phosphodiesterase, 10 mM tris pH 7.6 (HCl), and 10 mM MgCl_2 . This was incubated at 37°C for 30 minutes. Extensive phenol extraction was carried out to remove all traces of enzyme (this enzyme would itself inhibit protein synthesis), and this was followed by repeated ether extractions to remove phenol.

The degraded tRNA was dessicated using a Rotovap apparatus and applied to the Sephadex® G-100 gel filtration column used for tRNA purification. This species elutes in the same fractions as intact tRNA. The eluted fractions containing pure degraded tRNA were pooled, ethanol precipitated and taken up in H_2O at a concentration of 2

mg/ml; the degraded material was then analyzed by PAGE.

vi. 3'-Elongation of tRNA

tRNA was elongated by one nucleoside 3',5'-diphosphate at the 3'-terminus by the following method (Cribbs, 1982). The reaction contained 1.6 $\mu\text{g}/\mu\text{l}$ D. melanogaster tRNA, 2 mM pCp, 2 mM ATP, 15 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ BSA, 5 mM DTT, 50 mM HEPES pH 8.0 (KOH), 20% v/v DMSO, and 0.1 $\mu\text{g}/\mu\text{l}$ T4 RNA ligase, in a volume of 160 μl . The mixture was incubated overnight at 17°C. It was then ethanol precipitated, taken up in 100 μl H_2O , and a portion was analyzed by PAGE. The remainder was extracted three times with 1:1 phenol : chloroform, three times with ether, and ethanol precipitated.

The tRNA-pCp was applied to the Sephadex® G-100 gel filtration column used for purifying tRNA. Two peaks eluted from this column: The tRNA-pCp peak expected at a similar elution volume as tRNA, and a peak of totally included material, likely a mixture of residual phenol and pCp, widely separate from the tRNA-pCp peak. The tRNA-pCp fractions were pooled, ethanol precipitated, taken up in H_2O at a concentration of 50 $\mu\text{g}/\text{ml}$, and stored at -20°C.

vii. Reticulocyte Lysate Protein Synthesis Assays

To detect inhibition of protein synthesis by defective (degraded or elongated) tRNA, a rabbit reticulocyte lysate protein synthesis assay was used. Promega Biotec nuclease untreated lysate was aliquotted on arrival and stored at -70°C. Activity decreased somewhat after 4-5 months' storage.

A cocktail containing 105 mM creatine phosphate, 525 $\mu\text{g}/\text{ml}$ creatine

kinase, 53 mM HEPES pH 7.5 (KOH), 13 mM DTT, 0.42 mM ATP, 0.11 mM GTP, 19 unlabelled amino acids (excluding leucine) at 0.21 mM each, 0.13 mM hemin, 484 mM KOAc, and 5.72 mM $\text{Mg}(\text{OAc})_2$ was prepared and stored at -20°C (method from Promega Biotec, with minor modifications).

Before each experiment, a premix was made containing cocktail, ^3H -leucine (2 mCi / 0.0017 mg / 1.0 ml, New England Nuclear), Promega Biotec RNasin® (32 U/ μl), and H_2O in 5:2:1:4 proportions (v/v). For a typical time course experiment with 10 time points, each tube contained 15 μl lysate and 18 μl premix. Any extra whole, degraded, or elongated tRNA and $\text{Mg}(\text{OAc})_2$ to be added had already been dessicated in the appropriate tube before the lysate and premix were added. All tubes were incubated at 25°C for the indicated time.

Determination of ^3H -leucine incorporation was performed as follows: At the approximate time, 3 μl of the reaction mix was withdrawn and added to 1 ml 1 N NaOH / 2.0% H_2O_2 preincubated at 37°C . After a 10 minute incubation at 37°C , 4 ml ice cold 25% conc. HCl / 3.5% glacial HOAc / 2% casamino acids was added and the mixture was put on ice for 30 minutes. It was then precipitated onto Whatman® GF/C 2.4 cm filters under vacuum; the filters were washed liberally with ice cold 10% conc. HCl / 1.4% glacial HOAc and dried by washing under vacuum with acetone. Filtration results were much more consistent when the initial vacuum drawing the mixture through the filter was applied gently and intermittently (i.e., 5% error with intermittent vacuum vs. 17% error with continuous vacuum).

The dry filters were covered in scintillation vials with 700 μl Protosol® and incubated either overnight at room temperature or for 3 hours at 37°C . 58 μl glacial HOAc and 10 ml of a 0.4% PPO / 0.01%

Bis-MSB toluene-based fluor solution were added, and the vial contents were mixed well. The samples were then counted 2 minutes in an Isocap® 300 scintillation counter, discriminating for the 0.5 to 18 KeV energy range.

viii. Determination of Reticulocyte Lysate Endogenous tRNA

Determination of the amount of tRNA endogenous to the rabbit reticulocyte lysate used was performed as follows: 544 μ l lysate was diluted with 600 μ l H₂O in a siliconized glass tube, and 169 μ l (= 22 μ g) large rRNA purified on a Sephadex® G-100 column was added as a carrier. This was extracted twice with 700 μ l phenol, conserving the entire aqueous layer each time. The phenol was back extracted with 700 μ l H₂O, and the pooled aqueous phases were extracted with 1.4 ml ether. The aqueous phase was chromatographed on a Sephadex® G-100 column as described, and the amount of rabbit tRNA was quantified by integrating the area under the A₂₆₀ peak to give an estimate of 0.11 μ g endogenous tRNA/ μ l lysate.

A previous tRNA determination on less lysate (239 μ l), which included one more phenol extraction step (with greater losses at the interface), no carrier RNA, and ethanol precipitation prior to chromatography, gave a result 45% less than the more rigorous determination described above.

ix. One-Dimensional Polyacrylamide Gel Electrophoresis

A 42.9% acrylamide / 2.1% methylene bis-acrylamide stock solution was made and stored at 4°C. This was used to make 10% or 20% polyacrylamide gels (Fradin et al., 1975). Acrylamide at the desired concentra-

tion, 4 M urea, 1 x TBE, 0.05% ammonium persulfate, (and for some gels 1 $\mu\text{g/ml}$ EtBr) were mixed and degassed under vacuum. TEMED was added according to the acrylamide concentration and the presence or absence of EtBr: For a 10% gel, 0.1% TEMED was used, and for a 20% gel, 0.03% TEMED was used; these amounts were doubled if EtBr was present. The mix was poured between 35 cm x 20 cm glass plates (one plate siliconized), and allowed to set approximately one hour at room temperature. The gels were run in 1 x TBE (and 1 $\mu\text{g/ml}$ EtBr if present in the gel).

For 1.5 mm thick gels with 9 mm wide slots, up to about 75 μg of tRNA could be loaded in each slot; the tRNA sample was dessicated and taken up in 6 μl 50% (v/v) formamide / 13% sucrose / 0.3% xylene cyanol ff / 0.04% bromophenol blue loading solution. 0.4 mm thick gels were used when autoradiography was needed, to increase resolution. In this case, 3 μl samples in 9 mm slots gave good bands.

tRNA was spread over a larger range if the 10% gels were run at 4°C, as opposed to room temperature. In these gels, xylene cyanol runs at the leading edge of the tRNA. Maximum separation of tRNA was obtained by running the gel at approximately 450 V for > 20 hours, until the xylene cyanol was 1-2 cm from the edge of the gel.

For 20% gels, tRNA was spread well through the middle range of the gels if they were run at room temperature, 600-1,000 V, for 24 hours. If dye markers were desired throughout these 20% gel runs, 15 μl of loading solution could be applied to the top of the gel as the initial xylene cyanol in the sample reached the bottom. Electrophoresis was terminated when the second xylene cyanol band was about 3 or 4 cm from the end of the gel.

x. Two-Dimensional Polyacrylamide Gel Electrophoresis

tRNA could be typically separated into > 70 spots by two-dimensional PAGE, using 10% acrylamide / 4 M urea in the first dimension and 20% acrylamide / 4 M urea in the second dimension (Fradin et al., 1975; Mazabraud and Garel, 1979).

For these gels, the first dimension was run as for one-dimensional electrophoresis using a 1.5 mm thick 10% gel and 75 ug tRNA, until the leading edge of the tRNA was 1 cm from the end of the gel. A 12 cm x 1 cm strip was cut from the lowest 12 cm of the lane where the tRNA was run (using a cutter 1 cm wide made of sharpened plexiglass for this purpose), carefully lifted with a spatula, and laid horizontally at the top of one of the second-dimension plates. The gel apparatus was assembled, and care was taken to keep the gel strip moist so that no bubbles formed between it and the glass plates. The 20% acrylamide / 4 M urea gel was poured around the strip, allowed to set for approximately 1 hour, and run at about 550 V for 48 hours. New dye was added during the run as described, and the run was terminated when the second xylene cyanol band was 1-2 cm from the end of the gel.

xi. 5'-Labelling of tRNA with ^{32}P

The 5'-terminal phosphate group was removed from the tRNA to be studied using calf intestinal alkaline phosphatase (Boehringer, 28 U/ μl) (Cribbs, 1982). The enzyme was diluted by 1/1,000 in water immediately before use. A reaction tube with 0.25 $\mu\text{g}/\mu\text{l}$ pure D. melanogaster tRNA (larger RNA removed with Sephadex® G-100 gel filtra-

tion), 0.003 U/ μ l alkaline phosphatase, 20 mM tris pH 8.0 (HCl), and 0.2 mM EDTA (total volume 5 to 10 μ l) was incubated 15 minutes at 55°C; 1 μ l 50 mM nitrilotriacetic acid pH 7.0 was added to stop the reaction, and the mix was dessicated at room temperature.

To label the 5'-terminal nucleotide of susceptible tRNAs, T4 polynucleotide kinase (Boehringer, 4.5 U/ μ l) and [γ -³²P]ATP (New England Nuclear, 250 μ Ci / 25 μ l / 87 pmol) were used (Cribbs, 1982). A premix containing 40 mM tris pH 8.0 (HCl), 9 mM MgCl₂, 10 mM DTT, 0.01 mM ATP, 0.1 mg/ml BSA, 125 mM KCl, 0.2 U/ μ l T4 polynucleotide kinase, and 0.5 μ Ci/ μ l [γ -³²P]ATP was added to the dessicated mix, the volume being equal to that of the alkaline phosphatase reaction. This was mixed well and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of $\frac{1}{2}$ -volume of 25% sucrose / 0.2% xylene cyanol ff / 0.1% bromophenol blue in 98% formamide. 3 μ l of the resulting sample was analyzed by PAGE (20% acrylamide, 4 M urea, 0.4 mm thick). The gels were autoradiographed (Curex X-ray film) at 4°C for 3 days.

III. RESULTS AND DISCUSSION

Studies were undertaken both in vitro and on tRNA isolates from young and old Drosophila to attempt to determine the likelihood of partially 3'-degraded or elongated tRNAs accumulating during aging and acting as significant inhibitors of protein synthesis, by such mechanisms as blocking the binding of aminoacyl tRNAs to ribosomes. The in vitro studies involved enzymatic modification (partial 3'-degradation or elongation) of Drosophila tRNA and addition of this defective tRNA to a rabbit reticulocyte lysate protein synthesis system to study its effect on protein synthesis. Studies on tRNA extracts took the form of two-dimensional PAGE of young and old tRNA, and 5'-labelling of young and old tRNA to detect exposed 5'-ends of molecules partially degraded from the 3'-end.

i. Partial Enzymatic 3'-Degradation of tRNA

tRNA folded in the characteristic "cloverleaf" conformation typically has four single-stranded nucleotides at the 3'-terminus (see Figure 9, Part II). It has been shown that snake venom phosphodiesterase preferentially removes the single-stranded nucleotides at the 3'-end of tRNA, hydrolyzing only very slowly past this point unless the reaction is forced (Zubay and Takanami, 1964; von der Haar et al., 1971; Sprinzl et al., 1972; Addison, 1982).

A whole tRNA extract from Drosophila was used for degradation by this enzyme. Reaction conditions were chosen to maximize degradation of the four single-stranded nucleotides (somewhat elevated enzyme:tRNA ratio and 37°C temperature), and to minimize degradation beyond

this point (30 minute incubation), thus creating tRNA molecules essentially intact, but defective at the aminoacyl acceptor site. These partially degraded tRNAs were intended to mimic the tRNA^{Arg} fragment discussed (Introduction), where only five nucleotides have apparently been removed from the 3'-end (Cribbs, 1982).

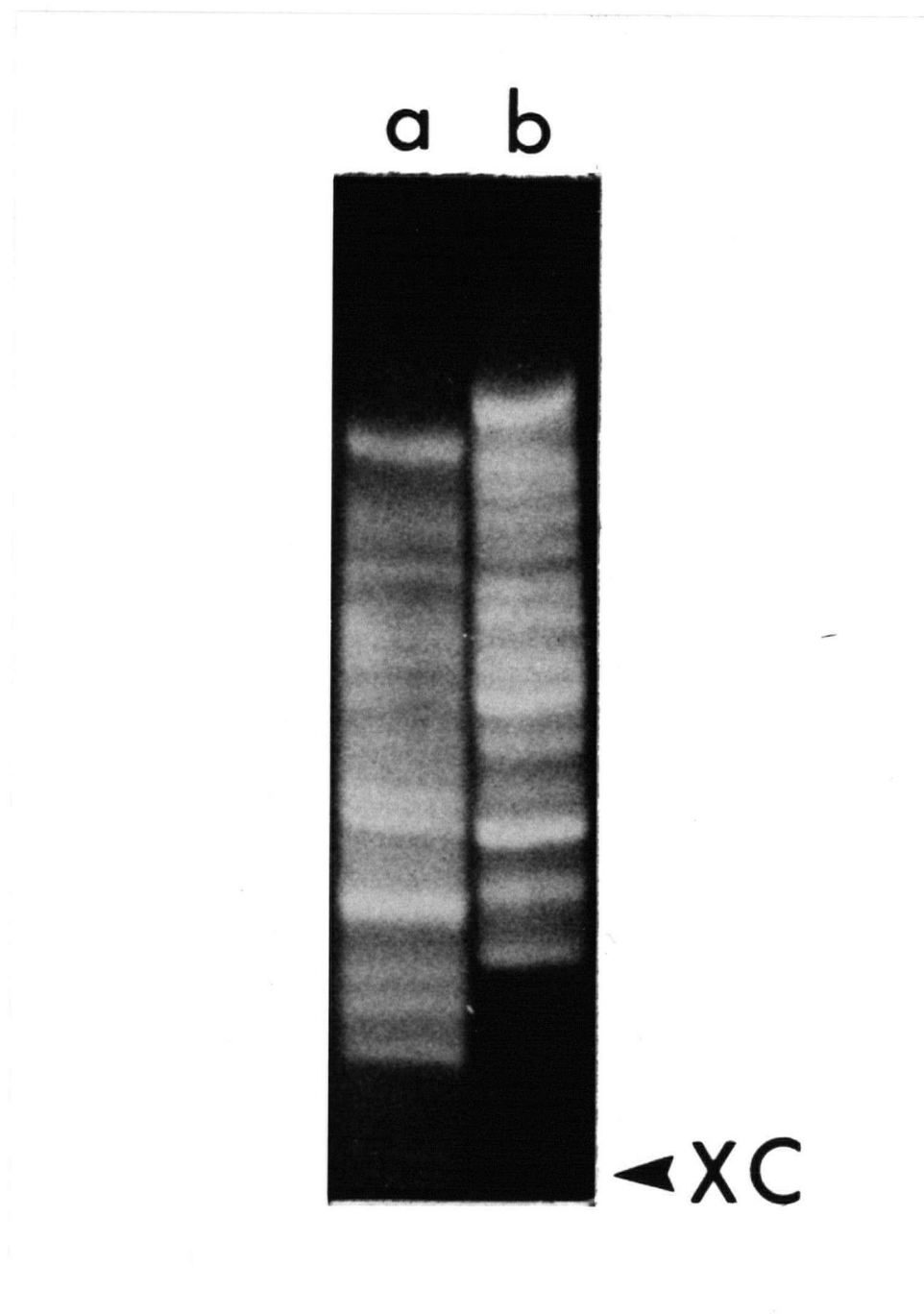
10% PAGE was used to assay for the extent of tRNA 3'-degradation after treatment with snake venom phosphodiesterase. The results showed a distinct shift of the entire tRNA profile downward in the gel, consistent with removal of a small number of nucleotides from the majority of tRNAs in the sample (Figure 1). Further digestion did not increase this shift, unless the reaction was forced, implying that the digestion stopped after removal of the four single-stranded nucleotides.

For comparison, tRNA digested with polynucleotide phosphorylase was also visualized by 10% PAGE (data not shown). This enzyme is known to function by a processive mechanism (Thang *et al.*, 1967), whereby digestion of an entire tRNA molecule takes place before the enzyme proceeds to the next molecule. In this case, a slight downward shift of the entire tRNA profile, as seen for snake venom phosphodiesterase digestion, was not apparent. Rather, the tRNA bands remained fixed and became more faint as digestion conditions became more vigorous.

Thus, on the basis of published studies on snake venom phosphodiesterase and the distinct downward shift seen on 10% PAGE, it is concluded that this tRNA has had only its single-stranded nucleotides removed by the enzyme, and that the majority of tRNAs present have been altered in this way.

Figure 1. Comparison of unaltered and partially 3'-degraded tRNA.

Drosophila tRNA partially degraded as described in Materials and Methods (lane a) and whole Drosophila tRNA (lane b) were electrophoresed using a 1.5 mm thick 10% polyacrylamide / 4 M urea gel. Fifteen μg of each RNA sample was loaded, and 430 V were applied for 23 hours at 4°C. Electrophoresis was terminated when the xylene cyanol ff dye (XC) was 2 cm from the end of the plate. The gel was immersed in 40% methanol / 1 $\mu\text{g/ml}$ EtBr for 20 minutes and photographed. The area shown in the figure is from the lower third of this gel.



ii. 3'-Elongation of tRNA

tRNA molecules elongated by one nucleoside 3',5'-diphosphate at the 3'-terminus were constructed by ligating pCp to whole tRNA extracted from Drosophila. The reaction stopped after addition of one monomer to each tRNA, as the enzyme will not use the newly added 3'-phosphate as a substrate. These molecules were designed to mimic tRNA precursors to the extent that the acceptor stem is not functional due to the presence of an elongated 3'-terminus, beyond the CCA end. They are also nonfunctional due to the absence of a 3'-hydroxyl moiety.

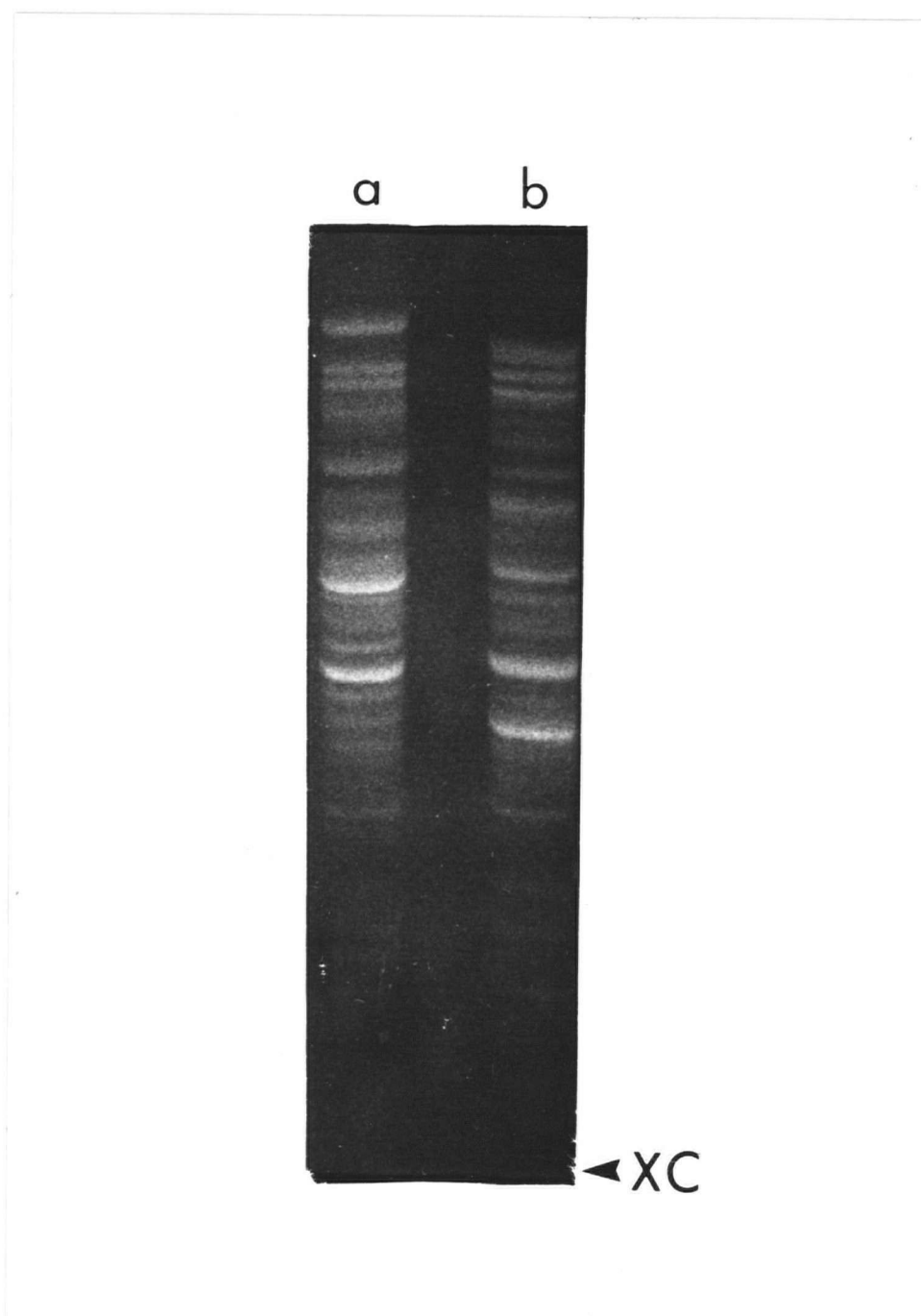
The ligation reaction appears to have modified the major portion of the tRNA, according to the moderate, distinct upward shift of the 20% PAGE tRNA profile seen in Figure 2. The major tRNA bands have certainly undergone a shift upon elongation, and most of the more faint bands also appear adjusted.

The discrete upward tRNA profile shift seen here, and the discrete downward shift of slightly larger magnitude seen for partially degraded tRNA (Figure 1), support each other as evidence that the desired minor adjustments have been made to the average tRNA size in both cases. Because these samples contain a mix of at least 99 different tRNA species (White et al., 1973), it is not possible to deduce the shift distance of each individual tRNA. However, the relative shift distances, when compared with the length of the tRNA profile itself, appear compatible with the removal of four nucleotides (Figure 1) or the addition of one nucleotide 3',5'-diphosphate (Figure 2).

These gels were run using 4 M urea as a denaturing agent; to fully denature the tRNA, the gels would need to be run at high tempera-

Figure 2. Comparison of unaltered and 3'-elongated tRNA.

Drosophila tRNA elongated by one nucleoside diphosphate at the 3'-terminus (lane a) and whole Drosophila tRNA (lane b) were electrophoresed using a 1.5 mm thick 20% polyacrylamide / 4 M urea gel containing 1 $\mu\text{g/ml}$ EtBr. Twenty μg of elongated and unaltered tRNA were loaded, and 600 V were applied for 24 hours at room temperature. Electrophoresis was terminated when the xylene cyanol ff dye (XC) was 1 cm from the end, and the gel was photographed. The area shown in the figure is from the middle third of this gel.



ture with 7 M urea present. However, the milder conditions were chosen because the influence of shape caused the separation of tRNA into a longer profile with more bands than would have been obtained under harsh denaturing conditions. Since individual bands cannot be matched between whole and altered tRNA profiles on the basis of these gels alone, total denaturation appeared unnecessary, and the wider spread of the tRNA on these partially denaturing gels seemed preferable for this qualitative analysis.

These analyses on partially degraded and elongated tRNA suggest that sufficient modification has taken place to render the majority of tRNA molecules in each sample ineffective, by altering the 3'-terminus.

iii. Inhibition of Protein Synthesis by Defective tRNA

A rabbit reticulocyte lysate nuclease untreated cell-free protein synthesis system was used to assay inhibition of protein synthesis by the partially degraded and elongated tRNAs described. This system was suitable for these initial studies, as it is readily available commercially, and it contains all the components necessary for translation, including mRNA. Unlike the more widely-used lysate treated with micrococcal nuclease to destroy endogenous mRNA (Pelham and Jackson, 1976), the untreated lysate was quite stable with respect to variations in Mg^{+2} concentration. Nuclease-treated lysate had an activity peak for a narrow range of added Mg^{+2} concentrations (0.6 mM to 0.8 mM), with the activity dropping off sharply on either side of this range, whereas untreated lysate was equally active from 0.6 mM to at least 3.6 mM Mg^{+2} .

Micrococcal nuclease is activated by Ca^{+2} , and deactivated by chelation of Ca^{+2} with excess EGTA. With an increase in the amount of Mg^{+2} present, enough Ca^{+2} is presumably displaced from chelation to re-activate the nuclease, causing a decrease in lysate activity [$k_a = 5 \times 10^{10}$ for Ca^{+2} and $= 2.5 \times 10^5$ for Mg^{+2} (Schmid and Reilley, 1957)]. Since tRNA does chelate Mg^{+2} to a certain extent, extra Mg^{+2} was added where extra tRNA was present at high levels. Thus, a lysate system much more stable with respect to Mg^{+2} concentration was needed for this series of assays. The nuclease untreated lysate was seen to be suitable in this respect, showing no activity change even upon addition of 0.3 μg whole tRNA/ μl lysate, with or without extra Mg^{+2} (Figure 3).

If the data had suggested that more detailed studies of inhibition should be pursued, a Krebs ascites cell tRNA-dependent protein synthesis system may have been suitable (Boime and Leder, 1972), as the amounts of both functional and non-functional tRNA can be controlled in that system. As seen in Figure 4 however, levels of exogenous partially degraded tRNA from 0.006 - 0.06 $\mu\text{g}/\mu\text{l}$ lysate produce absolutely no detectable change in lysate activity. This is an important negative result. It shows that even exogenous defective tRNA levels well within an order of magnitude of endogenous tRNA levels (0.11 $\mu\text{g}/\mu\text{l}$ lysate) do not inhibit protein synthesis in this system.

Two determinations of the level of endogenous lysate tRNA were made, as described. The most reliable experiment, in which care was taken to reduce tRNA losses to an absolute minimum at each stage, gave an estimate of 0.11 μg tRNA/ μl lysate. An earlier determination gave a value of 0.06 μg tRNA/ μl lysate. This earlier value is well

Figure 3

Effect of High Levels of Whole and Partially Degraded tRNA on Protein Synthesis

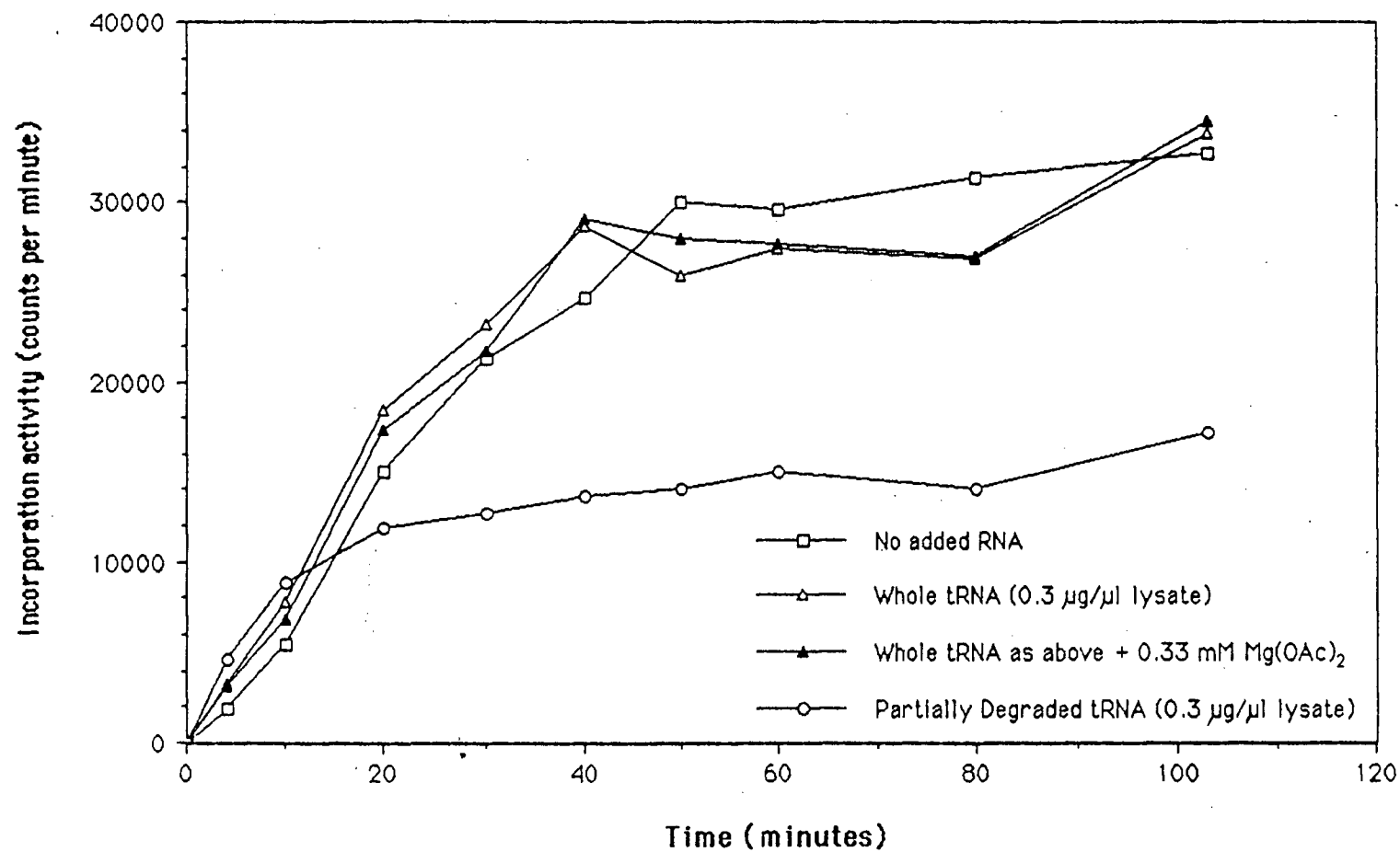
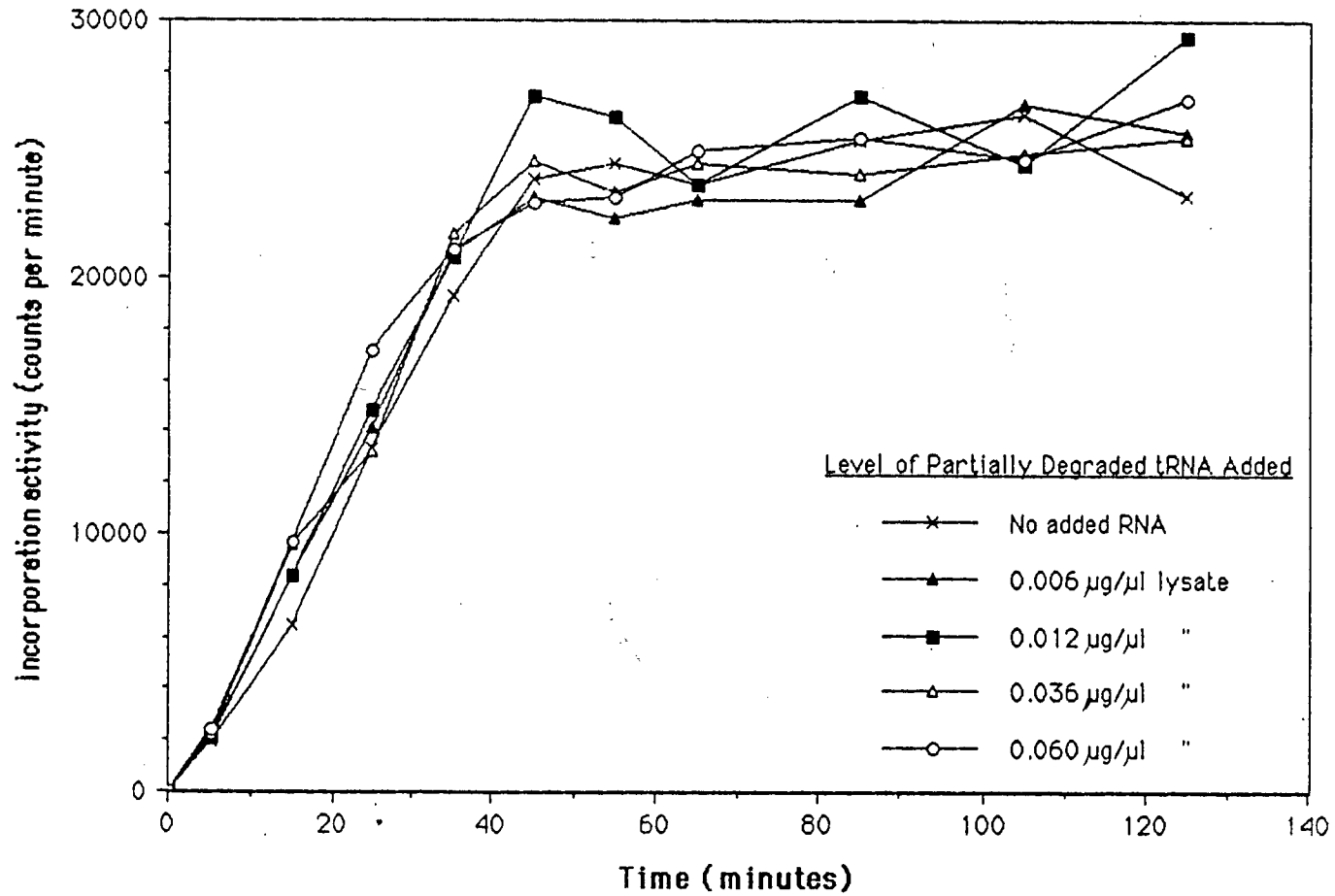


Figure 4

Effect of Low Levels of Partially Degraded tRNA on Protein Synthesis



within an order of magnitude of the more rigorously determined value, and shows quite good agreement with it considering the increased opportunities for tRNA loss in this earlier experiment (discussed in Materials and Methods).

If partially degraded tRNA were present in vivo at all, it would certainly not be expected to be found in a quantity approximating the level of functional tRNA present, but rather in much smaller amounts. Thus, the relevance of partially degraded tRNA to protein synthesis inhibition in vivo appears very doubtful from these data.

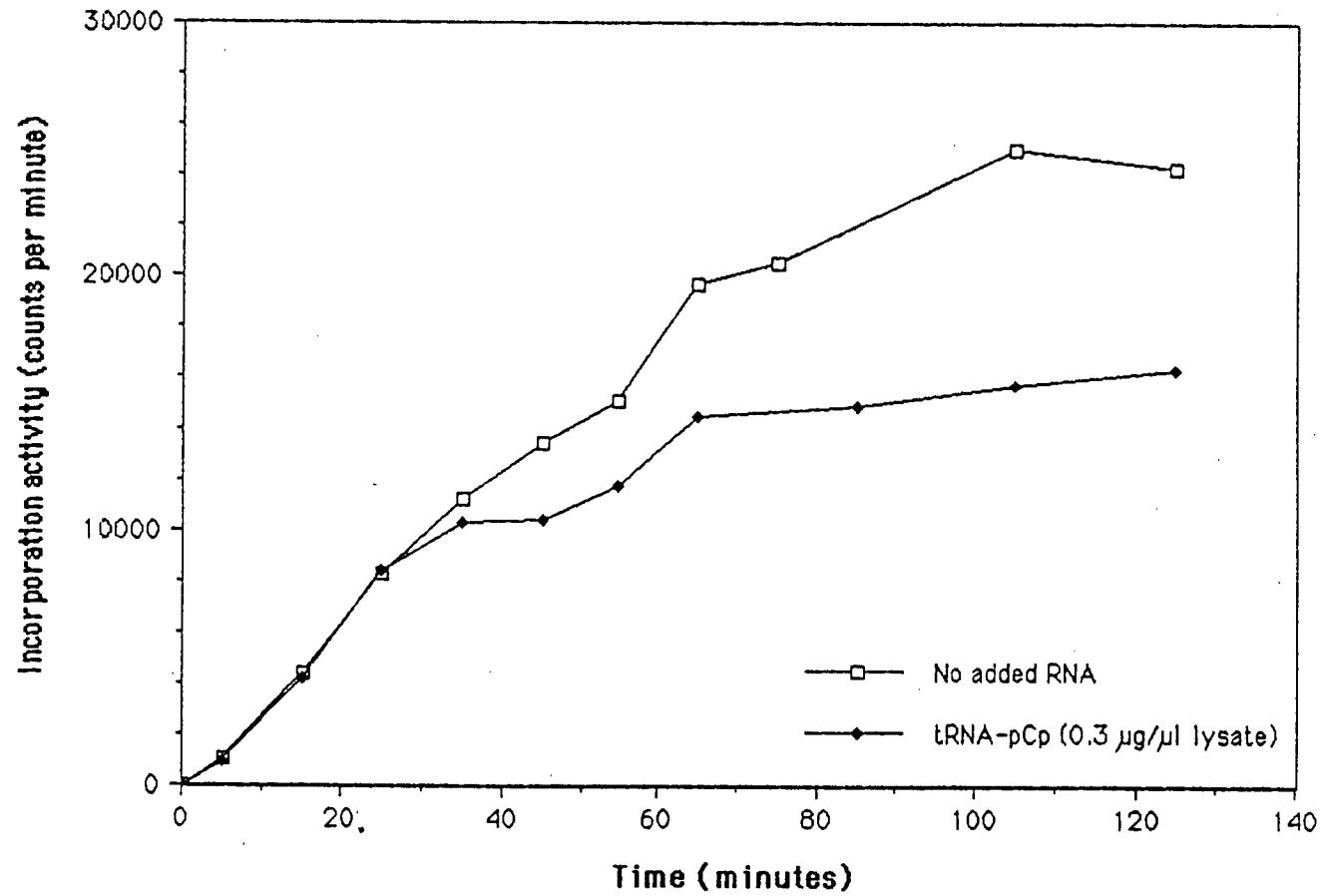
Higher levels (such as 0.3 $\mu\text{g}/\mu\text{l}$ lysate) of both defective tRNA types do produce appreciable inhibition of protein synthesis (Figures 3 and 5). This inhibition becomes most apparent after the first 20 to 40 minutes of synthesis, with the inhibited samples attaining only about 50% to 65% of the uninhibited ^3H -Leu incorporation after 2 hours. The initial synthesis rates appear comparable with or without defective tRNA present. In these preliminary results, elongated tRNA-pCp appeared to be a slightly less potent inhibitor than partially degraded tRNA. This could, however, be affected by the percentage of tRNA molecules modified in the respective tRNA samples, a percentage which was not measured quantitatively by the PAGE assays which were done.

The mechanism of this inhibition, although an interesting study in itself, was not investigated further here, due to the high levels of modified tRNA needed to produce detectable changes in protein synthesis, and the resulting apparent irrelevance to an in vivo situation.

To approach this study, one could prepare sufficient quantities

Figure 5

Effect of High Levels of Elongated tRNA-pCp on Protein Synthesis



of both purified tRNA₁^{Met} and a purified elongator tRNA, partially degrade them, and conduct inhibition studies. It would be interesting to see whether pure defective tRNA₁^{Met} could produce the same type of inhibition curve seen here, suggesting that these results signify inhibition at the initiation step. Further, the use of a pure degraded elongator tRNA species may illuminate any inhibition of elongation possibly masked here by the presence of defective tRNA₁^{Met}.

Elongation, and more specifically the binding of aminoacyl-tRNA to the ribosomes, is the step which shows the greatest activity decrease in aging Drosophila (Webster and Webster, 1982) and rat liver or kidney (Gabijs et al., 1983). A defective elongator tRNA would be a logical candidate as a competitive inhibitor of this step. Here, however, the ribosomes and/or elongation factors appear to possess efficient means of discriminating against excess defective tRNA approaching even a 1:1 concentration ratio with respect to functional tRNA; any small amounts of partially degraded tRNAs or unprocessed tRNA precursors that may be found in vivo would therefore not be expected, on the basis of these preliminary data, to be at all effective in inhibiting protein synthesis.

iv. Two-Dimensional Electrophoresis of tRNA from Young and Aged Drosophila

Whole tRNA extracts (by Method I) from newly-hatched and 32 day old Drosophila were compared using two-dimensional PAGE, which separates the samples into more than 70 spots. This approaches the 99 different major and minor tRNA species found by RPC-5 chromatography of Drosophila tRNA (White et al., 1973).

The 32 day old flies used in this study already show clear signs of aging, such as appreciably decreased vigor, as measured by the longer time taken to climb upward in a bottle after being forced to the bottom. Thus, this study was undertaken to see whether any major or minor tRNA spots can be seen to appear, disappear, or change as flies age. The tRNA populations from first- and third-instar larvae and newly-hatched adult Drosophila have previously been compared by RPC-5 chromatography to assay for developmental changes (White et al., 1973). Approximately one-third of the 99 detectable peaks showed some developmental alteration in that study.

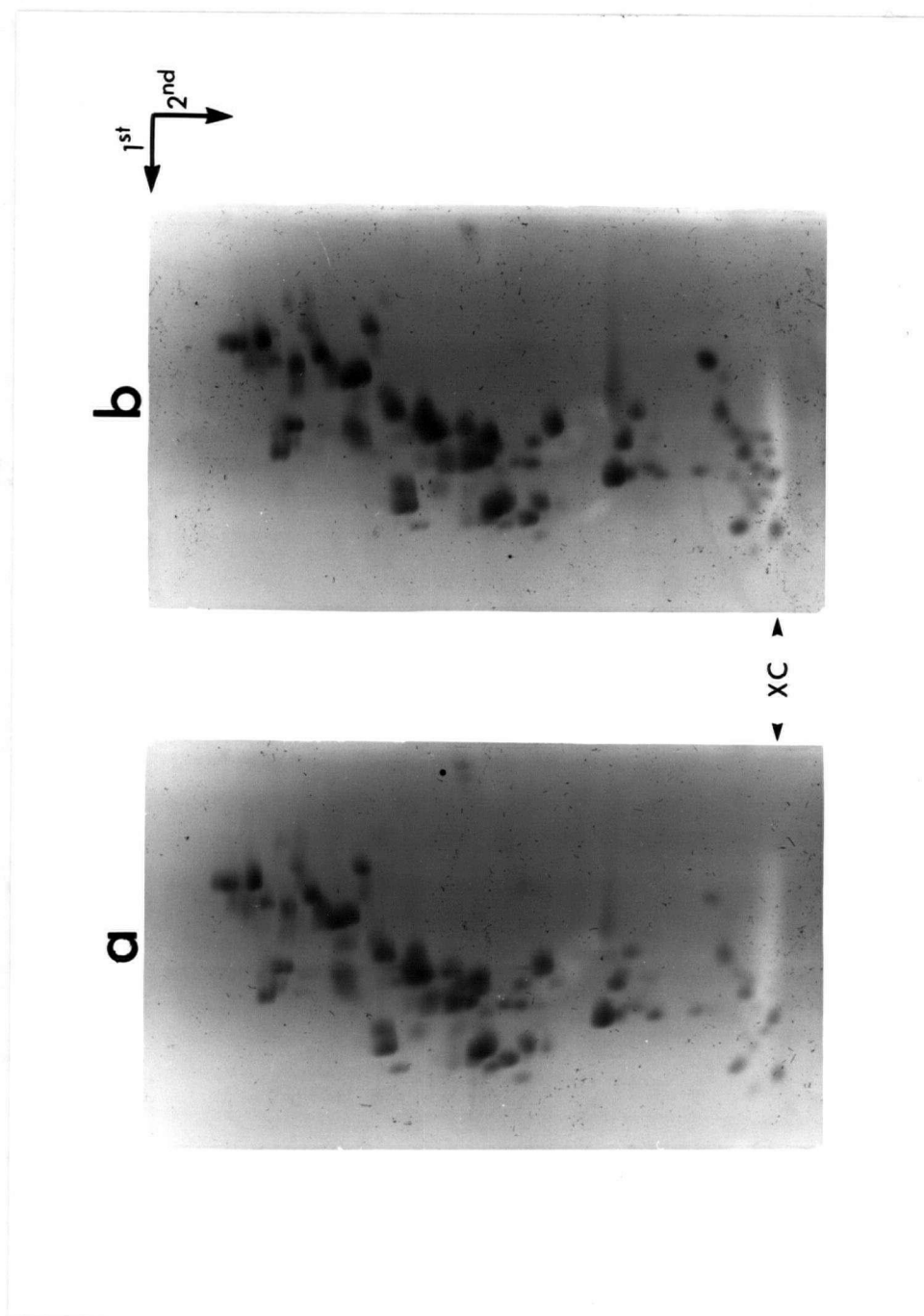
In the present study, it appears that the tRNA population remains essentially unchanged as the flies age, as assayed by two-dimensional PAGE (Figure 6). The lower limit of tRNA detection with these gels is estimated at less than 0.5 μ g, and the presence of more than 70 spots suggests that the majority of tRNAs are visualized here (bearing in mind that some spots overlap).

In the gel pair shown here, the isolate from aging flies has about four extra faint spots which run with the tRNA at the front of the sample (lower part of gel). These are not seen consistently however, and in other comparisons of tRNA from young and old flies this area of the gel is identical, and a few extra spots may appear among the larger tRNA which are not present here. The variable presence and position of these extra spots suggests they are probably artifacts resulting from degradation in some samples during tRNA isolation or handling, even though care was taken to prevent this.

Taken collectively therefore, the data from two-dimensional PAGE suggest that no appreciable change in tRNA population occurs.

Figure 6. Two-dimensional electrophoretic comparison of tRNA populations from newly-hatched and 32 day old Drosophila.

Entire tRNA populations from newly-hatched (part a) and 32 day old (part b) Drosophila melanogaster, isolated by Method I (Materials and Methods), were compared by two-dimensional PAGE. First dimension: 75 μ g of each of the two tRNA samples were electrophoresed in the same 1.5 mm thick 10% polyacrylamide / 4 M urea gel containing 1 μ g/ml EtBr. 450 V were applied for 19½ hours, and 700 V were then applied for 2 hours, all at 4°C. Second dimension: The lowest 12 cm of each lane were cut out, rotated through 90° and electrophoresed at room temperature using two matched 20% polyacrylamide / 4 M urea / 1 μ g/ml EtBr gels, at 550 V for 45 hours followed by 200 V for 7 hours. New xylene cyanol ff dye was added to each gel when the first aliquot of this dye reached the end of the gel, and each run was terminated when the second aliquot of the dye was 2 cm from the end of each gel (XC, shown in figure). Further staining for 10 minutes in an aqueous 1 μ g/ml EtBr solution was performed, and the lowest 23 cm of each gel was photographed and is presented here.



as Drosophila age, as assayed by this method.

Microheterogeneity between young and old tRNA populations would not be detected here. This is addressed to a certain extent, however, in the 5'-labelling studies which follow. In those studies, levels of RNA 1/100 - 1/1,000 times lower than in these spots should be detected, provided that the species is amenable to labelling at its 5'-end.

An interesting finding that emerged during these studies is that a much lower amount of tRNA seems to be available for isolation from aged flies than from young ones (typically about 60%). This figure is based on two isolations where the numbers of young and old flies were 102 in one experiment and about 200 in the other, both performed by the scaled-down version of Method I. In experiments using 1-10 gm of flies it also appeared that older flies yielded less tRNA, but these experiments were not as closely matched as to weight of young and old flies used.

It therefore appears either that older flies contain 40% less tRNA, or that tRNA is much less accessible for isolation in these flies. The former appears more probable, in view of the appreciable body tissue wastage and greatly reduced protein synthesis ability of older flies (Webster and Webster, 1979). However, even if the total amount of tRNA drops, it seems from these data that it is a fairly uniform drop, such that the tRNA pattern on two-dimensional PAGE remains relatively unchanged during aging.

v. 5'-Kinasing Studies of Young and Aged tRNA Populations

One possible inhibitor of protein synthesis in vivo was thought

to be tRNA with a few nucleotides removed from the 3'-end as discussed earlier, although the preceding in vitro data do not support this hypothesis. To assay for the presence of this partially degraded tRNA in vivo, radioactive 5'-labelling with polynucleotide kinase was employed.

The preferred substrate for both alkaline phosphatase and polynucleotide kinase is RNA or DNA with an exposed 5'-hydroxyl group; intact tRNA is generally a poor substrate, due to the omnipresent NCCA 3'-extension and the resulting steric hinderance. This is seen in the excellent removal of the 5'-phosphate and subsequent kinasing of a tRNA^{Arg} fragment missing five 3'-nucleotides, contrasted with the poor labelling of an intact tRNA^{Lys} species also present (Cribbs, 1982), as discussed. The 5'-terminus of the tRNA^{Arg} fragment had a one-nucleotide overhang, and gave a much darker band upon labelling than the intact tRNA^{Lys}, even though the latter was present in great excess.

Because of this differential labelling, a suitable assay for the buildup of partially 3'-degraded tRNA in aging Drosophila could be the appearance of tRNA bands which 5'-kinase intensely in the tRNA population of these aging flies. This is a sensitive assay, which should be able to detect picogram quantities of partially 3'-degraded tRNA. As well as being much more sensitive than two-dimensional PAGE of non-labelled tRNA, this is a more specific assay, in that it detects tRNA molecules with at least five nucleotides missing from the 3'-end in particular. Defects in the 3'-end may have an increased bearing on protein synthesis, due to the aminoacyl acceptance activity of this end.

a) Whole tRNA Extracts

The entire tRNA population from both newly-hatched and 30 day old Drosophila were treated with alkaline phosphatase and kinased as described, and then separated by 20% PAGE. The results are shown in Figure 7. Young tRNA isolated by both methods and old tRNA isolated by Method II are compared; it is seen that no prominent dark bands differentially appear in the tRNA fraction as flies age. The three bands which do appear slightly darker in the older population are of uncertain significance. They may be artifacts, but the middle one appears to correspond in position to a differentially labelling band from 25 day old HSS tRNA in Figure 8 (see below); the other two bands here do not appear to have similar correspondences. These bands may represent trace amounts of partially degraded tRNA molecules, but these very low amounts of defective tRNA would not be expected to affect protein synthesis, based on the preceding in vitro data. Elution and sequencing of the band which differentially labelled in the two independent experiments might provide information as to its origin and possible significance.

It should be noted at this point that the major differences between Methods I and II for tRNA isolation are the omission of the DEAE-cellulose chromatography step and the addition of 2% SDS and 1.5 M NaCl to the initial grinding buffer in Method II. The high salt concentration was added because large rRNA is relatively insoluble in high salt; with the absence of the DEAE-cellulose step, it was thought that this might decrease the relative amount of large rRNA applied to the Sephadex® G-100 column. However, for most purification

Figure 7. Comparison of 5'-kinasing susceptibility of tRNA from newly-hatched and 30 day old Drosophila.

Whole tRNA isolated by Method I from newly-hatched flies (lane a), by Method II from 30 day old flies (lane b), and by Method II from newly-hatched flies (lane c) was 5'-labelled with [γ - 32 P]ATP using alkaline phosphatase and polynucleotide kinase as described. A 0.4 μ g aliquot of each sample was electrophoresed using a 0.4 mm thick 20% polyacrylamide / 4 M urea / 1 μ g/ml EtBr gel, at room temperature, 1,000 V, for 22 hours. The gel was autoradiographed at 4°C for 3 days. The lower 25 cm of this gel is shown in the figure. Closed triangles (\blacktriangleright) mark the boundaries of the size range occupied by whole Drosophila tRNA; open triangles (\triangleright) delimit the size range of tRNA degraded by snake venom phosphodiesterase as described; arrowheads (\blacktriangleright) show the positions of the three bands differentially seen in tRNA from 30 day old flies (lane b), with a double arrowhead marking the extra band which also appears in Figure 8, lane b.

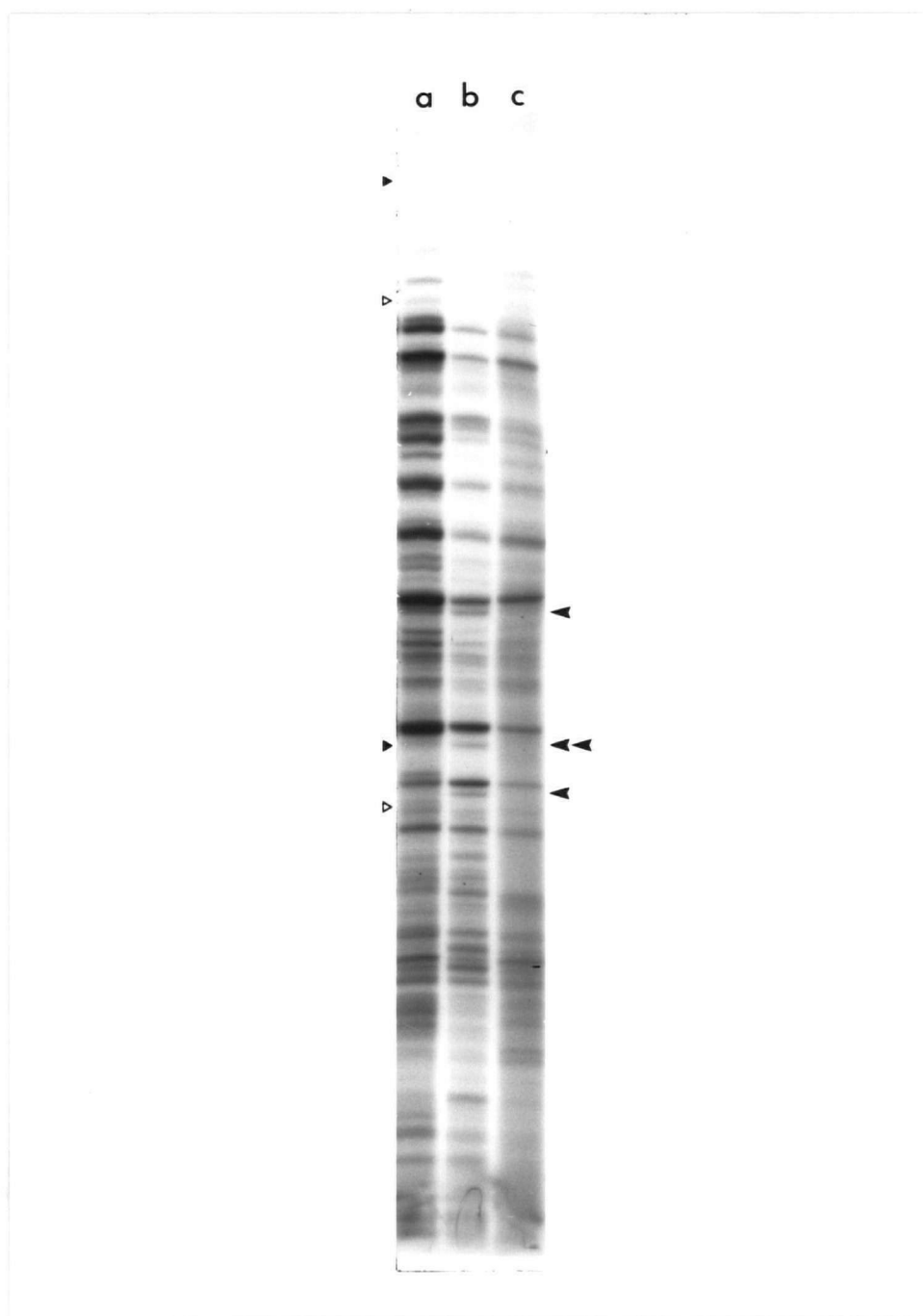
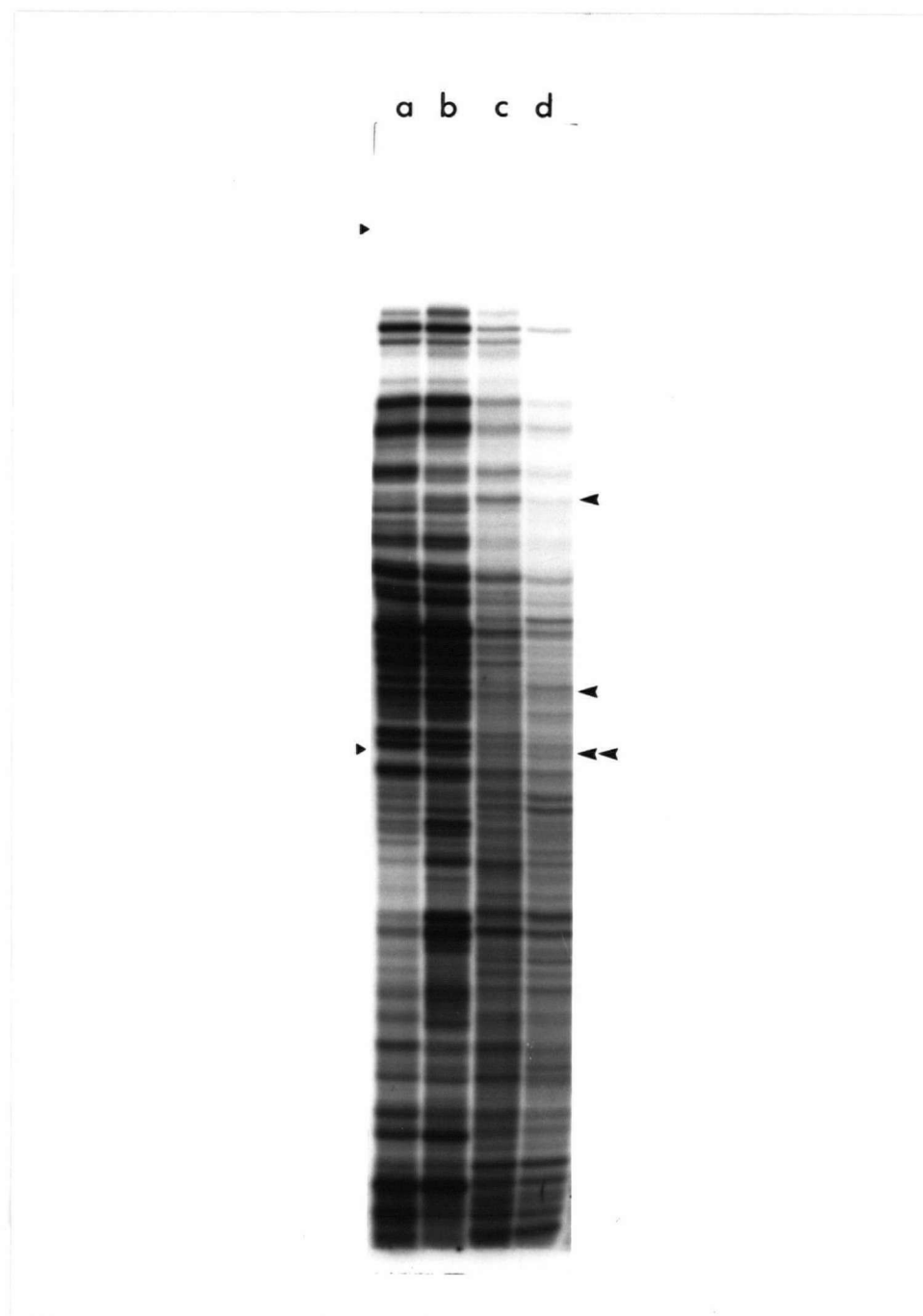


Figure 8. Comparison of 5'-kinasing susceptibility of ribosome-associated (HSP) vs. free (HSS) tRNA from newly-hatched and 25 day old Drosophila.

Susceptibility to 5'-kinasing was studied for tRNA populations derived from the high speed supernatant (HSS) centrifugal fraction of young (lane a) and 25 day old (lane b) Drosophila extracts, and from the high speed pellet (HSP) fraction of young (lane c) and 25 day old (lane d) extracts. The tRNA samples were isolated and 5'-labelled as described. A 0.4 ug aliquot of each sample was electrophoresed and autoradiographed as described in Figure 7. The lower 25 cm of this gel is shown in the figure. Closed triangles (▴) mark the boundaries of the size range occupied by whole Drosophila tRNA; arrowheads (▸) mark the positions of the three bands seen in 25 day old (lane b) but not young (lane a) HSS tRNA, with a double arrowhead marking the band which also appears in Figure 7, lane b.



experiments, it was found that relatively less high molecular weight material was obtained during gel filtration where a DEAE-cellulose column had been used first. For these labelling experiments, tRNA which had not been passed through DEAE-cellulose was included (i.e., Method II tRNA), in order to obtain a tRNA sample with the least chance of removal of any potentially interesting components in the tRNA size range by ion exchange chromatography.

b) Free and Ribosome-Associated tRNA

It has been found that the aminoacyl acceptance capacity of ribosome-bound tRNA from old rat liver is about 64% of that from young specimens (Mays et al., 1979; Lawrence et al., 1979). Thus, a kinasing study was done on tRNA from a salt-wash of young and 25 day old ribosomes (HSP tRNA), as well as relatively ribosome-free HSS tRNA from these two groups. If old ribosome-bound tRNA has 36% lower acceptance due even partially to appreciable 3'-degradation, it should, in theory, kinase very efficiently.

The results of the study, given in Figure 8, show that aged ribosome-bound tRNA does not appear to kinase significantly better than young ribosome-bound tRNA. It seems likely from this result that extensive 3'-degradation does not exist among the ribosome-associated HSP tRNA of aged flies, and if reduced aminoacyl acceptance were to be seen in this tRNA fraction, as in aged rat liver, it would have some other cause. Furthermore, there are no major differences between the young and old HSS tRNA labelling profiles. Three bands do appear slightly darker in the old HSS tRNA fraction, but only one of these apparently coincides with a differentially dark band

from aged whole tRNA extracts (as described above). However, this band is of similar intensity between the young and old HSP tRNA fractions. Thus, although the band has appeared darker in the old tRNA fraction in two independent experiments (Figures 7 and 8), it does not seem sufficiently intense to be a probable inhibitor of protein synthesis if, in fact, it is a partially degraded tRNA, based on the results of the in vitro studies done.

Furthermore, dramatic age-related changes are also absent from the labelling patterns of RNA fragments smaller than the tRNA size range. The largest difference is seen in RNA smaller than tRNA from HSS (Figure 8a and b), with about four bands in the 50-65 nucleotide range significantly darker in the 25 day old sample. This difference is not seen in the young vs. old whole tRNA labelling experiment however (Figure 7), and was not seen consistently in HSS electrophoresis; it is therefore probably an artifact of this particular electrophoresis experiment.

Overall, these data hint at possible alterations in the 5'-labelling pattern between young and old tRNA populations, but no prominent differences are seen. Defective tRNA molecules with exposed 5'-ends label many times more efficiently than intact tRNA, as a rule, making it unlikely that any minor changes in band intensity seen here are indicative of defective tRNA molecules abundant enough to significantly alter protein synthesis in vivo.

Part II

I. INTRODUCTION

The tRNA molecule forms the vital link which matches amino acids with their correct triplet codons in the mRNA during protein synthesis. The range of this function is attested to by the presence of at least 63 major and 39 minor chromatographically distinct tRNA species in Drosophila melanogaster (White et al., 1973). These are thought to be encoded by approximately 600-750 genes (Ritossa et al., 1966; Tartof and Perry, 1970; Weber and Berger, 1976). Bearing in mind that the majority of these minor tRNA species are thought to differ only in post-transcriptional modification, not in genetic origin, this gives an estimate of 10-13 copies for each tRNA gene contained in the Drosophila genome.

When total 4S RNA (containing the entire complement of tRNA) is hybridized in situ to Drosophila polytene chromosomes, it is seen to derive from at least 54 sites: 26 strong sites, and 28 weaker ones (Elder et al., 1980). Only one major site is found on the X chromosome (at 12E), and the rest are scattered seemingly at random throughout both arms of the two large autosomes; no tRNA sites are observed on the small autosome. However, it is now known that this procedure failed to detect some gene sites, such as the 19F site of tRNA^{Arg} (Newton, unpublished).

Cloning and sequencing of regions rich in tRNA genes has elucidated some of the general features of their chromosomal organization. The largest region examined by these techniques to date is a 94 kb sequence at 42A on the right arm of chromosome 2, containing a total

of 18 genes for tRNA^{Asn}, tRNA^{Arg}₂, tRNA^{Lys}₂, and tRNA^{Ile}, within 46 kb (Yen and Davidson, 1980). A second site at 90BC has also been studied, and is seen to contain 10 or 11 tRNA genes within 31 kb (DeLotto and Schedl, 1984). These include 6 sequenced genes for tRNA^{Val}, tRNA^{Pro}, tRNA^{Ala}, and tRNA^{Thr}, with other apparent tRNA genes present in this area not yet sequenced. Sequencing studies of these major tRNA hybridization sites, as well as smaller-scale endeavours (summarized by Leung, 1988) have shown that these sites can contain a number of genes for various unrelated tRNA species, that the genes are present in either transcriptional orientation, and that identical genes for a given tRNA isoacceptor can be found at more than one chromosomal hybridization site.

It has been found that approximately 20% of yeast tRNA genes contain introns, with the vast majority of these eukaryotic introns found thus far occurring one base pair 3' to the anticodon (Johnson and Abelson, 1983). Exceptions to this position do exist, however (Del Rey *et al.*, 1982). For Drosophila, only eight such genes have been found to date, suggesting that intron-containing tRNA genes may be somewhat less abundant in this species. Two closely-spaced Drosophila tRNA^{Leu}_{UUG} genes which derive from site 50AB (chromosome 2R) contain introns which are nearly homologous, and are 38 and 45 bp in length (Robinson and Davidson, 1981). These two genes are otherwise identical. Further, six out of eight otherwise identical Drosophila tRNA^{Tyr}_{UAC} genes contain introns of varying size (20-113 bp) and sequence (Choffat *et al.*, 1988).

A functional role in anticodon base modification seems apparent for those eukaryotic introns studied. For example, it has been found

that deletion of the intron from a yeast tRNA^{Tyr} ochre suppressor gene resulted in a lack of post-transcriptional modification of the middle nucleotide of the anticodon to Ψ, causing the in vivo suppressor activity of this tRNA to fall dramatically (Johnson and Abelson, 1983). Similarly, deletion of the Drosophila and Xenopus tRNA^{Tyr} introns prevents modification of the middle position of the anticodon to Ψ (Choffat et al., 1988). Further, loss of the intron from a yeast amber suppressor tRNA^{Leu} gene prevented modification of cytosine in the wobble position of the anticodon to 5-methylcytosine, leading to a reduction of suppressor activity (Strobel and Abelson, 1986).

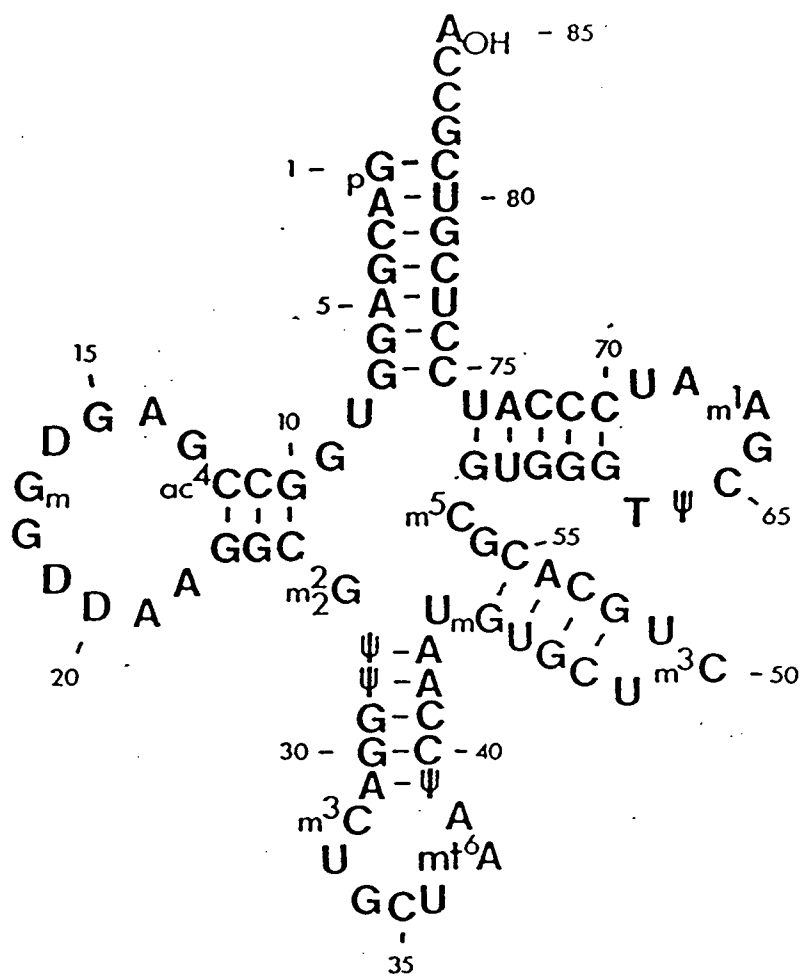
In our laboratory, a study has been undertaken of the Drosophila major isoacceptor tRNA₄^{Ser} (codon UCG) and tRNA₇^{Ser} (UCA, UCC, UCU) genes at their most prominent site 12DE on the X chromosome, and at the three minor sites 23E on chromosome 2L, 56D on chromosome 2R, and 64D on chromosome 3L (Hayashi et al., 1980; Cribbs et al., 1987b; Leung, 1988; Dr. D. Sinclair, unpublished). These two isoacceptors differ from each other at only three positions: #16, 34, and 77 (#34 being the wobble position of the anticodon), giving 96% homology between these two distinct isoacceptors which utilize different codons. This is unusual, as functionally distinct tRNA isoacceptors generally show 10 to 30% sequence divergence (Sprinzl et al., 1987), as is the case with tRNA_{2b}^{Ser} (AGC, AGU) which only shows 71 to 73% homology to tRNA_{4,7}^{Ser} (Cribbs et al., 1987a). Thus, tRNA₄^{Ser} and tRNA₇^{Ser} are thought to exhibit a high degree of concerted evolution.

In total, twelve tRNA_{4,7}^{Ser} genes have been cloned and sequenced thus far: Five for tRNA₇^{Ser}, three for tRNA₄^{Ser}, three genes that consist of hybrids between the tRNA₄^{Ser} and tRNA₇^{Ser} sequences, and

one copy of tRNA₄^{Ser} showing microheterogeneity with a C to T transition at position 50. This study has illuminated interesting features of tRNA gene organization and evolution, including the concerted evolution discussed above, the suggestion that recombination occurs between non-allelic tRNA genes giving rise to the observed hybrid gene structures, and the observation that mutations can accumulate at sites which are thought to be selectively neutral in tRNA genes which are otherwise highly conserved. Other examples of this microheterogeneity are found in Drosophila, with putative gene sequences which differ from corresponding tRNA sequences at 1 to 6 nucleotides being seen for tRNA₁^{Met} (Sharp et al., 1981), tRNA_{3b}^{Val} (Leung et al., 1984), tRNA₄^{Val} (Addison et al., 1982), tRNA₅^{Lys} (DeFranco et al., 1982), and tRNA^{Glu} (Hosbach et al., 1980). The relative transcriptional activity for each of these allogenes in vivo is not known; however, all of the genes whose activities have been assayed using various in vitro systems have shown activity in such systems (Leung et al., 1984).

The purpose of the present study was to extend this knowledge of the tRNA^{Ser} genes in D. melanogaster. The tRNA_{2b}^{Ser} isoacceptor has previously been highly purified by BD-cellulose / Sepharose® 6B / RPC-5 chromatography (Hayashi et al., 1982), and sequenced in our laboratory (Cribbs, 1982; Cribbs et al., 1987a). The sequence and cloverleaf structure of this molecule is given in Figure 9. This tRNA responds to the codons AGC and AGU, and is thus functionally distinct from both tRNA₄^{Ser} and tRNA₇^{Ser}.

By making use of this RNA sequence information, two DNA oligonucleotide probes were constructed and used to detect the tRNA_{2b}^{Ser}.



tRNA^{Ser}_{2b}

Figure 9. Cloverleaf structure of tRNA^{Ser}_{2b} [from Cribbs, 1982].

gene among fragments of the Drosophila genome selected by size. One such gene was isolated and sequenced. It is to be used in the future to study the distribution, organization, and architectural features of the tRNA^{Ser}_{2b} branch of the tRNA^{Ser} gene family in D. melanogaster.

In addition, one of these probes detected two other genes: A putative tRNA^{Ser}_{UCA} gene lacking correspondence to any tRNA characterized to date, and a tRNA^{Leu}_{CUG} gene which appears to correspond to tRNA^{Leu}₂, according to in situ hybridization results.

II. MATERIALS AND METHODS

E. coli strain DH5 α [F⁻, recA1, endA1, gyrA96, thi-1, hsdR17(r_k⁻, m_k⁺), supE44, λ ⁻, relA1, ϕ 80dlacZ Δ M15 (Leung, 1988)] was a gift from J. Leung. Cloning vector pUC 13 (Viera and Messing, 1982) was a gift from N. Seto. The tRNA_{2b}^{Ser} specific oligonucleotides GT8 and GT9 were synthesized by T. Atkinson in the laboratory of M. Smith. Sources of enzymes, chemicals, and Drosophila are as specified in Part I of this thesis.

i. Isolation of Genomic DNA

High molecular weight genomic DNA was obtained from adult Drosophila melanogaster (Ore R) by the method of McGinnis and Beckendorf (1983), as modified by J. Leung in our laboratory (Leung, 1988). Yields were typically in the range of 0.5 mg/gram of flies. This DNA was taken up in 0.5 ml TE [10 mM tris pH 8.0 (HCl) / 1 mM EDTA] and heated at 65°C for 30 to 60 minutes to aid dissolution. The genomic DNA was assessed by electrophoresis using 0.3% agarose gels (described below), which fractionate the 60 to 5 kb DNA size range (Maniatis et al., 1982). Genomic DNA isolated by this method was found to occupy a size range \geq 50 kb.

Before digestion with restriction endonucleases, this DNA was treated to remove contaminating RNA as follows: Pancreatic ribonuclease (Sigma, 95% type A / 5% type B), dissolved in TE at a concentration of 10 mg/ml, was added to a final concentration of 120 μ g/ml. This was incubated for 2-3 hours at 37°C. The DNA was then precipitated

by addition of 0.6 volumes of a 20% polyethylene glycol / 2.5 M NaCl solution and incubation on ice for no more than 15 minutes. After centrifugation and dessication, the genomic DNA was taken up in half the volume of TE it had previously occupied, to allow for dilution during restriction enzyme digestions.

ii. Agarose Gel Electrophoresis

Agarose submarine gels of the desired concentration (0.3% - 2.0%) were poured, allowed to set, and run in 0.5 x TBE, with EtBr variously present at 0.5 µg/ml in either the gel, the buffer, or both. A table of DNA fractionation ranges is given in Maniatis et al., (1982). Mini gels (10 cm long) were run at 100 V, generally for 1-2 hours. Large gels (25 cm long) were usually run at 50 V, 19 to 24 hours for a typical 0.9% gel. DNA was visualized and photographed using short wavelength (254 nm) ultraviolet light, except as noted.

iii. Restriction Endonuclease Digestion

Genomic DNA treated for RNA removal as described was digested with the restriction endonucleases HindIII, EcoRI, or PstI (Pharmacia) as follows: The reactions typically contained 0.3 mg/ml genomic DNA, 5 U enzyme/µg of genomic DNA, 100 µg/ml BSA, and 1 x Pharmacia H (EcoRI) or M (HindIII and PstI) buffers. This was incubated at 37°C for 3 to 5 hours, or, in the case of more delicate enzymes such as PstI, incubated 1 to 2 hours and given a repeat aliquot of the enzyme before further digestion for 1 to 2 hours. The digested DNA was extracted with 1:1 phenol/chloroform and ethanol/NH₄OAc precipitated. Plasmid DNA was digested less vigorously, with 3 U enzyme/µg

of DNA, 0.1 mg/ml DNA, and 2 hours incubation.

Such vigorous digestion conditions, with 2 to 3-fold excess of enzyme over that required for plasmid digestion, and significantly longer digestion times, appeared necessary for genomic DNA digestion. This achieved fairly complete digestion, as assayed by 0.6 to 0.9% agarose gel electrophoresis, whereas less vigorous conditions only gave partial digestion. Thus, a compromise was made between relatively complete digestion, and the risk of damage to single-stranded ends by any contaminating exonucleases.

iv. Genomic Southern and Filter Hybridizations

Genomic DNA digested with restriction endonucleases was electrophoresed using large 0.7% agarose gels as described, loading 20 μ g per lane. The electrophoresed DNA was denatured and neutralized by standard procedures (Maniatis *et al.*, 1982), and transferred to Hybond®-N (Amersham) according to the manufacturer's instructions.

The filter was prehybridized in a solution of 6 x SSC, 5 x Denhardt's solution, and 0.2% SDS, at 60 μ l/cm² of filter, for 3 to 18 hours, at 37 to 50°C, in a sealed bag.

The prehybridization solution was then poured off and the hybridization solution added at 40 μ l/cm² of filter. This solution consisted of 6 x SSC, 50 mM NaH₂PO₄ pH 7 (HCl), 20 μ g/ml *E. coli* tRNA, 5 x Denhardt's solution, 0.5% SDS, and 4 pmol oligonucleotide/ml hybridization solution, 5'-labelled with [γ -³²P]ATP as described below. Hybridization was carried out in a sealed bag overnight at 50 to 55°C.

The following day, the filter was washed with five changes of 6 x SSC / 0.1% SDS. The first three washes were done at room tempera-

ture, and the last two were done in a water bath heated to the specified temperature, for at least 15 minutes each. The washing solution was preheated to the appropriate temperature for these latter washes. The washed filter was autoradiographed at -70°C , using an intensifying screen, for 3 to 10 days.

v. Purification of DNA Fragments of Selected Size

Genomic DNA digested with the desired restriction endonuclease was electrophoresed on large 0.9% - 1.0% agarose gels with EtBr present, covering to exclude fluorescent light. Four or five lanes of 20 μg DNA each were run, and the DNA was viewed with long wavelength (366 nm) ultraviolet light, as briefly as possible. A gel slice 6-7 mm wide containing DNA of the desired size was excised. The DNA was electroeluted (Maniatis et al., 1982) in 0.5 x TBE using Spectropor.1 dialysis tubing, at 100 V for 30 minutes to 1 hour depending on the size of the DNA fragments. Following electroelution, the current was reversed and a 30 second pulse of 100 V was applied. The eluant was removed under subdued light, extracted with butanol until the volume was approximately 200 μl , extracted with 1:1 phenol/chloroform, and twice precipitated with ethanol/ NH_4OAc . The pellet was taken up in 20 μl TE, giving the DNA fragments an approximate concentration of 0.1 $\mu\text{g}/\mu\text{l}$.

vi. Oligonucleotide Preparation and Radioactive Labelling

Two synthetic oligonucleotides were used both to screen for, and to sequence the cloned tRNA genes. GT8 consists of nucleotides #1 to 23 of tRNA^{Ser}_{2b}, and GT9 complements nucleotides #62 to 82 of

the same tRNA (see Figure 9). For purification, the crude oligonucleotide was electrophoresed for 3 hours at 1,600 V in a 20% acrylamide / 7 M urea, 0.5 mm thick polyacrylamide gel as described in Part I. The desired band was excised and eluted by soaking overnight in a minimal quantity of 0.5 M NH_4OAc / 10 mM MgOAc . The eluant was filtered through siliconized glass wool and loaded onto a C_{18} Sep-Pak® column. The column was washed with 5 ml H_2O , and the oligonucleotide was then eluted with 20% HPLC grade acetonitrile. The first 2 ml of the acetonitrile wash was retained, dried $2\frac{1}{2}$ hours in a Savant Speed Vac® apparatus, taken up in H_2O at a concentration of 0.02 $\mu\text{g}/\mu\text{l}$, and stored at -70°C .

For hybridization experiments, the oligonucleotides were labelled at the 5'-end with [γ - ^{32}P]ATP and polynucleotide kinase. The reaction contained 40 pmol oligonucleotide, 40 mM tris pH 8.0 (HCl), 9 mM MgCl_2 , 10 mM DTT, 100 μCi [γ - ^{32}P]ATP (250 μCi / 25 μl / 87 pmol, New England Nuclear), and 1 μl polynucleotide kinase at 5-10 U/ μl (Pharmacia), in a volume of 20 μl . This was incubated 45 minutes at 37°C , and heated to 65°C for 10 minutes to inactivate the enzyme. This labelling mix was added directly to 10 ml hybridization fluid and used without further purification.

vii. DNA Ligation, Bacterial Transformation, and Colony Screening

pUC 13 (Viera and Messing, 1982) was used as the vector for cloning genomic fragments containing tRNA genes. The vector, propagated in E. coli strain DH5 α (Leung, 1988), was purified by equilibrium centrifugation in CsCl/EtBr as described below. It was digested with the appropriate restriction endonuclease, and the 5'-phosphate

groups were removed by adding approximately 1 U/ μ g calf intestinal alkaline phosphatase (Boehringer Mannheim, 19 to 25 U/ μ l), and incubating at 37°C for an additional 30 minutes. This limits recircularization of the vector during ligation.

Ligation efficiency was found to show extreme variation according to the relative concentrations of vector, insert, and especially enzyme. Levels found to be effective were as follows: 50-100 ng vector DNA, 20-100 ng insert (genomic DNA fragments purified as described), and 1.5 - 4 U T4 DNA ligase (Pharmacia, 7.5 U/ μ l), in a volume of 20 μ l. Optimum levels within these ranges varied between experiments. Ligation was carried out in 66 mM tris pH 7.6 (HCl), 6.6 mM MgCl₂, 10 mM DTT, 0.4 mM ATP, 10% polyethylene glycol, and 150 mM NaCl. The reaction was incubated at 4°C overnight (12-20 hours).

E. coli [strain DH5 α (Leung, 1988)] was used for transformation by the ligated DNA. Bacterial cells were made competent and transformed by the high efficiency method of Hanahan [Table 7 of Hanahan (1985)]. The cells could be frozen in 210 μ l aliquots at -70°C for a few months without appreciable loss of efficiency. The entire 20 μ l of ligation mix was used to transform a 210 μ l aliquot of competent cells. The ligation and transformation efficiency was such that at least half, and sometimes all of the transformed cell mix was plated per Petri dish. This was done by briefly centrifuging the cell mix, resuspending the pellet in 100-200 μ l SOB (Hanahan, 1985), and plating this on one or two 85 mm plates. Ampicillin at 50 μ g/ml was included in the plating medium to select against non-transformed cells. IPTG and X-Gal were added to the plates to provide an indication of the

number of transformants containing cloned inserts (Leung, 1988). Typical yields were 100-600 white colonies (generally containing plasmids with inserts), and 100-300 blue colonies (containing uncut or recircularized plasmids) per plate. This corresponds to a transformation efficiency of approximately $1 \times 10^4/\mu\text{g}$ DNA; cells transformed with uncut pUC 13 by this method gave an efficiency two to three orders of magnitude higher.

Duplicate colony lifts of each plate were made, using 82 mm Hybond®-N discs (Maniatis et al., 1982). The DNA was denatured with 1.5 M NaCl / 0.5 M NaOH for 5 minutes, neutralized with 1.5 M NaCl / 1.0 M tris pH 7.5 (HCl) for 2 x 5 minute intervals, and bonded to the filters by irradiation with 254 nm wavelength ultraviolet light for 4 minutes. Prehybridization, hybridization, and washing were carried out as described. Ten ml of hybridization fluid was used for up to 16 filters. Autoradiographs were exposed at -70°C with an intensifying screen for 2 to 4 days.

viii. Plasmid Isolation and Purification for Sequencing

Small-scale plasmid isolations were done by the alkaline lysis procedure of Maniatis et al., (1982). Plasmid DNA prepared by this method, and treated with ribonuclease and polyethylene glycol as described for genomic DNA, was sufficiently clean for restriction digests and agarose gel electrophoresis.

However, for sequencing, further purification by equilibrium centrifugation through CsCl gave the best results. Plasmid DNA was obtained from 250 ml of 2 x YT medium containing 50 $\mu\text{g/ml}$ ampicillin, by a scaled-up version of the above isolation procedure. After KOAc

precipitation, 0.7 volume of isopropanol was added to the supernatant to precipitate the nucleic acids. The pellet was taken up in 10 mM tris pH 7.5 (HCl), 10 mM EDTA, 0.84 g/ml CsCl, and 0.6 mg/ml EtBr, and centrifuged in a Beckman vTi65 rotor at either 65,000 rpm for 4 hours or 55,000 rpm for 17 hours. This was repeated, and the purified plasmid DNA was then extracted four times with butanol, and ethanol/ NH_4OAc precipitated three times. The final pellet was taken up in 0.5 ml TE to give 3-4 mg/ml of highly purified DNA.

ix. DNA Sequencing

Double-stranded plasmid DNA, which had been purified by CsCl centrifugation as described, was used as the template for sequencing. For each sequencing reaction, approximately 20 μg DNA was denatured in 20 μl of 0.15 M NaOH at room temperature for 10 minutes, and ethanol/ NH_4OAc precipitated.

Sequencing was carried out by the dideoxynucleotide termination method (Sanger et al., 1977), using two different procedures. The majority of sequencing was performed using the Sequenase kit (U.S. Biochemicals), by the protocol supplied with the kit. This was found to give somewhat clearer data than sequencing done with the Klenow fragment of DNA polymerase; the latter was used, however, where it was desirable to read sequences very close to the primer, and to support data generated by the Sequenase method.

The GT8 and GT9 oligonucleotides purified as described were used as primers to sequence all cloned tRNA genes in both directions wherever possible, with both sequencing methods.

For sequencing using the Klenow fragment (Newton, 1984), 16 ng

oligonucleotide was annealed to the denatured DNA in 50 mM tris pH 7.5 (HCl) / 10 mM NaCl / 10 mM MgCl₂, in a volume of 8 μ l, by heating to 75°C for 5 minutes and cooling gradually to 37°C. To this annealed DNA was added 2 μ l 15 μ M dATP, 3 μ l [α -³⁵S] dATP (New England Nuclear, 250 μ Ci / 20 μ l / 190 pmol), and 5 μ l of a premix consisting of 1 mg/ml BSA, 100 mM K₂HPO₄ pH 7.5, 10 mM DTT, and the Klenow fragment of DNA polymerase (Pharmacia, 5.0 - 7.5 U/ μ l) at 0.5 - 1.0 U/ μ l. 3 μ l of this mix was dispensed per G, A, T, and C reaction, and extension was started by adding 1 μ l of the appropriate ddNTP/dNTP mix (Appendix). This was incubated at 37°C for 10 minutes; 1 μ l of a solution containing 0.5 mM in each of dGTP, dATP, dTTP, and dCTP was added to each reaction, and a further incubation of 37°C for 10 minutes followed. Finally, 6 μ l of a solution of 10 mM EDTA, 0.1% xylene cyanol ff, and 0.1% bromophenol blue in 98% deionized formamide was added to stop the reactions. The samples were either electrophoresed immediately after heating to 75°C for 3 minutes, or frozen at -70°C for up to two weeks, heated as above, and electrophoresed.

Electrophoresis was performed using 6% polyacrylamide / 7 M urea wedge gels, mixed as described in Part I, with minor modifications: 0.08% TEMED was used, and the gels were run at 1,600 V for just over 1 hour, until the bromophenol blue dye was 1 cm from the end of the plates. The wedge was constructed by using 0.4 mm thick spacers along the entire length of the gel, and then adding two extra 0.3 mm thick spacers, 11 cm and 5 cm long, at the bottom of the gel. 2-3 μ l of sample was loaded in each 5 mm wide slot. Sequencing gels were fixed in 10% acetic acid / 12% methanol for 45 minutes to remove urea, and dried under vacuum for 1 hour at 80°C. Autoradiography

took place with the film in direct contact with the dried gel, at room temperature, for 1 to 3 days.

III. RESULTS AND DISCUSSION

i. tRNA^{Ser}_{2b} Gene Localization

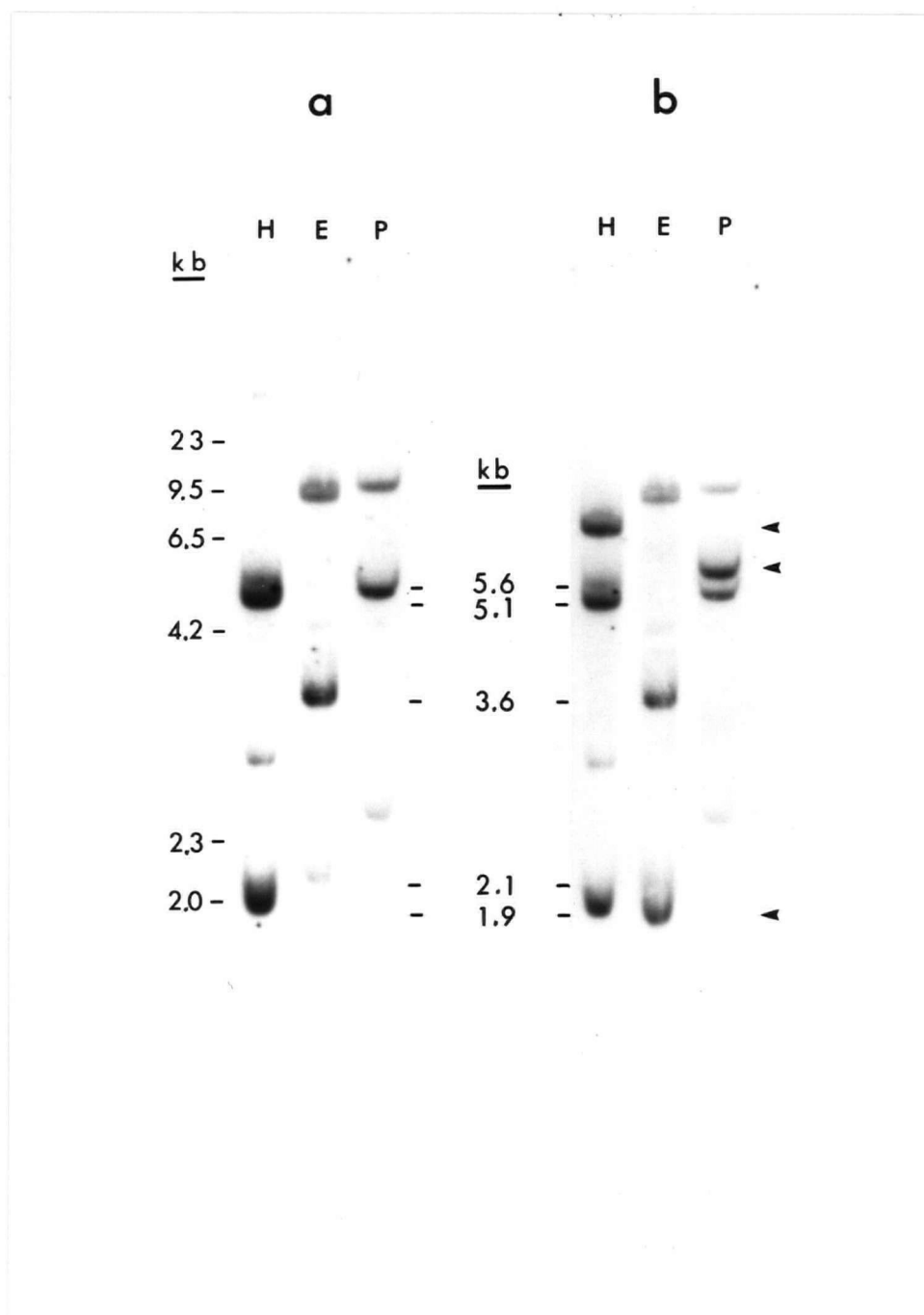
tRNA^{Ser}_{2b} was previously highly purified (Hayashi et al., 1982) and sequenced (Cribbs, 1982; Cribbs et al., 1987a) in our laboratory. The sequence of this molecule is shown in Figure 9. From the known sequence of this tRNA, two oligonucleotide probes were synthesized: GT8, a 23-mer identical to the 5'-end, with the sequence p-GACGAGGTGGCCG AGTGGTTAAG, and GT9, a 21-mer complementary to the 3'-end, with the sequence p-CGACGAGGATGGGATTGGAAC.

These probes were used to identify the genomic restriction fragments likely to contain copies of the tRNA^{Ser}_{2b} gene. Genomic DNA from Drosophila melanogaster (Ore R) was digested to completion with the restriction endonucleases HindIII, EcoRI, and PstI, and probed with the above oligonucleotides (Figure 10). When GT9 was used as the probe (Figure 10a), three major bands were seen in each lane; these were of dark, medium, and light intensity. For the hybridization using GT8 as a probe (Figure 10b), these same three bands were seen, and an extra dark band also appeared in each lane. This band must contain either a fragment of tRNA^{Ser}_{2b} from the 5'-end which lacks any homology to GT9, or a sequence very closely related by chance to GT8.

In situ hybridization studies (Hayashi et al., 1982) show purified tRNA^{Ser}_{2b} binding to three sites in the polytene chromosomes: It binds

Figure 10. Genomic Southern analysis of tRNA^{Ser}_{2b} genes.

Drosophila genomic DNA was digested with HindIII (lane H), EcoRI (lane E), and PstI (lane P) and electrophoresed using a large 0.7% agarose submarine gel at 50 V for 16 hours. The DNA fragments were transferred to Hybond-N and probed with the tRNA^{Ser}_{2b}-derived oligonucleotides GT9 (part a) and GT8 (part b), which were 5'-labelled as described. Washing was done in 6 x SSC / 0.1% SDS, at 63°C for GT8 and at 60°C for GT9. Autoradiograms were exposed at -70°C with an intensifying screen for 10-11 days. Size markers are derived from a HindIII restriction digest of lambda DNA, and are indicated at the left of the figure in kilobasepairs. Positions corresponding to regions which were targeted for cloning are indicated by size (kilobasepairs) in the middle of the figure. Arrowheads mark the three bands which hybridize to GT8 but not to GT9.



with similar intensity at sites 86A and 88A, and with slightly increased intensity at site 94A, all on the right arm of chromosome 3. These results suggest the presence of at least three or four (or multiples thereof) copies of the $\text{tRNA}_{2b}^{\text{Ser}}$ gene in the genome.

ii. Cloning of $\text{tRNA}_{2b}^{\text{Ser}}$, $\text{tRNA}_{\text{UCA}}^{\text{Ser}}$, and $\text{tRNA}_{\text{CUG}}^{\text{Leu}}$

The data obtained from genomic Southern blots was used to locate the size ranges of genomic digests to target and purify when attempting to clone the $\text{tRNA}_{2b}^{\text{Ser}}$ gene. When DNA is purified in this way before attempted cloning, the chances of cloning the desired gene are greatly increased, as most extraneous genomic fragments are eliminated by this procedure. Based on the ratio of the size of gel slice taken to the length of the entire track of electrophoresed DNA, by a very crude estimate the gene in question would be purified approximately 25-fold using this procedure. Thus, theoretically, to clone a specific 4 kb fragment from the 1.4×10^8 kb Drosophila genome by this method, an estimated 6,500 colonies would have to be screened to achieve a 99% probability of success (Kaiser and Murray, 1985). However, by this calculation, even screening 3,000 colonies would give an estimated 88% probability of success.

Initially, the gel slice containing EcoRI fragments of 1.9 to 2.1 kb, and that containing 3.6 kb fragments were cut out, and the associated DNA was electroeluted and cloned (see Figure 10). A total of 2,700 white colonies containing the 1.9 - 2.1 kb fragments were screened, but no tRNA genes were found. This is not surprising in retrospect, as the choice to clone this region was based on early

genomic Southern probes only with GT8. It was later found that the strong band at 1.9 kb does not, in fact, contain an intact tRNA^{Ser}_{2b} gene, as discussed earlier. One might have expected to find tRNA^{Ser}_{2b} in the faint 2.1 kb region which hybridizes to both GT8 and GT9, but since only 2,700 colonies were available for screening, such a tRNA sequence could have been missed by chance.

Very interesting results were obtained from the cloning of the 3.6 kb EcoRI region, however. A total of 4,900 white colonies derived from this region were screened using GT8, and two putative tRNA genes were cloned: a tRNA^{Leu}_{CUG}, and a tRNA^{Ser}_{UCA}. Neither of these two tRNA genes has close homology to tRNA^{Ser}_{2b}. tRNA^{Leu}_{CUG} was sequenced fully, and has only sporadic homology to tRNA^{Ser}_{2b}, other than an 18 nucleotide exact match through the TΨC arm [#56 to 73 of tRNA^{Ser}_{2b} (#57 to 74 of tRNA^{Leu}_{CUG}), Figure 11a and c]. At the 5'-end of tRNA^{Leu}_{CUG} only 16/23 bp match GT8 (the tRNA^{Ser}_{2b} probe that picked it out during screening), but a block of 12 homologous nucleotides in this region must have been sufficient to bind GT8 under the relatively less stringent conditions used (discussed below).

Only the 3'-portion of tRNA^{Ser}_{UCA} could be sequenced without further subcloning, as GT9 did not bind to this gene well enough to permit sequencing in the 5'-direction (only 14 to 16/21 bp homology with GT9, with no blocks of homology to this probe longer than 8 to 10 bp, Figure 11b). Of the region of tRNA^{Ser}_{UCA} that was sequenced, a 66 to 71% homology to tRNA^{Ser}_{2b} was found. These results suggest that the 5'-end of tRNA^{Ser}_{UCA} is probably only partially homologous to GT8, as was the case for tRNA^{Leu}_{CUG}. However, sufficient homology must exist in the region for GT8 to bind the gene during screening and sequencing.

Figure 11. Comparison of tRNA gene sequences.

The sequences of the nontemplate strands for a) tRNA^{Ser}_{2b}, b) tRNA^{Ser}_{UCA}, and c) tRNA^{Leu}_{CUG} are given, 5'-3' left to right. Structural genes or portions thereof are shown in bold print. Anticodons are underlined. R = purine; Y = pyrimidine; N = undetermined nucleoside.

	-50	-40	-30	-20	-10		10	20	30	40
a) Ser-2b	AAGAAAGT	GGTAGTTATG	GAGTGTANGA	AATNGTNATC	GATTTNGTGG	AAAAGAAAGT	GACGAGGTGG	CCGAGAGGTT	AAGGCGTTGG	ACTGCTAATC
b) Ser-UCA									ATGG	ACTTGAAATC
c) Leu-CUG							TAACA	GTCAGGATGG	CCGAGTGGTC	TAAGGCGCTG

	50	60	70	80	90	100	110	120	130	140
a) Ser-2b	CAATGTGCTC	TGCACGCGTG	GGTTCGAATC	CCATCCTCGT	CGAGTGGAAT	TTTTTNTTT	TTTCCATTNG	AATATTAACN	CAAAAAAGCC	ATGTGTGAAT
b) Ser-UCA	CATTGGGTTT	TACCCGCRCA	GGTTCRARTC	CTGTCCGCAG	CG					
c) Leu-CUG	GCAGTCTACT	CTGTAGGCGT	GGGTTTCAAT	CCCACTTCTG	ACAATRNYTT	TTTNTCCNAT	TT			

	150	160	170	180	190
a) Ser-2b	TACTTTATGG	AATATTTTAA	CTTGAATTTA	ACTTTTCAAT	TATACAATAT

The fact that these two genes were unexpectedly found using the tRNA^{Ser}_{2b}-specific probe GT8 is interesting, considering the lack of homology between the three genes. Screening was done at a low enough stringency (58°C, 6 x SSC) to allow hybridization of GT8 to these divergent sequences. This washing temperature was used even though the theoretically calculated temperature was 69°C for this oligonucleotide (Meinkoth and Wahl, 1984) because of initial unfamiliarity with the probe and its behaviour at various stringencies, and because it was desirable to obtain and test about 20 positives per screening.

The failure to find tRNA^{Ser}_{2b} among these 4,900 colonies derived from 3.6 kb EcoRI fragments is probably explained by the randomness of the search. The gene does appear to be contained within this region, according to the strong hybridization there of both GT8 and GT9 (Figure 10). Using the earlier calculation, the screening of 4,900 colonies would give an estimated 97% chance of obtaining the gene (Kaiser and Murray, 1985). However, the best presumption appears to be that the tRNA^{Ser}_{2b} gene was missed by chance in these screenings. Possible, but less likely, is that the gene contained in this 3.6 kb EcoRI region is not clonable.

The 5.1 kb region of a HindIII genomic DNA digest was targeted for tRNA^{Ser}_{2b} cloning, after repeated failure to obtain this gene from the 3.6 kb EcoRI region. This region was chosen because, in addition to the strong band at 5.1 kb, there is also a band of lesser intensity at 5.6 kb which hybridizes to both GT8 and GT9 (Figure 10). The gel slice containing these two bands was cut out, and the DNA was eluted and cloned. A total of 3,700 colonies were screened, and

one was found to contain a putative tRNA^{Ser}_{2b} gene.

In summary, three fragments containing at least one copy of a tRNA gene each were cloned into pUC 13: A 3.6 kb EcoRI fragment containing tRNA^{Leu}_{CUG}, a 3.6 kb EcoRI fragment containing tRNA^{Ser}_{UCA}, and a 5.1 kb HindIII fragment containing tRNA^{Ser}_{2b} (codons AGC, AGU).

iii. Sequencing and in situ Hybridization of tRNA^{Ser}_{2b}, tRNA^{Ser}_{UCA}, and

tRNA^{Leu}_{CUG} Clones

Preliminary sequence data (Figure 11) was obtained from the three pUC 13-based recombinant plasmids isolated as described. Double stranded DNA sequencing was used, and the oligonucleotides GT8 and GT9 were used as primers to sequence in opposite directions where possible. As mentioned earlier, this was not possible with the putative tRNA^{Ser}_{UCA} gene, due to insufficient homology with GT9; thus, only the 3'-portion of this gene was sequenced, using GT8 as a primer. tRNA^{Ser}_{2b} and tRNA^{Leu}_{CUG} were able to be fully sequenced using both primers. Thus, data was only obtained from one strand at the two ends of the genes where these primers hybridize, and in the flanking regions. The anticodon region was sequenced on both strands for both tRNA^{Ser}_{2b} and tRNA^{Leu}_{CUG}. Except for positions -58 to -2 and 98 to 195 of the tRNA^{Ser}_{2b} flanking regions, the data for all areas which were not sequenced on both strands was confirmed by at least two to four repeated experiments. These data appear quite reliable, according to their excellent correspondence to other known sequences, where such sequences exist (i.e., tRNA^{Ser}_{2b} and tRNA^{Leu}_{CUG}, as discussed below), and the exact agreement of all data generated from complementary strands.

None of the three cloned genes has the tRNA CCA 3'-terminus encoded in the DNA sequence. This is universally found to be the case for eukaryotic genes, the three terminal nucleotides being added post-transcriptionally by nucleotidyl transferase. All three genes do show good agreement with the 5'- and 3'-internal promoters described in the literature (Galli *et al.*, 1981), there being differences at only two positions at most. The putative internal promoters run from nucleotides 8 to 18, and 61 to 72 (62 to 73 for tRNA^{Leu}_{CUG}), Figure 11a to c. It is not surprising that these include the areas where GT8 and GT9 showed maximum cross-hybridization between the three genes.

a) tRNA^{Ser}_{2b}

The sequence of the cloned tRNA^{Ser}_{2b} gene is shown in Figure 11a. This gene had the actual tRNA sequence data available for comparison (Cribbs, 1982; Cribbs *et al.*, 1987a; Figure 9). The gene cloned here agreed with the tRNA data at every position except #16. This nucleotide, which appeared as a most unambiguous "dA" in the DNA sequence data of this clone, appeared quite clearly as dihydrouridine in the tRNA^{Ser}_{2b} sequence data (Cribbs, 1982), which would give a "dT" in the DNA at this position. If both sets of data are, in fact, correct, it appears that the tRNA^{Ser}_{2b} gene cloned here represents an allogene with a T to A transversion at position 16. Subcloning of the tRNA^{Ser}_{2b} gene contained in the 5.1 kb HindIII fragment, and sequencing of both strands would be necessary to confirm absolutely the existence of a sequence discrepancy at this nucleotide.

The occurrence of tRNA allogenes is well documented (see Introduction). For example, in previous studies of the tRNA_{4,7}^{Ser} gene cluster at 12DE on the X chromosome, as discussed earlier, a gene was found which was identical in sequence to tRNA₄^{Ser} except for a C to T transition at position 50 (Cribbs et al., 1987b). Both this gene and the tRNA_{2b}^{Ser} gene in question have tracts of oligo(dT) residues a short distance downstream from the 3'-end of the gene. These are thought to function as signals for termination of transcription (Adeniyi-Jones et al., 1984), and their retention may suggest that such genes are actively transcribed, the single mutations being neutral to natural selection.

The fact that allogenes do show in vitro transcriptional ability where these assays have been done, raises questions as to why the corresponding tRNA sequences show no variation at the microheterogeneous nucleotides. Perhaps the variant genes form minor chromatographic species which are separable from the major tRNA peaks studied; otherwise, it is possible that sequence microheterogeneity could affect the post-transcriptional modification of the variant tRNA species in such a way as to cause their rapid degradation, as suggested by Sharp et al. (1981).

The flanking sequences of this clone do not appear to show homology to any consensus sequence other than the 3'-oligo(dT) discussed, as is the general case for eukaryotic tRNA genes. In previous studies, however, regions around tRNA genes have been found to be quite AT-rich (Addison et al., 1982; DeLotto and Schedl, 1984; Glew et al., 1986; Suter and Kubli, 1988), and this is clearly seen here in the 64% AT content of the 5'-flanking sequence and the even higher 77% AT

content of the 3'-flanking sequence.

In situ hybridization results (Figure 12) show that the tRNA^{Ser}_{2b} clone described appears to derive from site 88A on the right arm of chromosome 3, one of the three chromosomal sites to which the tRNA^{Ser}_{2b} species also binds (Hayashi et al., 1982; Dr. S. Hayashi, pers. comm.). As both functional tRNA^{Ser}_{2b} and this apparent tRNA^{Ser}_{2b} allo gene appear to derive from 88A, it is possible that this chromosomal site may code for two or more tRNA^{Ser}_{2b}-related genes, or this may represent cross-hybridization. This site does not appear enriched for tRNA sequences, having less than four according to (Elder et al., 1980), but further molecular cloning and sequencing of the region would contribute to a comprehensive study of the tRNA^{Ser}_{2b} group of isoacceptors.

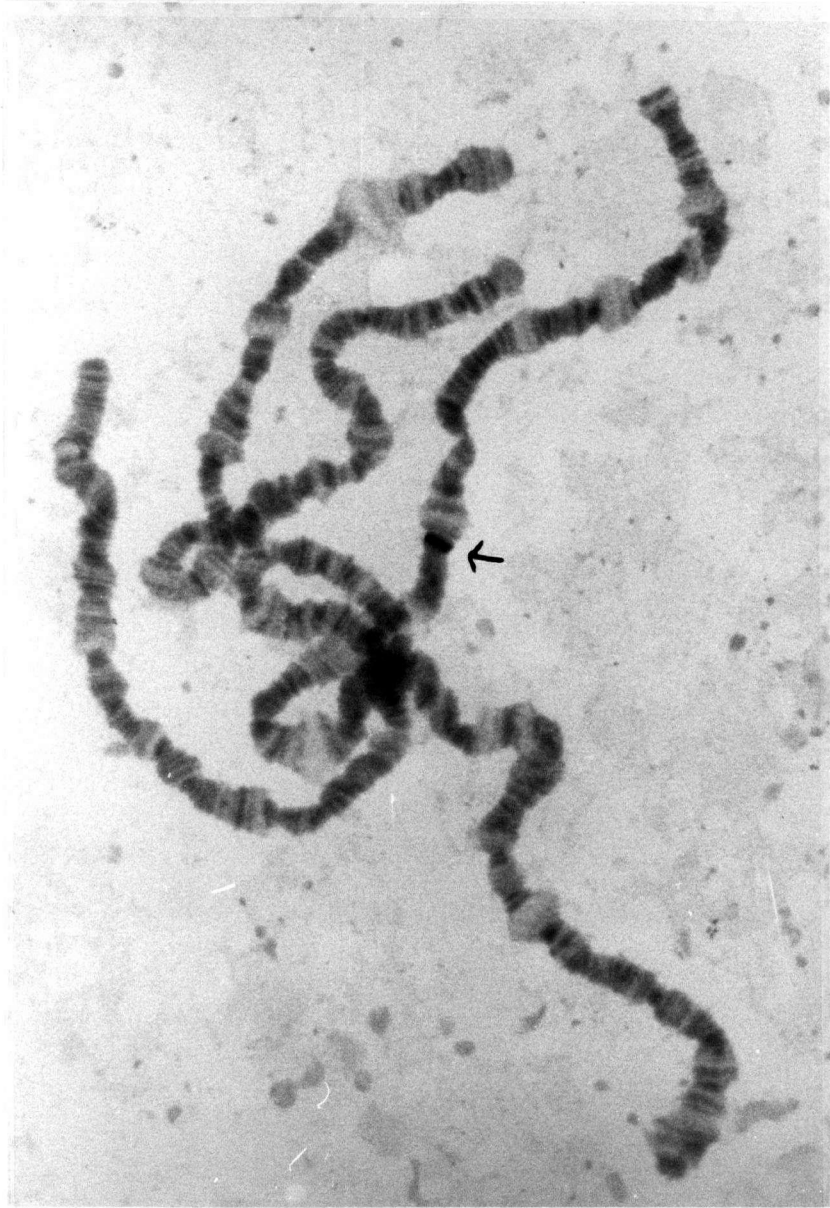
b) tRNA^{Ser}_{UCA}

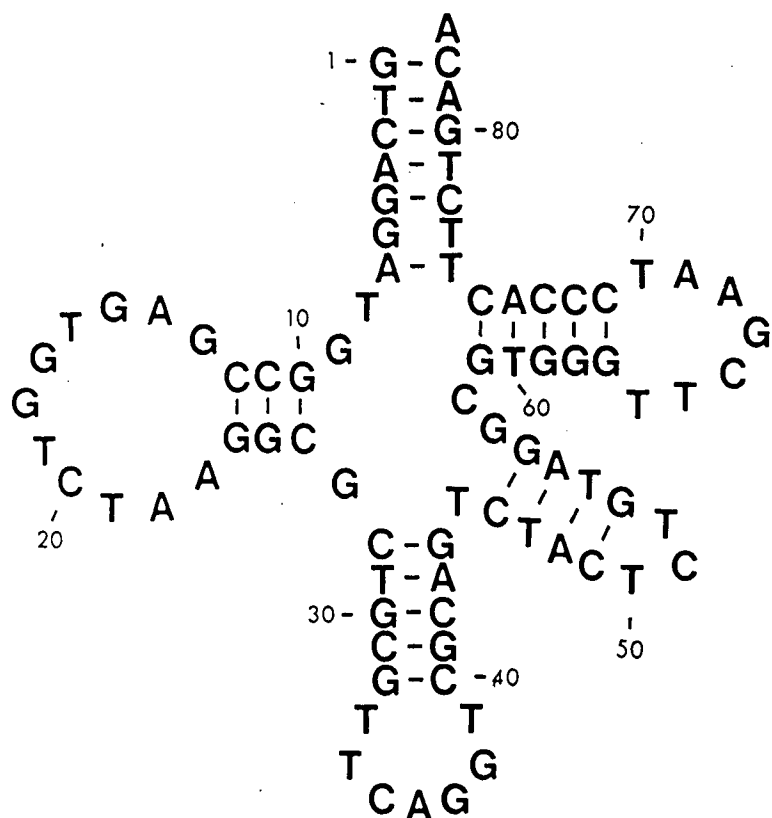
The partial sequence of a putative tRNA^{Ser}_{UCA} gene is given in Figure 11b. The sequenced portion is seen to fold readily into a standard tRNA cloverleaf structure (Figure 13b). This tRNA would be expected to recognize the codon UCG, in addition to UCA.

Interestingly enough, there are at least 14/21 (and possibly 16/21 matches between this gene and GT9 (positions 62 to 71, 74 to 76, 78, 81, and 82; Figure 11b) similar to the situation for the cloned tRNA^{Leu}_{CUG} gene. It could not, however, be clearly sequenced using this oligonucleotide as a primer, as could tRNA^{Leu}_{CUG}. Perhaps this is because the block of homology at the 3'-end of GT9 is only 8 to 10 nucleotides in length here, with the other 6 matches more dispersed.

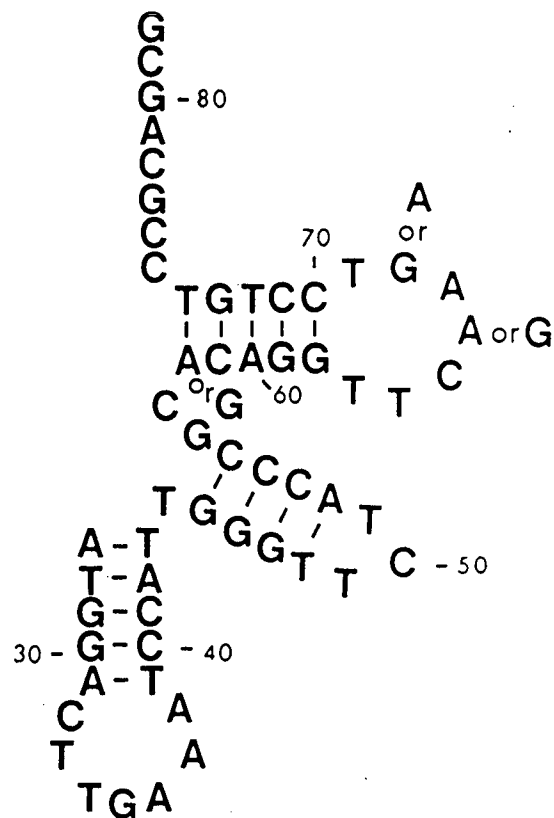
Figure 12. In situ hybridization of the tRNA_{2b}^{Ser} gene.

The pUC 13 recombinant plasmid containing the tRNA_{2b}^{Ser} gene within a 5.1 kb HindIII restriction fragment was labelled by nick translation with biotinylated dUTP (Bio-11-dUTP, Bethesda Research Laboratories), and hybridized to Drosophila third instar larvae salivary gland polytene chromosomes in 50% formamide / 6 x SSC at 37°C for 24 hours. Site of hybridization was determined using the BluGene nonradioactive detection system from BRL. Hybridization is seen at site 88A on the right arm of chromosome 3. Figure courtesy of Dr. S. Hayashi.





(a) c-tRNA^{Leu}_{CUG}



(b) Sequenced portion of c-tRNA^{Ser}_{UCA}
 (numbered as tRNA^{Ser}_{2b}, Fig.11)

Figure 13. Cloverleaf structures of cloned tRNA genes as predicted from the DNA sequence data.

The 5'-end of the gene proved to have enough similarity to tRNA^{Ser}_{2b} to allow GT8 to be used for its cloning and sequencing, but the amount of homology was not discovered, as this portion of the gene could not be sequenced from this recombinant plasmid. Again, subcloning and sequencing in both directions would be desirable here.

There is 66 to 71% homology between the known part of the sequence of tRNA^{Ser}_{UCA} and the corresponding portion of tRNA^{Ser}_{2b} (Figure 11a and b), which is similar to the 71 to 73% level of homology between tRNA^{Ser}_{2b} and tRNA^{Ser}_{4,7} (Cribbs et al., 1987a), and in agreement with the 10 to 30% sequence divergence generally found between functionally distinct tRNA isoacceptors (Sprinzl et al., 1987). Further, there is also 59 to 66% homology between the known part of tRNA^{Ser}_{UCA} and tRNA^{Ser}_{4,7} (Cribbs et al., 1987b). By contrast, the only notable homology between the sequenced portion of tRNA^{Ser}_{UCA} and the tRNA^{Leu}_{CUG} clone described is a 9 to 11 nucleotide match in the region of the TΨC loop from positions 62 to 72 of tRNA^{Leu}_{CUG} (61 to 71 of tRNA^{Ser}_{UCA}), Figure 11b and c. The heightened homology between this putative tRNA^{Ser}_{UCA} gene and tRNA^{Ser}_{2b,4,7} suggests that it may be an active member of the tRNA^{Ser} gene family.

The sequence data extending beyond the 3'-terminus of the tRNA^{Ser}_{UCA} structural gene appears quite degenerate (data not shown). This finding suggests that two or more copies of the tRNA^{Ser}_{UCA} gene may be present in this 3.6 kb EcoRI fragment, with these genes possessing divergent flanking sequences.

There is further evidence that two or more slightly different versions of the gene may be present: At three positions within the tRNA^{Ser}_{UCA} gene sequence, it is unclear whether the nucleotide is a

G or an A (Figure 11b). It appears more likely that these anomalies represent G to A transitions than sequencing artifacts, given the absence of such artifacts in other sequencing done here, their clear presence in each experiment carried out on tRNA^{Ser}_{UCA}, and the suggestion of the presence of more than one gene by the overlapping flanking sequence data above. Thus, this clone may contain yet another example of tRNA gene microheterogeneity.

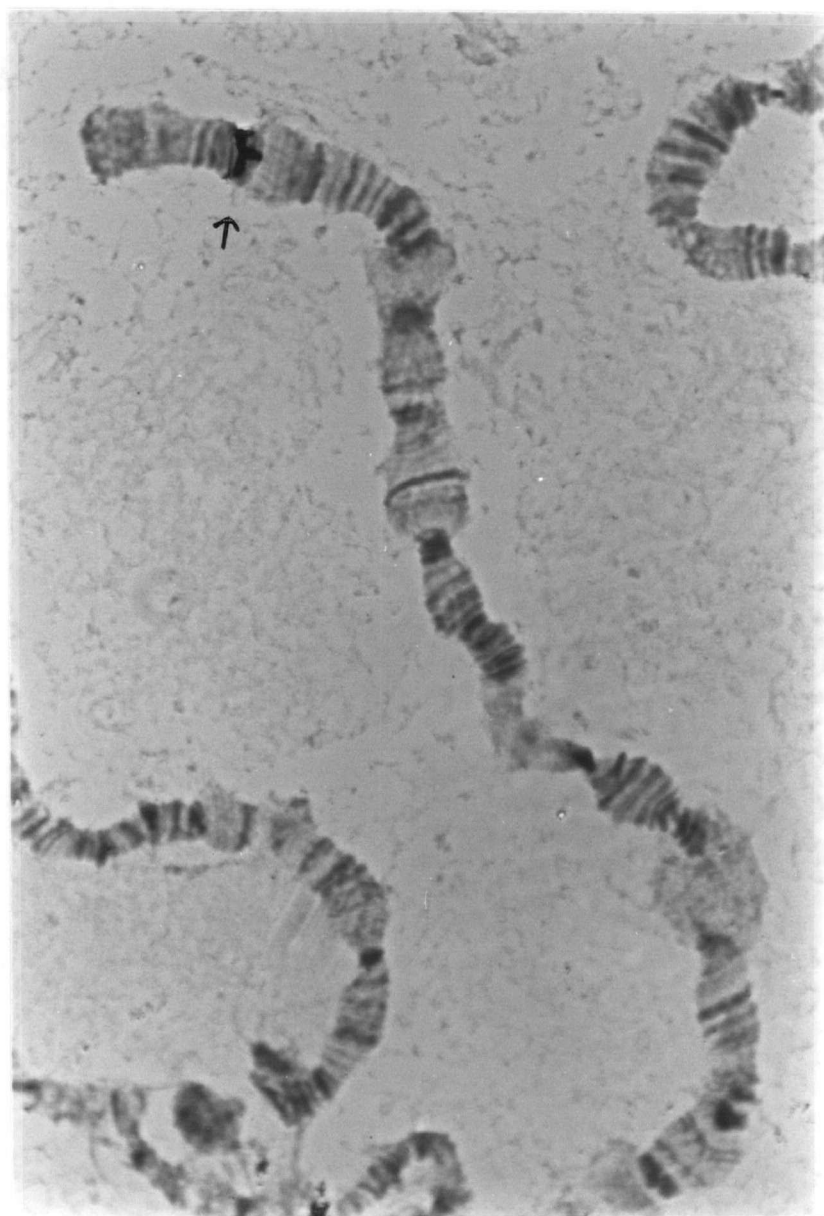
It should be noted that a dT-rich area extends from a position approximately 8 to 30 nucleotides past the 3'-terminus of the putative tRNA^{Ser}_{UCA} gene(s) (data not shown). The sequence data from at least two tRNA^{Ser}_{UCA} genes appears to overlap in this region as described above, however, and it is therefore unclear what oligo(dT) termination signal each individual gene possesses.

In a study of the four major serine accepting tRNA peaks (#2, 4, 5, and 7), separated by BD-cellulose chromatography, it was found that none of these major peaks bound significantly to the triplet UCA, except for a very low level of binding observed with tRNA^{Ser}₇ (White et al., 1975). It appears, therefore, that this serine accepting tRNA may form one of the more minor tRNA^{Ser} peaks, being present in the cell at lower levels than the major serine isoaccepting species.

The pUC 13 recombinant plasmid containing this 3.6 kb EcoRI fragment was also analyzed by in situ hybridization to D. melanogaster polytene chromosomes (Figure 14). It was found that this clone hybridized at 58AB on the right arm of the second chromosome. This is not a major site of tRNA genes, according to in situ hybridization experiments, being thought to have less than four putative tRNA genes altogether (Elder et al., 1980). However, the fact that tRNA does

Figure 14. In situ hybridization of the tRNA^{Ser}_{UCA} gene.

The pUC 13 recombinant plasmid containing at least one putative tRNA^{Ser}_{UCA} gene within a 3.6 kb EcoRI restriction fragment was labelled, hybridized to polytene chromosomes, and detected as in Figure 12. Hybridization is seen at site 58AB at the extremity of the right arm of chromosome 2. Figure courtesy of Dr. S. Hayashi.



bind at this site suggests that the clone obtained could correspond to a tRNA product which is uncharacterized at present. This chromosomal site also shows significant hybridization to tRNA^{Gly}_{GGA} (Hayashi et al., 1982).

Overall, the significant levels of homology between the sequenced portion of the tRNA^{Ser}_{UCA} gene cloned here and the known members of the tRNA^{Ser} gene family, the apparent presence of more than one tRNA^{Ser}_{UCA} sequence in this plasmid, and the possibility of microheterogeneity between these genes, all suggest that further study of this clone, and of the chromosomal region 58AB to which both it and tRNA^{Gly}_{GGA} hybridize, could provide an interesting addition to the body of knowledge already accumulated on the evolution of the tRNA^{Ser} gene family in Drosophila, and on tRNA gene organization within the genome of this organism.

c) tRNA^{Leu}_{CUG}

The sequence data for the tRNA^{Leu}_{CUG} gene contained in a cloned 3.6 kb EcoRI fragment is given in Figure 11c. The cloverleaf configuration of this sequence is shown in Figure 13a, suggesting that it is able to fold into a viable tRNA structure.

As noted earlier, it is interesting that this gene was found when screening with GT8, an oligonucleotide specific for tRNA^{Ser}_{2b}. There is a 12 bp block of homology from positions 8 to 19 (Figure 11a and c, bearing in mind that GT8 has a "dT" at position 16), and four matches to the sides of this block at positions 1, 3, 6, and 22, giving a total of 16/23 hybridizing nucleotides. The relatively

low washing stringency used (58°C, 6 x SSC), combined with the block of 12 homologous nucleotides, apparently created conditions sufficient to clone and sequence this gene using GT8.

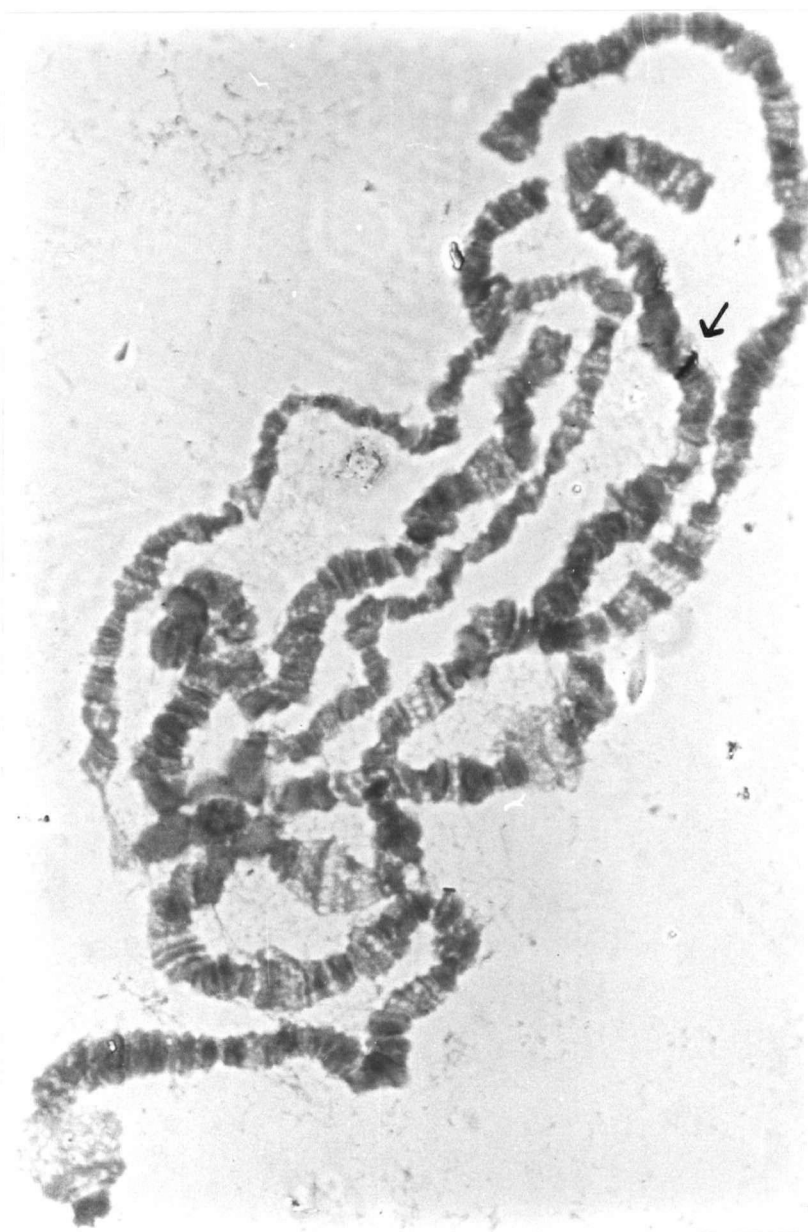
It also proved to be possible to sequence this gene in the reverse direction using GT9, due to a fortuitous block of homology 12 nucleotides long between tRNA^{Leu}_{CUG} and the 3'-end of GT9 (positions 63 to 74 of tRNA^{Leu}_{CUG}, Figure 11c). Two other nucleotides at positions 80 and 82 also show homology in this area, giving a total of 14/21 matches between GT9 and tRNA^{Leu}_{CUG}. As mentioned earlier, tRNA^{Ser}_{UCA} had 14 to 16/21 nucleotide homology to GT9, but could not be sequenced with that oligonucleotide. The longest block of homology in that case was 8 to 10 nucleotides. It thus appears that the 2 to 4 extra nucleotides in the block of homology between tRNA^{Leu}_{CUG} and GT9 contribute greatly to its ability to form a viable hybrid with that oligonucleotide under the conditions used.

It was impossible to obtain sequence data well into the flanking regions with this clone. This may indicate the presence of two or more identical copies of the tRNA^{Leu}_{CUG} gene in this recombinant plasmid, with somewhat divergent flanking sequences as discussed for the tRNA^{Ser}_{UCA} clone. When the plasmid was digested with AluI, at least three bands of approximate sizes 340, 390, and 500 bp hybridized to GT8 (data not shown). Attempts to subclone these fragments proved unsuccessful, but their existence does suggest the presence of more than one tRNA^{Leu}_{CUG} gene in this plasmid.

In situ hybridization showed the clone to derive from site 66B on the left arm of chromosome 3 (Figure 15); this is also the major site of hybridization for tRNA^{Leu}₂, suggesting the possible correspond-

Figure 15. In situ hybridization of the tRNA^{Leu}_{CUG} gene.

The pUC 13 recombinant plasmid containing at least one copy of the tRNA^{Leu}_{CUG} gene within a 3.6 kb EcoRI restriction fragment was labelled, hybridized to polytene chromosomes, and detected as in Figure 12. Hybridization is seen at site 66B on the left arm of chromosome 3. Figure courtesy of Dr. S. Hayashi.



ence of this clone to that tRNA (Hayashi et al., 1982; Dr. S. Hayashi, pers. comm.). As the tRNA^{Leu}₂ isoacceptor has not been sequenced to date, this cannot yet be verified. However, further support for this isoacceptor assignment comes from the fact that minor hybridization was occasionally observed at sites 44E (2R) and 79F (3L), the other two significant sites of tRNA^{Leu}₂ hybridization (Hayashi et al., 1982; Dr. S. Hayashi, pers. comm.). This hybridization is not visible in Figure 15.

A tRNA^{Leu}_{CUG} gene from D. melanogaster identical to this one has previously been cloned (Glew et al., 1986). However, comparing the available flanking sequence data of the present clone (Figure 11c) with that of the previous one shows that the two genes lack homology in both the 5'- and 3'-flanking regions, with the exception of the oligo(dT) terminator. Lack of flanking sequence homology between otherwise identical Drosophila tRNA genes has been a common finding (Robinson and Davidson, 1981; DeLotto and Schedl, 1984; Leung et al., 1984). It thus appears that at least two identical but distinct copies of the Drosophila tRNA^{Leu}_{CUG} gene have now been cloned. These two tRNA^{Leu}_{CUG} genes both derive from the 66B region (Figure 15; Glew et al., 1986). This region was seen to be one of the major sites of total tRNA hybridization to Drosophila polytene chromosomes, being thought to contain four or more tRNA genes (Elder et al., 1980).

The tRNA^{Leu}_{CUG} genes described show an extremely high degree of homology (93 to 95%) to tRNA^{Leu}_{CUG} genes or their tRNA transcripts cloned from a variety of organisms, including those from Xenopus laevis, mouse, rat, and cow mammary gland (Glew et al., 1986; Sprinzl et al., 1987). This is similar to the situation found for tRNA^{Lys} iso-

acceptors, where the tRNA^{Lys}_{AAG} genes from two widely divergent species, Drosophila and rabbit, have been found to be identical (Silverman et al., 1979; Raba et al., 1979), and the tRNA^{Lys}_{AAA} genes from these same species differ by only 5% (Cribbs, 1982). A fairly high degree of sequence conservation, 82%, also exists between these intron-less Drosophila tRNA^{Leu}_{CUG} genes and the intron-containing Drosophila tRNA^{Leu}_{UUG} genes described previously (Robinson and Davidson, 1981), with all but one of the nucleotide changes occurring within the anticodon stem and loop, and in the extra arm [sequences compared by Glew et al., (1986)].

The finding here of a second distinct tRNA^{Leu}_{CUG} gene copy deriving from the chromosomal region 66B, along with the high degree of sequence conservation between this gene and various other tRNA^{Leu} isoacceptors, and the major tRNA hybridization to this site, suggests that the chromosomal region 66B may contain an interesting cluster of tRNA^{Leu} genes, and possibly other tRNA genes as well.

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APPENDIXDideoxy/deoxynucleotide Mixes^(a)

	<u>G mix</u>	<u>A mix</u>	<u>T mix</u>	<u>C mix</u>
ddGTP	158	-	-	-
ddATP	-	114	-	-
ddTTP	-	-	549	-
ddCTP	-	-	-	546
dGTP	15.4	109	158	158
dATP	-	-	-	-
dTTP	158	109	15.4	158
dCTP	158	109	158	14.3

(a) concentrations given in μM . Originally obtained courtesy of Dr. Joan McPherson (see Newton, 1984) with some modifications.