The Genetic Origins of Transfer RNAs in Wheat (*Triticum aestivum*) Mitochondria

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Kathleen Elizabeth Glover

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Abstract

For this thesis the genetic origins of wheat mitochondrial tRNAs were studied. The primary focus of the research was the identification of the non-mtDNA-encoded tRNAs present in a wheat mitochondrial RNA preparation, as the wheat mtDNA-encoded tRNA population had already been extensively examined and found to contain far fewer than the number of tRNAs required for translation of mitochondrial messenger RNAs.

Sixteen tRNAs were identified through partial sequence analysis that show greatest sequence identity (70.5-100%) with plant cytosolic isoaccepting tRNA sequences and only 46-57.8% sequence identity with isoaccepting tRNAs encoded by plant mtDNA. These tRNAs are conventional in their primary sequences and secondary structures. Oligonucleotide probes specific for all of these tRNAs hybridized to both mitochondrial and cytosolic RNA preparations and the majority of these tRNAs were present in the mitochondrial RNA fraction at levels greater than determined for a cytosolic tRNA not required by wheat mitochondria for protein synthesis. Two of the tRNAs identified ($tRNA^{Asp}$ and $tRNA^{His}$) are redundant with respect to the tRNAs encoded within the mitochondrial genome. However, the amount of the cytosolic-like $tRNA^{Asp}$ present in the wheat mitochondrial RNA preparation could not be distinguished from that of a cytosolic tRNA not required for use in mitochondrial protein synthesis and therefore this tRNA may not function in mitochondrial translation. The presence of both a mtDNA-encoded $tRNA^{His}$ and a cytosolic-like $tRNA^{His}$ in the tRNA population may represent an intermediate stage in the evolution of the wheat mitochondrial tRNA population.

The wheat mitochondrial tRNA population as characterized to date includes 17 mtDNA-encoded tRNAs [10 "native" (tRNAs that have 65-80% sequence identity with homologous eubacterial and chloroplast tRNAs) and 7 "chloroplast-like" (tRNAs that have >90% sequence identity with chloroplast tRNAs)] and at least 13 "cytosolic-like" (nDNA-encoded) tRNAs. Assuming standard wobble rules for codon/anticodon base pair recognition, at least five tRNAs remain to be identifed. Moncot, dicot and liverwort mitochondrial tRNA populations have significant differences with respect to the genetic origin of their tRNA species.

List of Abbreviations

A, absorbance

A, adenosine

aa, amino acid

bp, basepair

C, cytidine

°C, degree Celsius

cyt, cytosolic

dNTP, 2'-deoxynucleoside-5'-triphosphate

ddNTP, 2',3'-dideoxynucleoside-5'-triphosphate

DMSO, dimethylsulfoxide

DTT, dithiothreitol

DNA, deoxyribonucleic acid

EDTA, ethylenediaminetetraacetate

g, gram

g, gravity

G, guanosine

h, hour

I, inosine

kbp, kilobase pair

µl, microliter

µM, micromolar

µg, microgram

mg, milligram

ml, milliliter

mM, millimolar

mmol, millimole

min, minute

mt, mitochondrial

M, molar

N, any nucleoside

n, nuclear

ng, nanogram

pmol, picomole

rpm, revolutions per minute

R, purine nucleoside

RNA, ribonucleic acid

r, ribosomal

s, sec

SDS, sodium dodecyl sulfate

T, thymidine

TEMED, *N,N,N'N'*-tetra-methylethylenediamine

Tris, tris(hydroxymethyl)-aminomethane

UV, ultraviolet

U, uridine

Y, pyrimidine nucleoside

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I. Introduction

When one surveys the coding functions of mitochondria from a range of animals, fungi, protists and angiosperms (flowering plants), it is clear that mitochondrial DNA (mtDNA) has the same basic function in all eukaryotes (Gray, 1992). In general, the mitochondrial genome encodes ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) to be used in synthesis of essential mtDNA-encoded polypeptide components of the mitochondrial electron transport chain. However, mitochondrial genomes are very diverse in structure and organization and in many aspects the angiosperm mitochondrial genome is distinct from those of animals, fungi and protists.

Plant mtDNAs represent the largest of the mitochondrial genomes, with the smallest known plant mitochondrial genome (187 kbp) being that of the liverwort, *Marchantia polymorpha* (a bryophyte or non-vascular plant) (Oda *et al.*, 1992a). The largest known plant mitochondrial genome (2400 kbp) is that of the muskmelon, *Cucumis melo* (Ward *et al.*, 1981). In comparison, animal mitochondrial genomes range in size from 13.8 to 42 kbp (Wolstenholme, 1992), whereas fungal mitochondrial genomes range in size from 17.6 kbp in the ascomycete, *Schizosaccharomyces pombe* (Zimmer *et al.*, 1984), to 176 kbp in the basidiomycete, *Agaricus bitorquis* (Hintz *et al.*, 1985).

Plant mitochondrial genomes differ from their non-plant counterparts not

only in size but in many other features as well. Whereas the animal mitochondrial genome consists primarily of coding sequence and is considered compact and efficient in genome structure (Wolstenholme, 1992) plant mitochondrial genomes appear to consist mostly of noncoding DNA, with minimal gene linkage (Lonsdale, 1989). Gene order is relatively constant within individual animal phyla, as compared to the significant genome rearrangements found within families of angiosperms (Gray, 1989). The rapid structural change of the angiosperm mitochondrial genome, thought to be the result of homologous recombination between the many direct and inverted repeats found within the genome, can be contrasted with its slow sequence divergence. In angiosperms, the mtDNA evolves 10-fold less rapidly in sequence than single-copy nuclear DNA (nDNA) (Wolfe *et al.*, 1987) whereas mammalian mtDNA evolves at 5-10 times the rate of single-copy nDNA in the same species (Wilson *et al.*, 1985). The point mutation rate of angiosperm mtDNA is only about one percent that of animal mtDNA (Wolfe *et al.*, 1987).

The active recombination system of angiosperm mtDNA and its low gene density have been cited as possible reasons for the ability of angiosperm mtDNA to readily incorporate "foreign" DNA (Gray, 1992), particularily chloroplast DNA. Although most of this promiscuous DNA appears to be largely nonfunctional, there is evidence that some plastid-derived tRNA genes are actively transcribed and processed to mature tRNAs (Joyce and Gray, 1989a).

Considering the large size of the angiosperm mitochondrial genome, one

might speculate that it encodes many more genes than do the smaller animal mtDNAs. The typical animal mitochondrial genome encodes 13 polypeptides involved in oxidative phosphorylation, large subunit (LSU) and small subunit (SSU) rRNAs, and 22 tRNAs. So far, however, only a few novel genes (e.g. 5S rRNA) have been identified in plant (specifically, angiosperm) mtDNAs in addition to a slightly different set of genes encoding respiratory chain proteins, LSU and SSU rRNAs, 20 or fewer tRNAs and a number of ribosomal protein genes. The presence of novel genes, repeated sequences, promiscuous DNA and introns can still not account for the large size difference between animal and plant mitochondrial genomes.

Examples of deviation from the universal genetic code are abundant in animal mitochondria (Wolstenholme, 1992). The translation of UGA as tryptophan is so far consistent among animal and most fungal mitochondria. Other deviations from the standard code include the use of not only AUG but also AUA and AUU and in some animals, AUC, GUG, UUG and GUU as initiation codons. Plant mitochondria on the other hand apparently use the universal genetic code and UGA is used to specify termination.

Not only do animal and fungal mitochondria exhibit deviations in translation of the genetic code, they also exhibit an expanded codon recognition pattern whereby a single tRNA is able to read all codons in a four-codon family using U:N wobble between the first base of the anticodon and the third base of the codon (Anderson *et al.*, 1981). Thus the minimal set of 22 tRNAs encoded by the animal

mitochondrial genome is sufficient to decode the mRNAs contained within these organelles. The majority of animal mitochondrial tRNAs are also aberrant in their primary sequences and secondary structures (Wolstenholme, 1992). The dihydrouridine and the T-pseudouridine-C loops may either be completely missing or severely truncated. In contrast, all plant mitochondrial tRNAs identified to date have standard primary sequences and secondary structures (Maréchal-Drouard *et al.*, 1993).

As yet, no angiosperm mitochondrial genome has been found to encode a set of tRNAs sufficient to decode the universal genetic code (Maréchal-Drouard *et al.*, 1993). Moreover, as will be discussed in following sections of this Introduction, evidence of tRNA import into plant mitochondria has been accumulating.

The only plant mitochondrial genome that has been completely sequenced is that of the liverwort, *M. polymorpha* (Oda *et al.*, 1992a). Its mitochondrial genome is different from that of flowering plants in a number of respects. The mitochondrial genome of liverwort is relatively small and apparently not highly recombined into smaller subgenomic molecules. There is no evidence of foreign ("promiscuous") DNA or mRNA editing. The gene organization is dense, with clustering of both ribosomal protein genes and tRNAs. A large number of introns (Group I and II) are present in the rRNA, tRNA and protein-encoding genes of *Marchantia* mitochondria. Although the complete sequence analysis of *Marchantia* mtDNA has revealed significantly more genes than have so far been identified in

angiosperm mitochondria, the 27 tRNA genes identified are still insufficient to translate all codons of the universal genetic code (Oda *et al.*, 1992b). The import of tRNA may eliminate the deficit.

The research presented in this thesis attempts to characterize the non-mtDNA-encoded tRNA population of wheat (*Triticum aestivum)* mitochondria with respect to tRNA identity, subcellular localization and genetic origin. This information can then be used in conjunction with previously identified mtDNA-encoded tRNAs to address questions such as: "Are the non-mtDNA-encoded and the mtDNA-encoded tRNA populations distinct?"; "Are any of the tRNAs redundant with respect to decoding of genetic information?"; "Must expanded wobble be invoked to decode the genetic code?"; and "Are the same tRNAs imported as in other plant mitochondria?".

I began this Introduction with a general overview of the mitochondrial genome of angiosperms and contrasted it primarily with animal and fungal mitochondrial genomes, as the mitochondrial tRNA populations specified by many of these are fully characterized. The remainder of the Introduction will discuss in detail what is known about plant chloroplastic, cytosolic and mitochondrial tRNAs, followed by a discussion of the import and export of nucleic acids in non-plant cell organelles and the mechanisms of tRNA import.

A. Plant tRNAs

(i). Chloroplast tRNAs

Chloroplast genomes of two angiosperms (tobacco and rice) (Sugiura and

Wakasugi, 1989; Hiratsuka *et al.*, 1989) and one gymnosperm (black pine (*Pinus thunbergii)*) (Tsudzuki *et al.*, 1994), have been completely sequenced, as has the chloroplast genome of *M. polymorpha* (Ohyama *et al.*, 1986). In tobacco and rice, the chloroplast genome encodes the same 30 tRNAs, whereas the *Marchantia* and black pine chloroplast genomes encode 31 and 32 tRNAs, respectively. The additional tRNA gene of the *Marchantia* chloroplast genome, as compared to tobacco and rice, is a $tRNA^{Arg}$(CCG). The black pine chloroplast genome encodes the 31 tRNAs found in *Marchantia* as well as a $tRNA^{Pro}$(GGG), not found in any of the angiosperm chloroplast genomes sequenced to date.

In general, plant chloroplast tRNAs possess the invariant and semi-invariant nucleotides characteristic of eubacterial and eukaryotic cytosolic tRNAs. They show little sequence similarity to eukaryotic cytosolic tRNAs and approximately 70% sequence identity to eubacterial tRNAs, as would be expected on the basis of their proposed endosymbiotic origin (Gray and Doolittle, 1982; Gray, 1992). The standard primary sequences and predicted secondary structures of these tRNAs intuitively preclude the existence of aberrant decoding of the genetic code in chloroplasts. However, the 30 chloroplast tRNAs of angiosperms are insufficient to decode the 61 sense codons found in chloroplast protein-coding genes using the standard wobble rules for base pairing between the codon and anticodon. A minimum of 32 tRNAs is required to decode the 61 sense codons of the genetic code. The rice and tobacco chloroplast genomes are apparently lacking tRNAs that would decode the GCU/C (Ala), CGC/A/G (Arg), CUU/C (Leu) and CCU/C (Pro)

codons. Because it is generally assumed that there is no import of tRNAs into chloroplasts, additional mechanisms of codon recognition were investigated. In an *in vitro* tRNA-dependent translation system from wheat germ, the chloroplast alanine (UGC), proline (UGG) and arginine (ICG) tRNAs are able to read all four alanine (GCN), proline (CCN) and arginine (CGN) codons, respectively (Pfitzinger *et al.*, 1990). The uridine in the wobble position of the UGC and UGG anticodons of $tRNA^{Ala}$ and $tRNA^{Pro}$ is modified. Because only an unmodified uridine has been shown to be able to base pair with all four nucleosides (Grosjean *et al.*, 1978), Pfitzinger *et al.* propose that a "two out of three" base pair recognition mechanism (Lagerkvist, 1978; Lagerkvist, 1986) is used. Similarly, the inosine nucleoside is not able to pair with guanosine according to the wobble hypothesis (Crick, 1966), so that a "two out of three" base pair recognition mechanism must be invoked in this case, as well. Again, using *in vitro* translation, Pfitzinger *et al.* (1990) were able to show that the chloroplast $tRNA^{Leu}(UAm^7G)$ is able to read the CUU/C codons, and they proposed U:U and U:C wobble pairing as the mechanism. Perhaps similar mechanisms of codon recognition exist for the GCU/C (Ala), CUU/C (Leu) and CCU/C (Pro) codons of *Marchantia* and for the GCU/C (Ala) and CUU/C (Leu) codons of black pine.

(ii). Cytosolic tRNAs

In contrast to mitochondrial or chloropast tRNA genes, relatively few plant nuclear tRNA genes have been isolated and sequenced. The identification of nDNA-encoded tRNA genes relies exclusively on known cytoplasmic tRNA

sequences, which can be used as probes for screening genomic DNA libraries. These sequences are relatively scarce due to the difficulty associated with the isolation of tRNAs and the laborious elucidation of tRNA sequences. Table 1 lists all of the plant nDNA-encoded tRNAs or tRNA genes sequenced to date.

Plant nDNA-encoded tRNAs are typical of other eukaryotic cytosolic tRNAs in that their primary sequences exhibit the standard invariant and semi-invariant nucleotides and they can be folded into the characteristic cloverleaf secondary structure. In general, plant nDNA-encoded tRNAs show greatest sequence similarity with those of animals and low sequence similarity with eubacterial tRNAs. The soybean $tRNA^{iMet}$ gene shares 85-90% sequence identity with $tRNA^{iMet}$ genes isolated from insect, amphibian and mammalian sources, whereas it shares only 70% sequence identity with those from fungi (Palmer and Folk, 1987). Similarly, $tRNA^{Trp}$ and $tRNA^{iMet}$ of wheat germ show 84% and 87% sequence identity with the respective vertebrate $tRNA^{Trp}$ and $tRNA^{iMet}$ (Ghosh and Ghosh, 1984). The wheat germ $tRNA^{Trp}$ shows only 68% sequence identity with $tRNA^{Trp}$ species of yeast and only 54% sequence identity with bacterial species.

As in animals and yeast, nuclear tRNA genes in plants seem to exist as multigene families that are either dispersed throughout the genome or clustered at single chromosomal sites (Maréchal-Drouard *et al.*, 1993). Other features in common with eukaryotic tRNA genes include: 1) a lack of coding sequence for the 3' terminal -CCA (Waldron *et al.*, 1985); 2) the presence of intervening sequences in certain tRNA genes (Fuchs *et al.*, 1992); 3) polymerase III-mediated

Table 1. Cytosolic transfer RNAs or their genes identified in plants

Amino acid	Anti-codon	PLANT SPECIES														
		WHT	BAR	RIC	ARA	RAP	SOY	LUP	TOB	PET	BEA	PEA	POT	SOR	SPI	CUC
Ala	UGC				+											
Arg	ICG	+														
Asn	GUU							+		+						
Asp	GUC						+									
Glu	UUC		+													
Glu	CUC		+					+								
Gly	GCC	+		+				+						+		
His	GUG							+								
Ile	IAU	+						+								
Leu	N/CAA										+		+			+
Leu	UAA							+								
Leu3	NAA										+					
Leu4	NAA										+					
Leu2	NAG										+					
Leu	IAG							+								
Leu	CAG							+								
Lys	UUU	+														
iMet	CAU	+			+		+	+			+					

Table 1. Continued:

Amino acid	Anti-codon	PLANT SPECIES														
		WHT	BAR	RIC	ARA	RAP	SOY	LUP	TOB	PET	BEA	PEA	POT	SOR	SPI	CUC
eMet	CAU	+					+	+								
Phe	GAA	+	+		+	+		+				+				
Pro	AGG										+					
Pro	UGG							+			+					
Ser	A/IGA				+				+						+	
Ser	UGA	+							+							
Ser	CGA							+	+							
Ser	GCU								+							
Trp	CCA	+			+											
Tyr	QUA	+														
Tyr	GUA	+			+			+	+							
Val	A/IAC				+			+								

WHT, wheat; BAR, barley; RIC, rice; ARA, *Arabidopsis thaliana*; RAP, rapeseed; SOY, soybean; LUP, lupin; TOB, tobacco; PET, petunia; BEA, bean (*Phaseolus vulgaris*); PEA, pea; POT, potato; SOR, sorghum; SPI, spinach; CUC, cucumber

Sequence for the majority of these tRNAs and/or their genes can be found in Steinberg *et al.* (1993) except for the following: wheat $tRNA^{Ile}$ (Kusama-Eguchi *et al.*, 1991); tobacco $tRNA^{Ser}$ (IGA), (UGA), (CGA) and (GCU) (Teichmann *et al.*, 1994); lupin $tRNA^{Leu}$ (UAA), (IAG) and (CAG) (Barciszewska *et al.*, 1990); and cucumber $tRNA^{Leu}$ (Jayabaskaran and Puttaraju, 1993).

transcription coupled with the presence of two internal transcriptional control regions that are essential for expression (Reddy and Padayatty, 1988); 4) the presence of oligo(T) stretches in the 3' flanking regions that may serve as transcription termination signals (Teichmann *et al.*, 1994); and 5) a maturation pathway involving precise 5' and 3' endonucleolytic cleavages followed by modification of most bases and finally intron excision (Stange and Beier, 1987).

Given the standard sequence and structure of the plant nDNA-encoded tRNAs and the fact that all sense codons of the genetic code appear in plant cytosolic proteins, it is likely that decoding of the genetic code occurs using the standard "wobble" rules for eukaryotic organisms. Therefore, a minimum of 33 tRNAs is required (including separate initiator and elongator methionine tRNAs). A complete set of tRNAs has not been characterized for any plant species (Table 1). Many of the tRNA isoacceptors constituting the minimal set of 33 have not been identified in any plant species. In fact, there is evidence that the plant nuclear genome encodes more than the minimal set of tRNAs. In *Nicotiana rustica*, four $tRNA^{Ser}$ isoacceptors with the anticodons UGA, CGA, IGA and GCU have been identified (Teichmann *et al.*, 1994). If the standard wobble rules are followed, only three isoacceptors are required to read all six serine codons; thus the tRNA with the UGA anticodon would not be required. Further evidence that more than the minimal set of tRNAs is encoded by the nuclear genome of plants comes from the first extensive study of a total cytosolic tRNA population. Total cytoplasmic tRNAs from cucumber hypocotyls were fractionated by two-

dimensional polyacrylamide gel electrophoresis (Jayabaskaran and Puttaraju, 1993). Of the 56 tRNA species isolated, 32 were identified by aminoacylation as tRNAs specific for 15 amino acids. However, until the sequencing of these tRNAs is completed, the exact number of unique isoaccepting tRNAs in the cytosol of plants cannot be determined.

(iii). Mitochondrial tRNAs

Plant mitochondrial tRNAs have been shown to have three genetic origins. Plant mitochondrial tRNAs considered "native" to the mitochondrion are mtDNA-encoded tRNAs that have 65-80% sequence identity with the homologous eubacterial and chloroplast tRNAs (Joyce and Gray, 1989a). The genes for these "native" tRNAs are assumed to have originated from the eubacteria-like endosymbiont(s) that provided the mitochondrial genome of plant and other eukaryotic cells (Gray and Doolittle, 1982; Gray, 1992). A second group of plant mitochondrial tRNAs with >90% sequence identity to chloroplast tRNAs has been identified. These mtDNA-encoded "chloroplast-like" tRNAs (Joyce and Gray, 1989a) are thought to be the result of incorporation of promiscuous chloroplastic DNA (Ellis, 1982) into the plant mitochondrial genome in the course of evolution (Stern and Lonsdale, 1982). The third genetic source of plant mitochondrial tRNAs is the nucleus. Plant mitochondrial tRNAs with virtually 100% sequence identity to cytosolic tRNAs have been identified in bean (Maréchal-Drouard *et al.*, 1988), potato (Maréchal-Drouard *et al.*, 1990) and wheat (Joyce and Gray, 1989a).

Prior to a detailed discussion of each of these distinct classes of plant

mitochondrial tRNAs, it should be noted that no nuclear DNA-encoded or "chloroplast-like" tRNAs identified to date are expendable with respect to protein synthesis in mitochondria. The nDNA-encoded or chloroplast-like tRNAs have no native mitochondrial counterparts and thus are not redundant (Dietrich *et al.*, 1992). Although no direct evidence is available that these tRNAs are functional in plant mitochondrial protein synthesis, they are processed to their mature size, contain modified nucleosides (Maréchal *et al.*, 1986), can be aminoacylated by mitochondrial aminoacyl-tRNA synthetases (Maréchal-Drouard *et al.*, 1990) and are substrates for 3' terminal -CCA addition by a tRNA nucleotidyltransferase isolated from wheat mitochondria (Joyce and Gray, 1989a). Additionally there is no evidence of selective codon usage in mitochondrial proteins (Schuster and Brennicke, 1985) or aberrant decoding of the genetic code. Therefore at least 33 tRNAs are expected to be required to function in protein synthesis.

"Native" plant mitochondrial tRNAs

"Native" mtDNA-encoded tRNAs and/or their genes have been identified in plants by direct sequencing (either complete or partial), by hybridization to unique oligonucleotide sequences and by aminoacylation. Unlike animal mitochondrial tRNAs, some of which show gross deviations from the standard form (Okimoto and Wolstenholme, 1990), plant mitochondrial tRNAs assume a highly conserved cloverleaf secondary structure and contain the characteristic invariant and semi-invariant nucleotides of the generalized tRNA model (Singhal and Fallis, 1979), with only minor deviations. The 3' terminal -CCA, present in all mature tRNAs,

is not encoded in any of the sequenced plant mitochondrial tRNA genes. Plant native mitochondrial tRNA sequences are substantially more similar to their eubacterial and chloroplast counterparts than to their homologues in fungal and animal mitochondria. Furthermore, native mitochondrial tRNAs appear to contain a low number of post-transcriptionally modified nucleotides, as in eubacteria and chloroplasts (Bjork and Kohli, 1990).

To date, through sequence analysis, hybridization to unique tRNA oligonucleotide sequences, or aminoacylation, 15 native mtDNA-encoded tRNAs have been identified in angiosperms (Table 2). In working with potato mitochondria, Maréchal-Drouard *et al.* (1990) found that a native mtDNA-encoded $tRNA^{Ile}$, identified as such by aminoacylation, hybridized to an oligonucleotide sequence complementary to a maize $tRNA^{"Met"}$(CAU) gene. In *E. coli* (Muramatsu *et al.*, 1988) and *Mycoplasma capricolum* (Andachi *et al.*, 1989), the $tRNA^{Ile}$(LAU) is transcribed from a gene with a methionine-specifying anticodon (CAU), but the cytosine base is post-transcriptionally changed to yield the novel nucleoside, lysidine (L). Subsequent work with the potato $tRNA^{Ile}$ showed the anticodon to be L^*AU (where L^* is a presumptive derivative of lysidine). This tRNA is encoded by a mitochondrial gene possessing a CAU anticodon (Weber *et al.*, 1990). It is likely that all plant mitochondrial native tRNAs identified by sequence similarity to the maize native mtDNA-encoded $tRNA^{"Met"}$(CAU) gene also contain the LAU anticodon, thereby representing isoleucine tRNAs. This group of tRNAs has been listed as $tRNA^{Ile}$(NAU) in Table 2.

Table 2. Mitochondrial transfer RNA or their genes identified in plants

	Amino acid	Anti-codon	PLANT SPECIES													
			WHT	MAI	POT	BEA	PET	SOY	LUP	ARA	TOM	OEN	SUN	SUG	RAP	RIC
Mitochondrial DNA-encoded "native" tRNAs	Asp	GUC	+	+	+											
	Cys	GCA			+		+				+		+	+		
	Gln	UUG	+	+	+		+									
	Glu	UUC	+	+	+		+	+		+						
	Gly	GCC			+		+		+			+				
	Ile[1]	NAU	+	+	+		+									
	Lys	UUU	+	+	+		+					+			+	
	fMet	CAU	+	+	+	+	+	+	+			+				
	Phe	GAA			+	+	+					+				
	Pro	UGG	+	+	+	+	+									
	Ser	GCU	+	+	+		+			+		+				
	Ser	UGA	+	+	+		+			+		+				
	Tyr	GUA	+	+	+	+	+			+	+	+	+			
	Val	nd[2]			a[3]											
	Val	nd			a											

Table 2. Continued:

	Amino acid	Anti-codon	PLANT SPECIES													
			WHT	MAI	POT	BEA	PET	SOY	LUP	ARA	TOM	OEN	SUN	SUG	RAP	RIC
Mitochondrial DNA-encoded "chloroplast-like" tRNAs	Asn	GUU	+	+	+	+	+		+		+		+			
	Cys	GCA	+	+												+
	His	GUG		+	+		+					+	+			
	eMet	CAU	+	+	+	+	+	+		+						
	Phe	GAA	+	+												
	Ser	GGA	+		+		+					+				
	Trp	CCA	+	+	+	+	+					+				
Nuclear DNA-encoded tRNAs	Ala	IGC			+											
	Arg	ICG			+											
	Arg	NCU			+											
	Gly	GCC	+													
	Ile	nd			a											
	Leu1	CAA	+		+	+										
	Leu2	NAG			a	+										
	Leu3	NAA			a	+										
	Leu4	NAA			a	+										
	Leu5	nd			a											
	Leu	AAG											+			
	Val	GAC	+													
	Thr1	nd			a											
	Thr2	nd			a											

Table 2. Continued:

WHT, wheat; MAI, maize; POT, potato; BEA, bean (*Phaseolus vulgaris*); PET, petunia; SOY, soybean; LUP, lupin; ARA, *Arabidopsis thaliana*; TOM, tomato; OEN, *Oenothera berteriana*; SUN, sunflower; SUG, sugarbeet; RAP, rapeseed; RIC, rice

Plant mitochondrial tRNAs appearing in this table were identified by a variety of techniques including aminoacylation, oligonucleotide hybridization and/or complete or partial sequencing of either the tRNA or its gene. [1] This tRNA is encoded by a mitochondrial gene possessing a methionine-specific CAU anticodon and has been identified as such in wheat and maize. However the base in the first position of the anticodon was shown in potato to be post-transcriptionally modified and the tRNA aminoacylated with isoleucine, not methionine. [2] If the anticodon was not determined in any of the plant species, 'nd' appears under the "anticodon" heading. [3] If a tRNA was identified by aminoacylation only, an 'a' appears across from the anticodon that most likely corresponds.

Plant mitochondrial tRNAs identified by partial sequence analysis, oligonucleotide hybridization and/or aminoacylation include: wheat $tRNA^{Ile}$, $tRNA^{eMet}$, $tRNA^{Gly}$, $tRNA^{Leu}$ and $tRNA^{Val}$ (Joyce and Gray, 1989); maize $tRNA^{Gln}$, $tRNA^{Asn}$ and $tRNA^{Phe}$ (Sangaré *et al.*, 1990); all potato tRNAs listed except $tRNA^{Ile}$ and $tRNA^{Leu}$ (Maréchal-Drouard *et al.*, 1990); all petunia tRNAs (Weber-Lotfi *et al.*, 1993); sugarbeet $tRNA^{Cys}$ and rice $tRNA^{Cys}$ (Izuchi *et al.*, 1990). The remaining tRNAs and/or their genes have been completely sequenced. Sequence for most of these tRNAs and/or their genes can be found in Steinberg *et al.* (1993) except for the following gene sequences: tomato $tRNA^{Tyr}$ and $tRNA^{Asn}$ (Izuchi *et al.*, 1990); *Oenothera berteriana* $tRNA^{Lys}$ (Binder *et al.*, 1990); and sunflower $tRNA^{His}$ (Ceci *et al.*, 1989), $tRNA^{Leu}$ (Ceci *et al.*, 1992), $tRNA^{Cys}$, $tRNA^{Tyr}$ and $tRNA^{Asn}$ (Ceci *et al.*, 1994).

Although 15 native mtDNA-encoded tRNAs have been identified in the 14 angiosperm species studied, only 10 of these tRNAs are common to both the monocotyledonous and dicotyledonous plant species studied. In the dicots, $tRNA^{Cys}$(GCA), $tRNA^{Gly}$(GCC), $tRNA^{Phe}$(GAA) and two $tRNAs^{Val}$ (anticodons unknown) are native mtDNA-encoded. These tRNAs have not been found as native tRNA species in monocots.

"Chloroplast-like" plant mitochondrial tRNAs

"Chloroplast-like" tRNAs of the plant mitochondrion represent a distinct group of the mtDNA-encoded tRNAs that are specifically related to tRNA genes of the chloroplast (>90% sequence identity to chloroplastic tRNA genes). Promiscuous chloroplastic DNA sequences are found widely distributed in plant mtDNAs (Stern and Palmer, 1984).

As with the native plant tRNAs, the chloroplast-like tRNAs are conventional in their secondary structures and contain the expected conserved primary sequence motifs of a typical tRNA (Joyce and Gray, 1989a). While some of the chloroplast-like tRNA genes have sustained inactivating mutations in the mitochondrial genome (Dron *et al.*, 1985), others have remained identical or almost identical to their chloroplast counterparts. Although there has been no formal demonstration that the chloroplast-like, mtDNA-encoded tRNAs that have been found are functional in protein synthesis in plant mitochondria, there is strong indirect evidence that this is the case. First, in many instances the mature tRNAs have been directly isolated from mitochondria. These tRNAs contain modified

nucleosides (Maréchal *et al.*, 1986), are substrates for -CCA addition by tRNA nucleotidyltransferase (Joyce and Gray, 1989a), and are present in the mitochondria at levels approximating those of native tRNAs. In addition, no native tRNAs corresponding to the chloroplast-like tRNAs have been found.

As with the native tRNAs the set of chloroplast-like tRNAs present in plant mitochondria varies between monocots and dicots. Of the seven chloroplast-like tRNAs presently identified in plants, five are found in both monocotyledonous and dicotyledonous plant species whereas two are unique to monocots (Table 2). Some of the chloroplast-like mtDNA-encoded tRNA genes may have been transferred before the divergence of monocotyledons and dicotyledons (Izuchi *et al.*, 1990). More data are required to determine whether or not the chloroplast-like tRNA genes common to both monocots and dicots were recruited independently and when during evolution these transfers of genetic information from chloroplasts to mitochondria occurred.

Nuclear DNA-encoded plant mitochondrial tRNAs

Evidence that the mitochondrial genome of angiosperms does not contain all of the tRNA isoaccepting species necessary for protein synthesis has been obtained for wheat (Joyce and Gray, 1989a), bean (*Phaseolus vulgaris)* (Maréchal-Drouard *et al.*, 1988), potato (Maréchal-Drouard *et al.*, 1990), maize (Sangaré *et al.*, 1990) and petunia (Weber-Lotfi *et al.*, 1993). In all instances, mitochondrial tRNAs that hybridize to mtDNA represent a subset of the total mitochondrial tRNA population required for mitochondrial protein synthesis. Although it is possible

that some mitochondrial tRNAs or their genes have escaped detection, it is unlikely that all the missing tRNAs are mtDNA-encoded. In wheat mitochondria, for example, 17 additional tRNAs are required to decode the universal genetic code according to the standard wobble rules.

The first "cytosolic-like" plant mitochondrial tRNA identified was a bean $tRNA^{Leu}(C^*AA)$. This tRNA has sequence identical to its cytosolic counterpart except for the presence of an $O^{2'}$-methylguanosine residue (Gm) at position 18 of the D loop instead of a G (Green *et al.*, 1987), and it was shown to hybridize to nDNA, not mtDNA. The importation of leucine tRNA isoacceptors into plant mitochondria is consistent with the absence of mitochondrial genes for leucine isoacceptor tRNAs in all plant species studied (Table 2). Other tRNAs for which no mitochondrial genes have been found include the alanine, arginine and threonine tRNA isoacceptors. However, as with the native and chloroplast-like tRNAs, it appears that differences between monocots and dicots exist with respect to the tRNAs they import (Table 2). Although limited data are available, cytosolic-like $tRNA^{Gly}$(GCC) and $tRNA^{Val}$(GAC) were identified in wheat mitochondria (Joyce and Gray, 1989a). In contrast, it has been determined that in potato mitochondria, these tRNAs are native (Maréchal-Drouard *et al.*, 1990).

The first intensive study of a plant mitochondrial tRNA population was conducted in potato. Maréchal-Drouard *et al.* (1990) were able to identify, through partial sequence analysis, hybridization and/or aminoacylation, 31 tRNA isoacceptors, 11 of which were cytosolic-like (Table 2). By postulating U:N

wobble, I:N wobble or a two-out-of-three codon recognition pattern (as inferred for chloroplasts; Pfitzinger *et al.*, 1990), these authors concluded that all sense codons can be read by the identified tRNAs. However, as only a single tRNA isoacceptor for the four-codon family of glycine was identified, and its anticodon sequence is GCC, at least one additional tRNA remains to be found.

Indirect evidence that plant mitochondrial tRNAs are indeed imported from the cytosol into the mitochondrion include: a mtDNA-encoded tRNA population insufficient to decode the universal genetic code by means of either standard or expanded wobble base pair recognition; the identification of mitochondrial tRNAs with virtually 100% sequence identity to cytosolic tRNAs; hybridization of mitochondrial tRNAs to nDNA; and the ability to aminoacylate these mitochondrial tRNAs with either mitochondrial or cytosolic enzyme extracts. The latter observation supports both the nuclear origin of these tRNAs and their ability to function in mitochondrial protein synthesis. Direct proof of import is limited to a single *in vivo* experiment (Small *et al.*, 1992). A nuclear gene encoding a bean $tRNA^{Leu}(C^*AA)$ was introduced into potato protoplasts by electroporation. The bean tRNA has two nucleotides in the variable loop that differ from the potato $tRNA^{Leu}(C^*AA)$ sequence. Transcripts of the introduced tRNA gene were detected in both the cytosol and the mitochondria, providing direct proof that a nuclear tRNA gene product can be imported. This weakens the argument that the cytosolic-like tRNAs of plant mitochondria are the products of cryptic mitochondrial genes that remain to be identified.

Mitochondrial tRNAs of Marchantia polymorpha

The complete nucleotide sequence of mtDNA from *M. polymorpha* has been determined (Oda *et al.*, 1992a). Despite the relatively small size (186,608 bp) of this mitochondrial genome compared to angiosperm mtDNA (~200-2400 kbp), the number of mtDNA-encoded tRNA genes is substantially greater in the former than in the latter. In liverwort mtDNA, 29 genes for 27 distinct species of tRNA were identified (Table 3). Most of the liverwort mitochondrial tRNA genes were found in five regions of a section of the circular mtDNA (Oda *et al.*, 1992b). Like other mitochondrial and chloroplast tRNA genes, the 3' terminal -CCA is not encoded in any of the liverwort mitochondrial tRNA genes. However, in contrast to the mitochondrial tRNA genes of angiosperms (in which no introns have been identified), the gene for $tRNA^{Ser}$(GCU) in liverwort mitochondria contains a group II intron. In comparing individual liverwort mitochondrial tRNA sequences with the available sequences of isoaccepting tRNA species of angiosperm mitochondria and chloroplasts, greatest similarity was with native mitochondrial tRNAs (76.2%-94.4% sequence identity). The lower sequence similarity with chloroplast tRNAs (50.3-77.2% sequence identity) led to the conclusion that the liverwort mitochondrial genome contains no chloroplast-like tRNA genes, implying that tRNA gene transfer from angiosperm chloroplasts to mitochondria occurred after the divergence of flowering plants from bryophytes.

All 61 sense codons are used in the genes of known proteins in the liverwort mitochondrial genome. Allowing G:U and U:N base pairing (expanded wobble),

Table 3. Transfer RNA genes and potential codon recognition pattern in the liverwort mitochondrial genome

UUU	Phe	gaa	UCU	Ser	uga	UAU	Tyr	gua	UGU	Cys	gca
UUC	Phe	"	UCC	Ser	"	UAC	Tyr	"	UGC	Cys	"
UUA	Leu	uaa	UCA	Ser	"	UAA	ter		UGA	ter	
UUG	Leu	caa	UCG	Ser	"	UAG	ter		UGG	Trp	cca
CUU	Leu	uag	CCU	Pro	ugg	CAU	His	gug	CGU	Arg	acg
CUC	Leu	"	CCC	Pro	"	CAC	His	"	CGC	Arg	ucg
CUA	Leu	"	CCA	Pro	"	CAA	Gln	uug	CGA	Arg	"
CUG	Leu	"	CCG	Pro	"	CAG	Gln	"	CGG	Arg	"
AUU	Ile	~	ACU	Thr	ggu	AAU	Asn	guu	AGU	Ser	gcu
AUC	Ile	~	ACC	Thr	"	AAC	Asn	"	AGC	Ser	"
AUA	Ile	cau	ACA	Thr	~	AAA	Lys	uuu	AGA	Arg	ucu
AUG	Met	cau	ACG	Thr	~	AAG	Lys	"	AGG	Arg	"
	fMet	cau									
GUU	Val	uac	GCU	Ala	ugc	GAU	Asp	guc	GGU	Gly	gcc
GUC	Val	"	GCC	Ala	"	GAC	Asp	"	GGC	Gly	"
GUA	Val	"	GCA	Ala	"	GAA	Glu	uuc	GGA	Gly	ucc
GUG	Val	"	GCG	Ala	"	GAG	Glu	"	GGG	Gly	"

Codons are in uppercase letters and anticodons of the corresponding tRNA genes, identified by Oda *et al.* (1992b), are in lowercase letters. The codon recognition pattern allows both G:U and U:N wobble base pairing.

~ appears where a tRNA corresponding to the codon has not been identified.

the 27 identified tRNAs can read all but the ACR (threonine) and AUY (isoleucine) codons. However, in two instances, neither G:U nor U:N wobble is required in liverwort. In the UUR leucine codon family, there are two $tRNA^{Leu}$ species with the anticodons UAA and CAA, making U:G recognition unnecessary. In the GGN glycine codon family, two $tRNA^{Gly}$ species with the anticodons GCC and UCC exist, making U:N wobble unnecessary. Allowing U:N wobble, another redundant tRNA of liverwort mitochondria is the $tRNA^{Arg}$(ACG). Therefore, if U:N and U:G wobble are used in translation in liverwort mitochondria, extra tRNAs exist. However, knowing that even with U:N and U:G wobble two additional tRNAs are required to decode the genetic code, it is reasonable to suggest that tRNA import may be occurring in liverwort mitochondria, as in angiosperm mitochondria. Oda *et al.* (1992b) considered several possibilities for recognition of the ACR and AUY codons: 1) the $tRNA^{Thr}$(GGU) and $tRNA^{Ile}$(CAU) recognize the ACR and AUY codons, respectively, by a two-out-of-three mechanism; 2) the genes are present in the mtDNA but were not identified because they do not form the standard cloverleaf structure, or because of the presence of a large intron in the coding region; 3) the genes are not present in the main mitochondrial genome but are located in a plasmid, as in the case of the maize mitochondrial $tRNA^{Trp}$ gene (Leon *et al.*, 1989), although no plasmids have been identified in *M. polymorpha* mitochondria; and 4) the genes are encoded in the nucleus and the tRNAs are imported into the mitochondria.

Although the liverwort mitochondrial genome does not have all the required

tRNA isoacceptors for recognition of the 61 sense codons of the genetic code, it does encode native tRNA genes corresponding to all amino acids. In contrast, no tRNA genes for alanine, arginine, leucine and threonine have yet been found in the mitochondrial genomes of angiosperms. The liverwort mitochondrial genome also encodes native tRNA genes that are chloroplast-like in angiosperms.

Plant mitochondrial tRNA processing

Detection of plant mitochondrial tRNA precursor transcripts by primer extension analysis has proven difficult. Recently Binder and Brennicke (1993b) successfully detected (using guanylytransferase to cap primary transcripts) an *Oenothera* mitochondrial tRNA precursor transcript containing $tRNA^{Phe}$ and $tRNA^{Pro}$ genes, verifying that plant mitochondrial tRNAs are indeed transcribed as larger precursor molecules. In wheat, *in vitro* T3 or T7 RNA polymerase-mediated transcripts containing either native or chloroplast-like mitochondrial sequences have been shown to undergo single specific 5' and 3' endonucleolytic cleavages when incubated in the presence of a mitochondrial soluble extract (Hanic-Joyce and Gray, 1990). The tRNA products have 3' hydroxyl and 5' phosphoryl termini at the expected positions. The $-CCA_{OH}$ terminus is added to the processed 3' termini of tRNAs by a nucleotidyltransferase present in the wheat mitochondrial extract.

Although plant mitochondrial tRNA genes display strong sequence similarity with their eubacterial counterparts, the tRNA genes do not encode the $-CCA_{OH}$ terminus, and the single specific 5' and 3' endonucleolytic cleavages that occur

resemble those observed for nuclear tRNA precursors. This finding is not unexpected as mitochondrial tRNA precursors from animals and yeast are processed in a similar fashion (reviewed by Altman, 1989). In both eubacteria and eukaryotes the 5' leader sequence is removed by the endonuclease RNase P. However in eubacteria the 3' terminus is generated by an endonucleolytic cleavage that occurs at some distance from the 3' terminus, with subsequent trimming by a exonuclease.

The mechanisms for tRNA processing observed in plant mitochondria are also similar to mechanisms described for tRNA processing in chloroplasts (Marion-Poll *et al.*, 1988). It is possible that the same enzymes are involved in tRNA processing in both chloroplasts and mitochondria. The processing enzymes involved may be encoded by single nuclear genes and imported into both organelles (Hanic-Joyce and Gray, 1990).

Transcription of mitochondrial DNA-encoded tRNAs

Plant mitochondrial tRNA genes are generally not clustered or closely linked to other genes (Binder and Brennicke, 1993a). This suggests that most transcription units are monocistronic. The existence of two distinct classes (native and chloroplast-like) of tRNA genes in plant mtDNA and the fact that many of the tRNA genes are distant from any recognizable gene, raises questions about the number, location and nature of promoter elements. Sequence comparisons have so far failed to reveal any eubacteria-like regulatory signals flanking native wheat mitochondrial genes (Joyce *et al.*, 1988). Joyce *et al.* (1988) did identify a 12

nucleotide purine-rich motif beginning a variable distance upstream (70-131 nucleotides 5' of the tRNA coding sequence) of 5 wheat mitochondrial tRNA genes and proposed a consensus sequence (AAGAANRR; R=purine) that might play a role in the initiation of transcription. The same motif has subsequently been found upstream of a number of mitochondrial tRNA genes in other plant species (Maréchal-Drouard *et al.*, 1993). There is, however, no direct evidence that this motif plays a role in the expression of plant mitochondrial tRNA genes (Gray *et al.*, 1992).

In *Oenothera* mitochondria, Binder and Brennicke (1993b) determined the transcription initiation site of the above-mentioned guanylytransferase-capped primary transcript containing $tRNA^{Phe}$ and $tRNA^{Pro}$ genes. This $tRNA^{Phe}$-$tRNA^{Pro}$ gene locus is present in the mtDNA of *Oenothera* in several different arrangements. The sequence surrounding the transcription initiation site for the $tRNA^{Phe}$ gene was compared to putative promoter motifs identified for rRNA and protein-coding genes in *Oenothera* (Binder and Brennicke, 1993b) and soybean (Brown *et al.*, 1991) and was found to be very similar. This observation suggests that the transcription initiation site identified upstream of the gene for $tRNA^{Phe}$ represents the functional promoter for the primary bicistronic transcript of both genes. Binder and Brennicke (1993b) comment that the high sequence similarity of transcription initiation sites of the mRNA, rRNA and native tRNA genes suggests that all three types of RNA molecules can be transcribed by the same RNA polymerase-transcriptional cofactor system in plant mitochondria. In yeast

mitochondria, some tRNAs can be transcribed singly, from their own promoters, whereas other tRNAs are transcribed in clusters or together with protein-coding or rRNA sequences (Schinkel and Tabak, 1989). A single promoter motif is used for the different types of genes and transcriptional units in yeast mtDNA. However in plant mitochondria, investigations by *in vitro* capping and *in vitro* transcription have revealed the presence of several distinct promoter regions for rRNA and protein-coding genes (Gray *et al.*, 1992), with only limited correspondence between mitochondrial transcription initiation sites in different monocots (wheat versus maize) and even less between monocots and dicots (wheat/maize versus soybean).

The internal consensus sequences for an RNA polymerase III-type promoter are largely conserved within plant mitochondrial tRNA genes (Joyce *et al.*, 1988). However, eubacterial and chloroplast tRNA genes also contain these consensus sequences but eubacterial and some of the chloroplast tRNA genes are transcribed from external promoters (Maréchal-Drouard *et al.*, 1991). As sequence comparisons have not revealed any eubacteria-like regulatory signals flanking native wheat mitochondrial genes, it is unlikely that the typical bacterial '-10' and '-35' motifs found upstream of at least some of the chloroplast-like tRNA genes are functional in the mitochondrion (Joyce and Gray, 1989a).

B. Import and export of nucleic acids in non-plant cell organelles

(i). RNA import/export in animal cell organelles

In general, the vertebrate mitochondrial genome has been found to encode all of the RNA molecules necessary to conduct protein synthesis within the

organelle. Therefore it is generally assumed that import of RNA does not occur in vertebrate mitochondria. This is especially true for tRNAs, as most animal mitochondrial genomes contain the 22 tRNA species required to decode their modified genetic code. However there are several examples of RNA import into the mitochondria of animals and one example of export.

Wong and Clayton (1986) isolated from human KB cells an enzyme fraction containing DNA primase and DNA γ polymerase activities; enzymes involved in mtDNA replication. The physical properties of the mitochondrial primase are distinct from those of its nuclear counterpart and thus primases from these two compartments could be distinguished. In contrast to the nuclear primase, the mitochondrial primase is readily separable from the DNA polymerase γ, has a greater sedimentation rate and a nuclease-sensitive component. The major RNA species found in the glycerol density gradient fraction containing the peak DNA primase activity was sequenced and identified as the nDNA-encoded 5.8S rRNA. Nuclear DNA-encoded 5.8S and 5S rRNAs have always been found in substantial quantities in RNAs isolated from animal mitochondrial preparations and had always been attributed to contamination from the cytosol (Tapper *et al.*, 1983). However, increasing mitochondrial purity by sedimentation in density gradients and removal of the outer mitochondrial membrane with digitonin have failed to reduce significantly the amount of 5.8S RNA present in the mitochondrial preparation (Wong and Clayton, 1986).

In 1987, Chang and Clayton characterized a mammalian site-specific

endoribonuclease that cleaves RNA near one of the transition sites of primer RNA synthesis to DNA synthesis, at the leading-strand origin of mitochondrial replication. This endonuclease, termed MRP (mitochondrial RNA processing), was shown to contain a nDNA-encoded RNA component that is essential for its enzymatic activity. However, only a fraction of the total cellular MRP RNA or MRP RNase activity was subsequently shown to be associated with the mitochondrion (Karwan *et al.*, 1991). Most of the MRP RNA is found in the nucleus and MRP RNase isolated from nuclei is involved in processing of 5.8S rRNA in nucleoli (Chu *et al.*, 1994). Whether the level of MRP RNase found in isolated mitochondria is biologically significant or whether it is an extramitochondrial contaminant of the mitochondrial preparation is being debated (Kiss and Filipowicz, 1992; Topper *et al.*, 1992).

Recently, a nDNA-encoded 5S rRNA was identified in bovine mitochondria (Yoshionari *et al.*, 1994). This RNA was found in purified mitochondrial preparations as well as in mitoplasts, even following treatment of the mitochondria or mitoplasts with RNase A. The authors concluded that this RNA species is contained within the mitochondrial inner membrane. The 5S rRNA was also seen in rabbit and chicken mitochondria.

Using *in situ* hybridization techniques, the mitochondrial large subunit rRNA (mtlrRNA) of *Drosophila melanogaster* was found to be localized outside of the mitochondria, in the polar plasm (the posterior pole region of oocytes and cleavage embryos) (Kobayashi *et al.*, 1993). This RNA is believed to have a role in pole

cell formation because: the injection of mtlrRNA into embryos damaged by ultraviolet radiation enables them to resume pole cell formation; the RNA is localized to the polar plasm region of the cell; and the amount of extramitochondrial mtlrRNA decreases during development from the cleavage to the blastoderm stages. Sense probes for the mtlrRNA were not able to detect any extramitochondrial mtlrRNA nor were signals obtained for other mitochondrial rRNAs (e.g., small subunit rRNA). Although mtlrRNA is obviously present in *Drosophila* mitochondria, no hybridization signals within mitochondria were obtained. As hybridization preceded embryo sectioning in this study, mtlrRNA probe was not accessible to mtlrRNA within the mitochondria. The authors speculated that export of mtlrRNA from the mitochondria to the cytosol occurs only in the polar plasm, as this is the only location where hybridization signals were found.

(ii). tRNA import/export in non-plant cell organelles

A requirement for import of tRNAs into the mitochondria of unicellular eukaryotes (protists) is now well documented. In *Chlamydomonas reinhardtii*, a unicellular green algae, only three tRNA genes were found within a sequenced 12.35 kbp region of the 15.8 kbp mtDNA (Boer and Gray, 1988). No additional tRNA genes were found when sequencing of the mitochondrial genome was completed (Vahrenholz *et al.*, 1993). Examination of six protein-coding genes in *C. reinhardtii* reveals the use of 53 codons and no deviations from the standard genetic code. Even assuming a two-out-of-three codon recognition mechanism, a

total of 23 different tRNAs is still required, assuming that separate initiator and elongator $tRNAs^{Met}$ are used.

In the mtDNA of the trypanosomatid protozoa, *Trypanosoma brucei* (Hancock and Hajduk, 1990) and *Leishmania tarentolae* (Simpson *et al.*, 1989), tRNA genes appear to be completely absent. Neither isolated mitochondrial tRNAs nor a variety of nuclear tRNA gene probes, shown to hybridize with both mitochondrial and cytosolic tRNAs, hybridize with *T. brucei* or *L. tarentolae* kinetoplast DNA. Although there may exist cryptic mtDNA-encoded tRNA genes whose transcripts are extensively edited to produce the mature tRNAs, sequences of kinetoplastid nuclear tRNA genes (to which mitochondrial tRNAs hybridize) reveal a typical eukaryotic primary sequence and secondary structure. Extensive computer-assisted searches of the maxicircle sequences have revealed no significant similarities to any of the nDNA-encoded tRNAs (Lye *et al.*, 1993). For both *L. tarentolae* and *T. brucei*, the mitochondrial tRNA population appears to represent a selected set of nDNA-encoded tRNAs.

When mitochondrial tRNAs were isolated from the ciliate protozoan, *Tetrahymena pyriformis* and separated by two-dimensional urea-polyacrylamide gel electrophoresis, at least 36 spots were resolved (Suyama, 1986). However when mitochondrial tRNAs hybridizing specifically to mtDNA were recovered and subjected to a similar electrophoretic procedure, only ten spots were resolved. The tRNA species that did not hybridize to mtDNA could be isolated from nuclease-treated mitochondria and could be aminoacylated with either mitochondrial or

cytosolic enzymes. These tRNAs are presumed to be cytoplasmic tRNAs imported into the mitochondria. Only 7 distinct tRNA genes have been identified in *T. pyriformis* mtDNA, which has now been completely sequenced (G. Burger and M. W. Gray, personal communication).

Except for a few hundred base pairs near the replication terminus, the mitochondrial genome of another protozoan ciliate (*Paramecium aurelia*) has been completely sequenced (Pritchard *et al.*, 1990). Again, far fewer than the minimal set of tRNAs required for protein synthesis were found. Genes for only 3 of the 7 distinct tRNA genes identified in *T. pyriformis* mtDNA were found in *P. aurelia* mtDNA. With the exception of a possible $tRNA^{Met}$ gene (Heinonen *et al.*, 1987), no other tRNA genes were identified.

The complete sequence of the mitochondrial genome of the amoeboid protozoan, *Acanthamoeba castellanii*, has been determined (Burger *et al.*, 1995). Sixteen tRNA genes specific for 13 amino acids have been identified. As 62 sense codons representing all 20 amino acids are found in the protein-coding genes of *A. castellanii* mitochondria, the genome encodes fewer than the minimal number of tRNA species required to support mitochondrial protein synthesis. Additional tRNAs are thought to be imported from the cytosol.

In *A. castellanii* mitochondria a modified genetic code is used in which the UGA codon specifies tryptophan rather than termination. The only mtDNA-encoded $tRNA^{Trp}$ found in *A. castellanii* has a CCA anticodon and should not be able to decode the UGA codon. Because there is no evidence that UGA is used

as a tryptophan codon in *A. castellanii* nDNA-encoded mRNAs, if a nDNA-encoded $tRNA^{Trp}$(UCA) exists, it would presumably not be used in the cytosol but only imported into the mitochondria. Experiments are in progress to determine whether the first anticodon position of the $tRNA^{Trp}$(CCA) is post-transcriptionally modified, possibly allowing for recognition of the UGA codon (Burger *et al.*, 1995).

In a comparison of a two-dimensional urea-polyacrylamide gel electrophoretic pattern of yeast (*Saccharomyces cerevisiae)* mitochondrial tRNAs and a similar electrophoretic pattern obtained from mitochondrial tRNAs that hybridized to mtDNA, only a single difference was observed (Martin *et al.*, 1979). The total mitochondrial electrophoretic pattern contained an additional tRNA species, which was identified as a nDNA-encoded $tRNA^{Lys}$(CUU). Again, extensive purification of the mitochondria, and isolation of tRNA from mitoplasts, failed to remove this tRNA from the resulting mitochondrial RNA preparation. This tRNA is not acylated by the mitochondrial aminoacyl-tRNA synthetase preparation and its function is unknown.

C. Mechanisms of tRNA import

As evidence has accumulated supporting the existence of tRNA import into mitochondria, so have questions surrounding the mechanism. Due to the apparent selective nature of the tRNA import mechanism and the well documented pathway of protein import into mitochondria, it has been postulated that the aminoacyl-tRNA synthetases might serve as carrier proteins in this process (Suyama and

Hamada, 1978). As support for this hypothesis, it was demonstrated in yeast that a single-stranded or double-stranded DNA oligomer (24 nucleotides long) could be imported into the mitochondrion when it was covalently linked via its 5' end to the C-terminus of a mitochondrial precursor protein (Vestweber and Schatz, 1989). However, the fact that the cytoplasmic lysyl-tRNA synthetase is not found in the mitochondria of yeast suggests that the synthetase may not be involved in the transport of the single nDNA-encoded mitochondrial $tRNA^{Lys}$ of yeast (Martin *et al.*, 1979). In *T. brucei*, *in vitro* experiments with a mutant $tRNA^{Tyr}$, which remains unspliced, showed that the tRNA could not be aminoacylated (Schneider *et al.*, 1994a). However, when the corresponding tRNA gene was transfected into the nuclear genome, the mutant tRNA was recovered in the mitochondrial fraction. Recovery of the unspliced, mutant tRNA in the mitochondria of *T. brucei* provides more direct proof of tRNA import in this organism and argues against the alternative explanation that the tRNAs are created by extensive modification of transcripts from cryptic mitochondrial genes. The fact that the unspliced tRNA mutant is imported with comparable efficiency to that of the spliced wild-type $tRNA^{Tyr}$ suggests that the import system tolerates significant deviations from the classic tRNA structure, at least within the anticodon loop. Small *et al.* (1992) showed in a transgenic potato plant that a $tRNA^{Leu}$ gene, mutated by a 4 nucleotide addition to the anticodon loop, was expressed and the product imported into mitochondria. Similar results were obtained in cells of *L. tarentolae* stably transfected with a *Leishmania* plasmid containing either the wild-type nuclear

$tRNA^{Thr}$ gene, a mutant $tRNA^{Thr}$ gene with a 6 nucleotide insertion in the anticodon loop, or a mutant $tRNA^{Thr}$ gene with a 4 nucleotide insertion in the variable arm (Chen *et al.*, 1994). In this case, it was found that the mutated tRNA containing the anticodon loop insertion was imported into mitochondria, although at a lower level than the wild-type suggesting that the import machinery is capable of discriminating against the mutant RNA. In contrast, the extra nucleotides in the variable arm blocked import, suggesting the involvement of this region in the import process. However, the loss of import could also be attributed to an altered tertiary structure, as the mutant tRNA showed a drastic change in gel mobility.

In other work using *Leishmania*, a nDNA-encoded $tRNA^{Gly}$(CUG) was found not to be imported into the mitochondria, whereas two other nDNA-encoded tRNAs were (Lye *et al.*, 1993). The $tRNA^{Gly}$ gene occurs singly and is unusual in that the putative RNA polymerase III transcription temination signal is a string of eight T residues followed by an A and seven C's, whereas the genes for the two imported tRNAs are closely linked and have a putative transcription termination signal of only four T's. The possibility of a relationship between the solitary $tRNA^{Gly}$ gene organization and unusual transcription termination signal on the one hand and the absence of tRNA import on the other was suggested. However in subsequent sequencing of a 2600 bp nDNA fragment containing 10 tRNA genes, no correlation between location of the tRNA gene within the cluster and import was found (Shi *et al.*, 1994).

Other evidence for the lack of aminoacyl-tRNA synthetase involvement in

tRNA import comes from the detection of nDNA-encoded precursor tRNAs in the mitochondria of *T. brucei* (Hancock *et al.*, 1992). These precursor tRNAs could be processed by RNase P and tRNA nucleotidyltransferase and were not found in the cytosol, although corresponding mature tRNA transcripts were. The identification of precursor tRNAs within the mitochondria provides evidence that a precursor tRNA is the substrate for import and it is unlikely that these long precursor tRNAs interact with cognate synthetases.

Several factors involved in the transport of the nDNA-encoded $tRNA^{Lys}$ of yeast into isolated mitochondria *in vitro* have been elucidated. Tarassov and Entelis (1992) tested various fractions of total cellular extract and found that the addition of the S-100 supernatant to the reaction mixture makes the $tRNA^{Lys}$ inaccessible to RNase. This result was not obtained in the absence of mitochondria. Fractionation of the S-100 extract on heparin-Sepharose produced a heparin-binding fraction that alone could not direct import of the tRNA but could enhance the efficiency of import when added to the S-100 extract or to the fraction not retained on heparin. As heparin-binding properties usually correlate with RNA-binding properties, the involvement of an RNA-binding protein is implied, in addition to other protein components. The transport of the cytoplasmic $tRNA^{Lys}$ also required the presence of ATP and an ATP generation system, thereby showing import is an energy-dependent process.

In contrast to the apparent specificity of the yeast tRNA import system, import of small ribosomal RNAs (srRNAs), 5S rRNA and tRNAs into *Leishmania*

mitochondria was demonstrated in an *in vitro* system that required only an isolated mitochondrial suspension, RNA and ATP (Mahapatra *et al.*, 1994). Preincubation experiments indicated that the rate-limiting step in the *in vitro* import system involved the interaction of the RNA with mitochondria, which was followed by rapid import of the RNA species in the presence of ATP. Because soluble cytoplasmic proteins were not required in this system, a membrane-bound RNA binding protein was postulated to be acting as an import receptor.

Through gel retardation assays and competition experiments a factor(s) in *Leishmania* cytoplasm was shown to specifically interact with srRNAs and tRNAs (Ghosh *et al.*, 1994). This RNA binding factor was originally fortuitously identified by its ability to bind the 5'-untranslated region of a β-tubulin antisense transcript. Because this β-tubulin antisense transcript was also shown to be imported into *Leishmania* mitochondria in the above mentioned *in vitro* import assay (Mahapatra *et al.*, 1994), the involvement of this RNA binding factor in importation of RNA, possibly serving as a import receptor, was suggested. When a 27 nucleotide region of the β-tubulin antisense transcript containing a series of hexapurine repeats was deleted, a decrease in both affinity of the RNA for the binding factor and import efficiency was observed. Apparently extracts of *Leishmania* mitochondria also contain this RNA binding factor as an RNA/protein complex was detected by a gel retardation assay following incubation of the ^{32}P-labelled β-tubulin antisense transcript with extracts of purified mitochondria (Mahapatra *et al.*, 1994).

In the early work identifying tRNA species imported into plant mitochondria, a series of bean nDNA-encoded mitochondrial leucine tRNAs were shown to differ from their cytosolic counterparts by a single post-transcriptional modification ($O^{2'}$-methylguanosine (Gm) at position 18 of the mitochondrial tRNA) (Maréchal-Drouard *et al.*, 1988). This modification was postulated to serve as an import signal. However, since then, some cytosolic counterparts of imported mitochondrial tRNAs have been found to contain the Gm_{18} nucleoside (Dietrich *et al.*, 1992). More recently three nDNA-encoded mitochondrial tRNAs of *T. brucei* and their cytosolic counterparts were sequenced and shown to differ by a single post-transcriptional modification of the mitochondrial tRNAs at the conserved C_{32} residue (Schneider *et al.*, 1994b). However, the modified cytidine is not required for import because an unspliced mutant $tRNA^{Tyr}$, purified from the mitochondria of transformed cells, was not modified at that position. The modification was therefore suggested to be involved in adapting the imported tRNAs to specific requirements of the mitochondrial translation machinery.

II. Material and Methods

A. Isolation of wheat embryo mitochondria

(i). Fractionation of wheat embryos from seed

Viable wheat (*Triticum aestivum*) embryos were prepared from pedigree seed of the variety, Katepwa, obtained from the Alberta Wheat Pool, Alberta. The procedures used for fractionation of the wheat seed and purification of the embryos were according to Spencer *et al.* (1992). Basically the wheat seed was ground briefly in a commerical Waring blender and sieved to remove intact seed and fragments of seed both larger and smaller than intact embryos. Bran remaining with the crude embryos was blown away with a forced air stream. The embryos were then further purified by an organic solvent (cyclohexane/carbon tetrachloride; 10:27, v/v) floatation procedure, which separated endosperm fragments from the embryos. The embryos were allowed to air dry and then sieved to remove broken embryo fragments. Any residual bran was blown off. Embryos were kept dry through use of a dessicant and stored at 4°C.

(ii). Germination of wheat embryos

Normally 24 g of embryos were germinated by spreading ten, 2.4 g lots of embryos on 13.5 cm petri plates containing a single layer of Whatman 3MM filter paper that had been prewetted with 16 ml of 1% dextrose. The embryos were allowed to germinate in the dark at room temperature for 24 h.

(iii). Isolation of wheat embryo mitochondria

Mitochondria were isolated from germinating wheat embryos according to the procedure detailed by Spencer *et al.* (1992). I will, however, explain the method used, indicating with bold font the fractions sampled for liquid scintillation counting in experiments that are described in a following section.

All solutions were prepared one day prior to use except that the β-mercaptoethanol and BSA were added immediately before use. All procedures were performed at 4°C.

Germinated embryos (initially 24 g) were ground in a chilled mortar for 5 min with 100 ml of homogenizing medium (0.44 M sucrose, 0.05 M Tris-HCl (pH 8.0), 0.003 M EDTA-Na, 0.001 M β-mercaptoethanol, and 0.1% BSA) and the homogenate was then filtered through 4 layers of cheesecloth. The ground embryos were returned to the mortar and ground again with 100 ml of fresh homogenizing medium and the homogenate filtered as above. This step was repeated a third time. The combined filtrate was filtered through one layer of Miracloth. This filtrate was centrifuged at 1,000*g* for 6 min and the resulting supernatant was centrifuged for 6 min at 2,000*g* in an IEC centrifuge, model PR-6. These two centrifugations are referred to as the **first and second low-speed spins** in pertinent discussions. The pellet of the first low-speed spin is enriched in nuclei and was kept for nDNA extraction as described below.

The supernatant of the second low-speed spin was centrifuged for 20 min at 18,000*g* (**first high-speed spin**) in an IEC B20A centrifuge (872 rotor). The

supernatant of this centrifugation contains cytosolic RNA and is referred to as the **post-mitochondrial supernatant**. Extraction of cytosolic RNA from this fraction is described below.

The crude mitochondrial pellet obtained from the first high-speed spin was resuspended in 70-75 ml homogenizing medium using a Potter-Elvehjem tissue grinder (glass tube and Teflon pestle). The mitochondrial suspension was then centrifuged again at 1,000*g* and then at 2,000*g* as described above. These represent the third and fourth low-speed spins, respectively. The crude mitochondria were then pelleted again by centrifugation at 18,000*g* (**second high-speed spin**) for 20 min as described above.

The mitochondrial pellet was resuspended in a total of 9 ml of homogenizing buffer again using a Potter-Elvehjem tissue grinder and then distributed onto three discontinuous sucrose gradients consisting of a 7.5 ml lower layer of 1.55 M sucrose in Buffer E (0.050 M Tris-HCl, 0.003 M EDTA-Na (pH 8.0), 0.001 M β-mercaptoethanol and 0.1% BSA) and a 15 ml upper layer of 1.15 M sucrose in Buffer E. The gradients were centrifuged for 1 h at 22,500 rpm in a Beckman SW25.1 swinging bucket rotor using a Beckman L8-M ultracentrifuge. The mitochondria form a compact yellow/brown band at the interface of the 1.15 and 1.55 M layers. The upper part of the gradient was removed by aspiration and the purified mitochondria were collected using a bent-needle syringe. The purified mitochondrial fraction was diluted slowly over a 10 min period with 2 volumes of Buffer C (0.05 M Tris-HCl and 0.02 M EDTA-Na (pH 8.0)). The mitochondrial

suspension was then centrifuged at 18,000*g* for 20 min (**third high-speed spin**).

If mtDNA was to be isolated, the crude mitochondrial pellet (from the second high-speed spin) was first treated with DNase to eliminate nDNA contamination. The crude mitochondrial pellet was resuspended in 30 ml homogenizing medium containing 10 mM $MgCl_2$ using the Potter-Elvehjem tissue grinder. DNase I (Sigma) was added to a final concentration of 50 μg/ml. After 1 h incubation at 4°C the suspension was diluted slowly with 2 volumes of Buffer B (0.44 M sucrose, 0.05 M Tris-HCl (pH 8.0), 0.02M EDTA-Na, 0.001 M β-mercaptoethanol and 0.1% BSA). The mitochondria were pelleted by centrifugation at 18,000*g* for 20 min. The DNase-treated mitochondria were resuspended in 60 ml of Buffer B and centrifuged again at 18,000*g* for 20 min. The mitochondrial pellet was then prepared for the sucrose gradient as described above.

(iv). Isolation of wheat embryo nuclei

The crude nuclear pellet obtained from the first 1,000*g* centrifugation during mitochondrial isolation (see above) was washed two times by resuspending the pellet in 25 ml of Buffer A using a Dounce homogenizer and centrifuging at 2,000*g* for 6 min. The pellet(s) was resuspended in 9 ml of Buffer A and layered on top of three discontinuous sucrose gradients and centrifuged as in the mitochondrial purification procedure. Nuclei purified on these sucrose gradients appear as pellets at the bottom of the tubes. The supernatants were gently aspirated and the pellets were each resuspended in 30 ml of Buffer E. (0.05 M

Tris-HCl (pH 8.0), 0.003 M EDTA (pH 8.0), 0.001 M β-mercaptoethanol and 1% BSA)).

B. Isolation of nucleic acids

(i). Isolation of RNA from purified mitochondria

Purified mitochondrial pellets were suspended in 9 ml of Buffer D (0.01 M Tris-HCl (pH 8.5), 0.05 M KCl, and 0.01 M $MgCl_2$; Leaver and Harmey (1973)) and 1 ml of 20% Triton X-100 in Buffer D was added. The sample was vortexed vigorously for 30 sec and then placed on ice for 30 sec. This was repeated 5 times. The mixture was then centifuged at 10,000*g* for 10 min. The supernatant was collected and an equal volume of 2X detergent mix (2% sarkosyl [sodium N-lauroylsarcosinate] and 0.1 M NaCl) was added. Then an equal volume of phenol/cresol mix (phenol/cresol/8-hydroxyquinoline; 500:70:0.5; Parish and Kirby (1966)) which had been equilibrated with 10-50 mM Tris-HCl (pH 7.5 - 8.0) was added. This mixture was vortexed and the phases clarified by centrifugation at 2,000*g* for 5 min. To the collected aqueous phase, solid NaCl was added to a final concentration of 0.5 M. The tube was vortexed until the salt dissolved and the phenol extractions were repeated until insoluble material at the aqueous/phenol interface was no longer evident. Two volumes of 95% ethanol were added to the final aqueous phase and the mitochondrial RNA was precipitated overnight at -20°C. The precipitated RNA was pelleted by centrifugation for 10 min at 12,000*g*, redissolved in 0.5 ml of dH_2O, and extracted with phenol until no material was observed at the interface. To remove residual phenol the mtRNA was

generally precipitated at least twice, then washed with 80% ethanol, dried and redissolved in dH_2O. For long term storage an equal volume of 95% ethanol was added and the RNA was stored at -20°C.

(ii). Isolation of cytosolic RNA

Cytosolic RNA was isolated from the supernatant of the first 18,000*g* centrifugation during isolation of mitochondria (see above). Cytosolic RNA was prepared in a similar fashion to mitochondrial RNA beginning with the first detergent/phenol extraction of mitochondrial RNA.

(iii). Isolation of total cellular RNA

Total cellular RNA was prepared for use in the slot blot hybridization procedure. Embryos (2.5 g) were germinated as described for mitochondrial isolations. The imbibed embryos were ground in a mortar with 10 ml of Buffer C (0.05 M Tris-HCl (pH 8.0) and 0.02 M EDTA (pH 8.0)) and 10 ml of phenol/cresol mix. The homogenate was centrifuged at 18,000*g* for 20 min in an IEC B20 centrifuge. The supernatant was collected and an equal volume of phenol/creosol mix was added. This was then extracted on a horizontal platform shaker for 10 min at 4°C followed by centifugation at 18,000*g* for 10 min. Solid NaCl was dissolved in the aqueous phase to a final concentration of 0.05 M and phenol extraction was repeated until no more material was visible at the interface. The RNA was precipitated with ethanol and washed with 80% ethanol. The RNA was then redissolved in 3 ml of dH_2O, made 0.3 M sodium acetate with 3 M sodium acetate, and extracted with a equal volume of phenol/cresol mix. Phenol

extractions were repeated until no material was visible at the interface and then the RNA was precipitated twice with ethanol.

(iv). Isolation of mitochondrial DNA

The pellets of the DNase-treated, sucrose gradient purified mitochondria were resuspended in 4.7 ml Buffer C and then 0.1 ml of Buffer C containing 1.25 mg of self-digested Pronase (Calbiochem) was added. To lyse the organelles 1.2 ml of Buffer C containing 10% w/v sodium sarkosyl was added . The tube was mixed very gently and incubated at room temperature for 30 min. Following the addition of 2.0 ml of Buffer C containing 4 M CsCl the tube was mixed gently and placed on ice for 1.5 h. The tube was then centrifuged at 2,000*g* for 30 min. To the supernatant 3.2 g of solid CsCl were added and dissolved by inversion.

The addition of ethidium bromide (0.2 ml of a 10 mg/ml solution) was performed in subdued light as were all subsequent steps. The ethidium bromide-containing solution was then layered on a 3.0 ml cushion consisting of Buffer C, 0.2 ml of 10 mg/ml ethidium bromide solution and 1.01 g/ml CsCl. The tube was then centrifuged at 25,000 rpm at 20°C for 36 h in an SW41 rotor in a Beckman L8-70M ultracentrifuge. A long-wave UV (365 nm, black light) lamp was used to detect the DNA band, which was recovered and mixed with an equal volume of water-saturated butanol. The layers were allowed to separate, the butanol phase was discarded and the extractions were repeated until all traces of dye were eliminated. The solution was then diluted two-fold with TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)) and the DNA was precipitated by addition of 2.5 volumes

of 95% ethanol. The precipitated DNA was pelleted by centrifugation at 1,000g for 30 min. The pellet was washed with 80% ethanol, dessicated briefly, and redissolved in TE. The mtDNA was extracted with phenol and re-precipitated from ethanol two times.

(v). Isolation of nuclear DNA

Nucleic acids were extracted from sucrose gradient purified nuclei resuspended in Buffer E, with addition of SDS to 1%, sodium acetate to 0.3 M and an equal volume of phenol mix, followed by ethanol precipitation from the resulting aqueous phase. The precipitated DNA was redissolved in Buffer C (to a final volume of 10.4 ml) and 10.5 g CsCl were added. The tubes were mixed by gentle inversion. Prior to the addition of 0.2 ml of 10 mg/ml ethidium bromide solution the DNA-containing solutions were moved to subdued light. The dye and DNA sample were mixed and transferred to quick seal polyallomer tubes. The DNA samples were then centrifuged at 40,000 rpm for 44 h at 20°C in a Ti50 fixed angle rotor in a Beckman L8-70M ultracentrifuge.

The DNA band was visualized using a long-wave UV lamp and recovered. Butanol and phenol extractions and ethanol precipitations were conducted as described for mtDNA.

C. Radioactive end labelling of RNA and oligonucleotides

(i). 3' end-labelling using RNA ligase

Crude [γ-^{32}P]ATP (7,000 Ci/mmol) was used to synthesize [5'-^{32}P]pCp using polynucleotide kinase (Walseth and Johnston, 1979). RNA was then labelled

at the 3' end using [5'-^{32}P]pCp and RNA ligase according to Peattie (1979).

(ii). 3' end-labelling using wheat tRNA nucleotidyltransferase

Wheat tRNA nucleotidyltransferase was isolated from sucrose gradient purified mitochondria according to Hanic-Joyce and Gray (1990). As this enzyme specifically labels tRNA, total mitochondrial RNA could be used in the reaction to obtain 3' end-labelled tRNA. To label 80 µg of total mitochondrial RNA the RNA was dissolved in 150 µl of dH_2O and 20 µl of reaction mix (20 mM Tris-HCl (pH 8.0), 0.1 mM CTP, and 10 mM $MgCl_2$) were added. Approximately 2 mCi of [α-^{32}P]ATP (specific activity 800 mCi/µm) were desiccated, redissolved in 150 µl of dH_2O and added to RNA mixture. The wheat tRNA nucleotidyltransferase was then added (80 µl of a DEAE-Sephacel column fraction). The reaction mixture was then placed in a 25°C waterbath for 2 h. Following incubation the solution was made 0.3 M sodium acetate and extracted with an equal volume of phenol/cresol mix. The collected aqueous phase was extracted with phenol again and then precipitated twice with ethanol.

The same procedure was used to specifically end label cytosolic tRNAs. In experiments to determine the amount of radiolabelled cytosolic tRNA in various fractions of a mitochondrial preparation, the labelled cytosolic tRNA (following phenol extractions) was separated from unincorporated [α-^{32}P]ATP by centrifugation through a G50 Sephadex column. The eluate obtained was centifuged through a second G50 Sephadex column before the final ethanol precipitations.

For the dot blot hybridizations mitochondrial tRNA labelled with wheat tRNA nucleotidyltransferase was dissolved in 10 µl of dH_2O and 10 µl of loading buffer (Peattie, 1979), heated at 65°C for 5 min and electrophoresed in a 6 % polyacrylamide, 7 M urea gel (20 cm by 20 cm by 0.15 cm) in 1X TBE (25 mM boric acid, 5 mM EDTA and 25 mM Tris-HCl (pH 8.3)). The gel was prerun at 350 V for 2 h and run 1 h at 500 V. The tRNAs were detected by autoradiography and extracted from the gel according to Rubin (1973). Basically this involved homogenizing the gel slice in 2 ml of a high salt buffer (0.01 M Tris-HCl (pH 9.1), 0.5 M NaCl and 10 mM EDTA-Na_2) and 2.0 ml of phenol/cresol mix, followed by shaking for 20 min at 4°C. The aqueous phase was collected following centrifugation and re-extracted several times with phenol/cresol mix before ethanol precipitations.

(iii). 5' end-labelling of oligonucleotides

Oligonucleotides specific for tRNAs were purchased from the Regional DNA Synthesis Laboratory, University of Calgary, Canada. They were generally 20 nucleotides long and contained 5'-hydroxyls that were labelled using polynucleotide kinase and [γ-^{32}P]ATP under standard conditions (Geliebter, 1987), except that spermidine was used at a final concentration of 1 mM. Sequences of the oligonucleotides are presented in Table 4.

D. 2-D and 3-D gel electrophoresis

(i). Method I (radiolabelled tRNAs)

Transfer RNAs that were radiolabelled could be separated in a two-

Table 4. Sequences of tRNA-specific oligonucleotides used for the slot blot analysis and/or reverse transcriptase sequencing procedures

tRNA	Complementary position in tRNA	Sequence of oligonucleotide 5' ---------- 3'
Phe(GGA)	72-53	CAAGCTAGGTGCGAGTGGCG
Pro(UUG)	73-54	TCAAGGTGACAGGATTCGAA
Ala(GGc)	73-55	TGGAGAUGTGGGGTATCGA
Ala1(uGX)	69-55	GATGAGGGGGATCGA
Ala2(XGX)	71-55	GAGGTGCGGGGAATCGA
Arg(ICG)	73-54	CGACTCCGCTGGGGATCGAA
Arg(CCU)	72-53	GCGCCAGGTAGGGGTCGAAC
Asp(GUC)[1]	73-54	CGCCGTTGCCGGGGATCGAA
Gly(GCC)	50-30	GGGTCTGTACCGTGGCAGGG
Gly(UCC)	73-54	TGCGTCTGCCGGGAGTCGAA
His(GUG)[1]	70-51	GGCTGCTGGGATTCGAACCC
Ile(GAU)	71-52	GCCCATACAGGTCTUGAACC
Leu(GAG)	72-53	ACAAGAGUGGGAUTTGAACC
Leu(UAG)	72-53	GACAGCTGTGGGATTTGAAC
Leu1(XAA)	72-53	TCAGAAGTGGGGTTTGAACC
Leu4(XAA)	72-53	CTGGCTGTGGGGTTCGAACC
Val(GAC)	73-54	TGGCTTCGCCCGGGTTCGAA
Val(UAC)	71-52	TCTGAGCCCAGGTTCGAACT
Asp(GUC)[2]	73-54	CGGGGAAAACGGGGTTCGAA
His(GUG)[2]	73-54	GGCGAACGACGGGAATTGAA
His(GUG)AS[3]	73-54	TTCAATTCCCGTCGTTCGCC

[1]and [2] denote the nDNA- and the mtDNA-encoded tRNAs, respectively

[3] denotes the antisense oligonucleotide sequence specific for the mtDNA-encoded $tRNA^{His}$. This sequence is identical to the tRNA sequence.

dimensional (2-D) gel electrophoresis procedure developed for yeast tRNAs (Fradin *et al.*, 1975). A 10% polyacrylamide, 4 M urea gel (32 cm by 40 cm by 0.4 mm) was used to electrophorese up to 5 μg radiolabelled tRNA. (All gels described in this section were prepared in 1X TBE (50 mM boric acid, 1 mM EDTA and 50 mM Tris-HCl (pH 8.3)) and polymerized using ammonium persulphate and TEMED). The gel was run at 500 V until the xylene cyanol marker dye had migrated 35 cm (~ 26 h). After autoradiography, the region of the gel containing the tRNAs was excised as a single slice. The slice was then placed on a glass plate (40 cm by 32 cm) approximately 5 cm from the top of the plate and another glass plate was placed on top. The two glass plates were then clamped together in a manner similar to regular gel preparation. A 20% polyacrylamide, 4 M urea gel solution was then poured between the glass plates avoiding the gel slice. The gel solution was poured until it reached within 2 cm of the gel slice. A small amount of TBE was gently layered on top of the 20% gel solution to ensure proper polymerization. Polymerization generally took 10 to 15 min using 650 μl 10% ammonium persulphate and 20 μl of TEMED per 100 ml of gel solution, as catalysts. The TBE was then removed using a filter paper wick and a 10% poylacrylamide, 4 M urea gel solution was poured on top of the 20% polyacylamide gel and around the tRNA-containing gel slice. Following polymerization this gel was electrophoresed at 4°C for 160 h at 500 V. An autoradiograph showing the 2-D electrophoretic profile could then be obtained.

(ii). Method II (unlabelled tRNAs)

The method above could be used to separate tRNAs for sequence analysis; however, only a very small amount of each tRNA is obtained and if one does not know which particular tRNA is of interest, a large number of tRNAs must be sequenced in a very short time as the tRNAs are already radioactively labelled.

To obtain a larger amount of each tRNA, a 2-D gel electrophoresis procedure based on the method of Burkard *et al.* (1982) was adapted for use. Both the 10% and 20% gel solutions described above were used for the first and second dimensions, respectively; however, the gels were 0.75 mm thick and stacking gels were employed. For the first dimension, the 10% polyacrylamide, 4 M urea gel solution was poured between two glass plates, leaving approximately 5 cm at the top of the gel for the 5% stacking gel solution (5% polyacrylamide, 4 M urea and 0.5 mM Tris-HCl (pH 6.7)), which was poured following the polymerization of the 10% polyacrylamide gel solution.

In order to achieve good resolution of a large amount of tRNA, wheat mitochondrial RNA was salt fractionated. This involved dissolving total mitochondrial RNA in dH_2O to a concentration of 4-5 mg/ml. Solid NaCl was added to a final concentration of 1 M and the solution was stored at $4^{o}C$ for at least 8 h. The RNA solution was then centrifuged for 10 min at 3,000*g* and the salt-insoluble RNA was precipitated again, as above. Following centrifugation the supernatants containing the salt-soluble RNAs (5S and tRNA) were combined, precipitated twice with ethanol, extracted with phenol and precipitated with ethanol two more times.

Approximately 125 µg of salt-soluble RNA was loaded on the gel for the first dimension electrophoresis. The RNA was dissolved in 10 µl of dH_2O and 10 µl of loading buffer (Peattie, 1979). The gel was electrophoresed for 26 h at 500 V. From a knowledge of the 2-D gel electrophoretic pattern obtained with radiolabelled tRNA, it was possible to delineate the region containing the tRNA and excise as it as a single gel slice. However, the tRNAs could also be located using ultraviolet light.

The gel slice from the first dimension was then placed on a glass plate in a similar manner to that described above. The 20% poylacrylamide gel solution was poured below the gel strip, allowed to polymerize and then a 10% stacking gel solution (10% polyacrlyamide, 4 M urea, 0.5 mM Tris-HCl (pH 6.7)) was poured on top of the 20 % polyacrylamide gel solution and around the gel slice. The gel was run at 4°C for 160 h at 500 V. The gel was stained with ethidium bromide solution (1 µg/ml) and the spots visualized under ultraviolet light. The tRNA-containing spots in the gel were excised and placed in eppendorf tubes containing 400 µl elution buffer (10 mM magnesium acetate, 0.5 M ammonium acetate and 1 mM EDTA) and 400 µl phenol/cresol mix (Schnare *et al.*, 1985), and 10 µg linear polyacrylamide carrier (Gaillard and Strauss, 1990) were added. These tubes were shaken at 4°C for 20 h, then centrifuged for 5 min and the aqueous phases moved to clean eppendorf tubes. The tRNAs were then precipitated by addition of 2 volumes ethanol. The tRNAs could be stored in this form at -20°C but were precipitated with ethanol again before [5'-^{32}P]pCp end-labelling.

Following radioactive end-labelling of the tRNAs, they were electrophoresed in a 6% polyacrylamide, 7 M urea gel run at 1600 V for 3 h (the xylene cyanol migrates approximately 25 cm in 3 h). Following autoradiography the tRNA bands were excised and eluted from the gel slice as described above.

E. Sequence analysis

(i). Chemical sequencing of 3' end-labelled tRNA

Chemical sequencing of the 3' end-labelled tRNAs was based on the procedures of Peattie (1979). This method generates base specific modifications, following which strand scission is induced by treatment with aniline acetate.

The labelled tRNAs were dissolved in 26 µl dH_2O and 50 µg of *E. coli* tRNA was added as carrier. The RNA samples were heated at 65°C for 5 min and five, 5 µl aliquots were taken for the G, C, A, U and alkali reactions. The aliquots were placed on ice and remained on ice unless otherwise stated. All precipitations were at -70°C.

For the A reaction, 200 µl of 50 mM sodium acetate (pH 4.5) were added to the 5 µl of RNA followed by the addition of 1 µl of diethylpyrocarbonate. The mixture was incubated at 90°C for 3.5 min. The reaction products were precipitated by the addition of 50 µl 1.0 M β-mercaptoethanol, 1.5 M sodium acetate and 750 µl 95% ethanol. Following centrifugation the pellet was dissolved in 200 µl 0.3 M sodium acetate and precipitated with 3 volumes of 95% ethanol.

For the G reaction, 200 µl of 50 mM cacodylic acid (pH 5.5) were added to the 5 µl of RNA followed by the addition of 1 µl of dimethylsulfate. The

mixture was incubated at 90°C for 1 min. The reaction products were precipitated by the addition of 50 µl 1.0 M β-mercaptoethanol, 1.5 M sodium acetate and 750 µl 95% ethanol. Following centrifugation the pellet was dissolved in 200 µl 0.3 M sodium acetate and precipitated with 3 volumes of 95% ethanol. The pellet was then washed with 80% ethanol, desiccated, and dissolved in 5 µl dH_2O. Ten µl of 1.0 M Tris-HCl (pH 8.0) and 5 µl 0.53 M sodium borohydride were added and the sample was placed in the dark for 30 min. Following addition of 100 µl of 3 M acetic acid (titrated to pH 4.7 with NaOH) the sample was precipitated with 300 µl of 95% ethanol.

For the C reaction, the 5 µl aliquot containing the RNA was frozen at -70°C and then lyophilized. The lyophilized RNA was dissolved in 10 µl 3 M NaCl in hydrazine, incubated on ice for 20 min and the products precipitated with 0.5 ml of 80% ethanol. Following centrifugation the pellet was dissolved in 200 µl 0.3 M sodium acetate and precipitated with 3 volumes of 95% ethanol.

For the U reaction, the 5 µl aliquot containing the RNA was frozen at -70°C and then lyophilized. The lyophilized RNA was dissolved in 10 µl of 50% hydrazine and incubated on ice for 8 min. The reaction products were precipitated by the addition of 200 µl 0.3 M sodium acetate and 750 µl 95% ethanol. Following centrifugation the pellet was redissolved in 200 µl 0.3 M sodium acetate and precipitated with 3 volumes of 95% ethanol.

For strand scission the RNA pellets obtained following centrifugation were washed with 80% ethanol, dessicated, redissoved in 6.4 µl dH_2O and 13.6 µl of

aniline acetate (aniline:glacial acetic acid:dH_2O; 1:3:3.5) were added. The samples were incubated at 65°C for 20 min in the dark. All samples were lyophilized several times, dissolved in gel loading buffer and stored at -70°C until used.

For the alkali reaction an equal volume of 95% ethanol was added to the 5 µl RNA sample and the mixture was then dessicated. The tRNA was resuspended in 20 µl 0.15 M ammonium hydroxide, incubated at 90°C for 1 min and then chilled on ice. The sample was frozen at -70°C, lyophilized, redissolved in 20 µl dH_2O and lyophilized again. The RNA was then dissolved in gel loading buffer.

(ii). Reverse transcriptase sequencing

Dideoxynucleotide sequencing of RNA using ^{32}P end-labelled oligonucleotide primers was as described by Geliebter (1987). Mitochondrial RNA (~10 µg) was annealed to 5 ng of the radiolabelled oligonucleotide at 5°C below the Td, where Td = 4(G+C) + 2(A+T) (Zeff and Geliebter, 1987). The annealed oligonucleotide then served as a primer for avian myeloblastosis virus (AMV) reverse transcriptase and in the presence of dNTPs and ddNTPs the cDNA transcript was directly sequenced. The dNTPs and ddNTPs were included in the reaction mix at the concentrations indicated by Geliebter (1987) whereas actinomycin D was omitted. The stop solution used was 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol.

(iii). Sequencing gel electrophoresis

All sequencing reactions for the tRNAs were electrophoresed in a 10% polyacrylamide, 7 M urea, 40% (v/v) formamide, 1X TBE2 (90 mM Tris-HCl, 90

mM boric acid, 2 mM EDTA (pH 8.0)) gel. The gels were prerun for 1 h at 2000 V and a 1-2 µl sample of the RNA sequencing reactions (heated at 80°C for 5 min) was loaded per well. The gels were run at 65 W (3000 to 3500 V) until the xylene cyanol reached 17 cm (~2 h). The temperature of the gel as measured on the outside of the glass plate was 50°C. (Tempered glass plates were used for sequencing gel preparation.)

(iv). Thin layer chromatography

Thin layer chromatography was used to determine the anticodon sequence of a wheat mitochondrial $tRNA^{Arg}$. This tRNA was partially hydrolyzed in alkali as described above except that no *E. coli* tRNA was added as carrier and hydrolysis was for 30 sec. The 3' fragments (reaction products) of the partially hydrolyzed tRNA have 5' hydoxyls that can be end-labelled using [γ-^{32}P]ATP and polynucleotide kinase (PNK). This involved dissolving the lyophilized RNA reaction products in 4.4 µl of TE and adding 1 µl of 10X kinase buffer (500 mM Tris-HCl and 100 mM $MgCl_2$ (pH 7.6)) and 0.4 µl of 25 mM spermidine HCl. This mixture was heated at 65°C for 3 min and cooled quickly on ice. Following the addition of 2 µl DMSO, 1 µl DTT and 0.6 µl [γ-^{32}P]ATP (7000 Ci/mmol), 0.5 µl PNK (~5 units) was added and the reaction incubated at 37°C for 45 min. The mixture was then made 2 M ammonium acetate, 2 µg of *E. coli* tRNA was added as carrier and the products were precipitated with 40 µl of 95% ethanol. The products were then precipitated twice more before being electrophoresed in a formamide-containing sequencing gel as described above.

Following autoradiography of the gel, the bands in the region of the anticodon sequence were excised and eluted as described for tRNAs resolved in the 2-D gel electrophoresis procedure except that 5 μg of *E. coli* tRNA was added as carrier instead of linear polyacrylamide. The RNA reaction products were then completely digested to nucleoside 5'-monophosphates (pN) with ~100 ng nuclease P1 in 10 μl 50 mM ammonium acetate by incubation at 50°C for 2 h (Beier *et al.*, 1984).

The 5' nucleotide of each eluted fragment was identified by cellulose thin layer chromatography using a two solvent system. Two cellulose coated plastic plates (Eastman Chromagram) containing fluorescent indicator were first dipped in a 10% saturated ammonium sulfate solution and dried before use. The hydrolysates of eluted tRNA fragments and marker nucleotides (pG, pA, pU, pC and pI) were applied to the cellulose plates. One plate was then chromatographed in a 95% ethanol:dH_2O (4:1) solvent (Lane, 1963) and the other plate was chromatographed in a saturated ammonium sulfate:2-propanol (40:1) solvent (Singh and Lane, 1964). Migration of the marker nucleotides was visualized with a short-wave UV lamp and ^{32}P-labelled terminal nucleotides were detected by autoradiography.

F. Southern hybridizations

(i). Dot blot hybridizations

Nuclear and mitochondrial DNA were hydrolyzed with either *Eco*RI (GIBCO, BRL) or *Hin*dIII (Pharmacia) using the conditions specified by the

enzyme suppliers except incubation time was a minimum of 4 h. Following hydrolysis, the reaction mixtures were made 0.3 M sodium acetate, extracted with an equal volume of phenol/cresol mix and precipitated with ethanol. Three nDNA blots each containing 80 μg of *Eco*RI digested DNA, 80 μg of *Hin*dIII digested DNA and 20 μg of uncut DNA were prepared by dissolving the nDNA in 15 μl of 0.4 M NaOH and spotting onto a 2 cm diameter disc of nylon membrane (Biotrans, ICN Biomedicals Inc.) in 1.5 μl applications. Similarly, two blots each containing 40 μg of *Eco*RI digested mtDNA, 40 μg of *Hin*dIII digested mtDNA and 20 μg of uncut mtDNA, were prepared. The blots were placed on filter paper saturated with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 5 min and then placed on filter paper saturated with neutralizing solution (3 M sodium acetate (pH 5.5)) for 5 min. The blots were allowed to air dry and were then baked at 80°C for 1 h.

Southern hybridizations of mitochondrial RNA to the dot blots were performed as suggested by the membrane supplier (ICN) using gel purified mitochondrial RNA labelled using [α-^{32}P]ATP and wheat tRNA nucleotidyltransferase as described above.

The blots were prehybridized for 2 h at 42°C in 0.5 ml of hybridization solution (5X Denhardt's solution (0.1% Ficoll, polyvinylpyrrolidone, BSA), 5X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 250 μg/ml herring sperm DNA and 50% v/v formamide). The hybridization buffer was removed following the prehybridization and 0.5 ml

of fresh hybridization buffer containing the labelled tRNA was added to each blot. Hybridizations were performed in a water bath for ~16 h at 42°C. The blots were washed at 42°C, twice in 1 ml of 2X SSC and 0.1% SDS, for 30 min each time and then washed in 0.1X SSC and 0.1% SDS for 30 min.

Following the washes the tRNAs that had hybridized to the membrane-bound DNA were removed by washing the blots in 500 µl of 1X TE containing 0.1% SDS at 85°C for 1 h with shaking. The blots were removed and the eluted tRNA precipitated. The blots were hybridized twice more and the tRNA obtained was pooled from the respective mtDNA or nDNA blots. The precipitated tRNA contained some DNA that was removed from the blot during tRNA extraction. To eliminate the possibility that the DNA might interfere with the subsequent 2-D gel electrophoretic profile the tRNA was again purified by gel electrophoresis as described previously.

(ii). Southern hybridization analysis with tRNA-specific oligonucleotides

Wheat nDNA and mtDNA were digested with restriction endonucleases (*Eco*RI (GIBCO, BRL); *Hin*dIII (Pharmacia); and *Sal*I (Boehringer Mannheim)), according to the instructions supplied by the manufacturer. For each reaction 0.25 µg of mtDNA and 25 µg of nDNA were incubated with the appropriate enzyme for at least 7 h at 37°C. The reaction mixtures were then extracted with phenol, precipitated with ethanol and resolved in a 0.7% agarose gel (17.7 cm by 20.3 cm by 0.5 cm) in 90 mM Tris-HCl, 90 mM boric acid and 2 mM EDTA-Na_2 (pH 8.0). Electrophoresis was for 20 h at 25 V. The gel was stained for 30 min in ethidium

bromide (1 μg/ml), destained for 15 min and photographed. Transfer to a nylon membrane was according to the manufacturer's instructions (Biotrans, ICN Biomedicals). Briefly, the gel was incubated at room temperature for 10 min in 0.25 M HCl. This was repeated with fresh HCl, following which the DNA was denatured by incubating the gel in 0.5 M NaOH and 1.5 M NaCl for 45 min at room temperature with agitation. The gel was then neutralized by incubating in 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.4), again at room temperature with gentle agitation for 30 min. DNA transfer proceeded overnight in 20X SSC and the membrane was baked at 80°C for 2 h.

The blots were rinsed in 2X SSC and then prehybridized for 2 h at the hybridization temperature (see below) in 5 ml of hybridization solution (10X Denhardt's solution (0.2% Ficoll, polyvinylpyrrolidone, BSA), 5X SSC, 20 mM sodium phosphate (pH 6.5), 7% SDS, and 100 μg/ml herring sperm DNA). The oligonucleotide probe (0.1 μg) was 5' end-labelled as described above, heated at 80°C for 5 min and added to the hybridization solution containing the blot. Then 1 ml of 50% dextran sulphate was added. Hybridizations were performed in a Hybaid oven for ~16 h at 5 to 7°C lower than the calculated dissociation temperature (Td) of the oligonucleotide probe (Zeff and Geliebter, 1987).

The blots were washed for 60 min in 50 ml of 3X SSC, 10 mM sodium phosphate (pH 6.5), 10X Denhardt's and 5% SDS, at the hybridization temperature, in the Hybaid oven. This was followed by two, 30 min washes in 50 ml of 1X SSC, 1% SDS, at the same temperature.

Hybridizations and washings at lower temperatures were tried in an attempt to obtain a signal with the oligonucleotides specific for nDNA-encoded tRNAs. Oligonucleotides were also 3' end-labelled using terminal transferase (Promega) and [α-^{32}P]dATP (6000Ci/mmole). Oligonucleotide (1 µl of ~2 pmole/µl) was mixed with 20 pmoles (6 µl) of [α-^{32}P]dATP, 6 µl of 5X TdT buffer (Promega), 3 µl of 2 mg/ml BSA and 13 µl of dH_2O. Ten to 20 units of terminal transferase were then added and the reaction mixture incubated at 37°C for 30 min. To prevent hybridization of the poly A tail to T tracts in the DNA, poly A at a final concentration of 50 µg/ml was added to the hybridization solution during the prehybridization period.

G. Slot blot preparation, hybridization and analysis

Slot blots were prepared using mitochondrial, cytosolic and total cellular RNA. As a control some of the blots contained cytosolic and total cellular RNA that had been treated with DNase. For DNase treatment, RNA at a ratio of 1 µg to a least 1 unit of DNase I (35 units/µl; RNase-free) was incubated in 50 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$ in a total volume of 100 µl at 37°C for 20 min. Following incubation, samples were diluted with 100 µl dH_2O and extracted with phenol. The RNA was precipitated with ethanol three times, dissolved in dH_2O and quantified by measuring ultraviolet absorbance (A_{260}). To confirm the success of the DNase I treatment RNA samples were resolved in agarose gels and stained with ethidium bromide (1 µg/ml).

To prepare RNA samples for the slot blot, 12 µg of RNA were dissolved

in 50 µl of TE and added to 250 µl 20X SSC, 100 µl 37% (v/v) formaldehyde and 100 µl dH_2O. This mixture was heated at 65°C for 15 min and immediately cooled on ice. The RNA-containing solution was then serially diluted seven times adding an equal volume of 10X SSC each time to produce 1:2 to 1:128 fold dilutions. RNA samples on the resultant slot blot ranged from 6 µg to 0.047 µg. The RNA samples were applied to a Biotrans nylon membrane (ICN Biomedicals) using a Bio-Dot SF blotting apparatus (Bio-Rad) according to the instructions supplied by Bio-Rad, using 10X SSC as the wash solution. The membrane was baked for 2 h at 80°C.

Hybridization using oligonucleotides and washing of the blots were as described above for the Southern hybridization with oligonucleotides.

The blots were autoradiographed using Kodak X-Omat K X-ray film without intensifying screens. The autoradiographs were scanned using an Apple Colour One scanner and Ofoto 2.0 program. The intensity of each band was then determined using the NIH Image Analysis program and the linear regression coefficients were calculated using MacCurveFit.

III. Results

A. Purity of wheat mitochondrial tRNA

(i). Estimation of the amount of cytosolic tRNA present in mitochondrial RNA preparations

To assess the level of cytosolic tRNA present in mitochondrial RNA isolated from wheat embryos, 40 μg of wheat cytosolic RNA (3' end-labelled with 1 mCi of [α^{32}P]ATP and wheat mitochondrial tRNA nucleotidyltransferase) were added to homogenizing medium containing 1 g of wheat embryos that had been germinated for 24 h. The embryos were then ground and taken through the standard protocol for wheat mitochondria isolation and RNA extraction as outlined in sections A and B of the Material and Methods except volumes and amounts of reagents used were reduced to reflect the initial weight of embryos (1 g instead of 24 g). The results of scintillation counting of various preparative fractions for two experiments are presented in Table 5A. The majority of the radioactivity present initially is lost through the first three centifugations of the embryo homogenate (1000, 2000 and 18,000*g* spins). The mitochondrial pellet of the first high-speed spin (18,000*g*) contains less than 10% of the initial radiolabelled RNA.

Cytosolic RNA was extracted from the post-mitochondrial supernatants of the first high-speed spins and the specific activities determined (Table 5B). From the specific activities, the amount (μg) of cytosolic RNA in the mitochondrial RNA

Table 5A. Scintillation counting of subcellular fractions and cytosolic and mitochondrial RNA preparations following addition of radiolabelled cytosolic RNA to a wheat embryo mitochondrial RNA isolation procedure[1].

	Total counts per minute [2]						
	Initial [3]	Supernatant [4]				cyt RNA [5]	mt RNA [6]
Experiment		1st or 2nd low-speed spin	1st high-speed spin	2nd high-speed spin	3rd high-speed spin		
1	2,131,596	1,463,920	1,349,729	15,872	344	419,805	941
2	4,581,800	3,325,000	2,744,820	33,726	1,085	741,980	1,025

[1] The procedure used to isolate wheat embryo mitochondrial RNA is detailed in the Material and Methods, sections A and B. Radiolabelled cytosolic RNA was added to germinated wheat embryos as described in the text.

[2] Total counts per minute were determined through liquid scintillation counting of ^{32}P-labelled cytosolic RNA present in the RNA preparations or supernatants as described below.

[3] Total counts per minute in the ^{32}P-labelled cytosolic RNA (added to the wheat embryos).

[4] Total counts per minute in the supernatants of either the first or second low-speed spin (1,000 or 2,000*g*) and the first, second and third high-speed spins (18,000*g*). See Material and Methods, section A(iii) for details. The fractions for which the total number of counts were determined are highlighted in the Material and Methods with bold font for ease in identification.

[5] and [6] Total counts per minute in the final, isolated cytosolic and mitochondrial RNA preparations, respectively. See sections B(i) and (ii) of the Material and Methods.

Table 5B. Determination of the percent cytosolic RNA content of mitochondrial RNA preparations

Experiment	Yield		Specific activity of cyt RNA[3] (cpm/µg)	cyt RNA in mt RNA[4] (µg)	Percent cyt RNA in mt RNA[5]
	cyt RNA[1] (µg)	mt RNA[2] (µg)			
1	5,848	55	72	13	24
2	4,992	38	149	7	18

[1] and [2] Yield (as determined from A_{260}) of the final, cytosolic and mitochondrial RNA preparations, respectively. See sections B(i) and (ii) of the Material and Methods.

[3] Specific activity of the cytosolic RNA was determined by taking the total counts per min or the cytosolic RNA preparation (see Table 5A) and dividing by the yield of cytosolic RNA (µg); *e.g.* 419,805 ÷ 5,848 = 72.

[4] Amount of cytosolic RNA (µg) in the mitochondrial RNA was determined by taking the total counts per min for the mitochondrial RNA preparation (see Table 5A) and dividing by the specific activity of the cytosolic RNA; *e.g.* 941 ÷ 72 = 13.

[5] Percent cytosolic RNA in mitochondrial RNA = {(µg cyt RNA in mt RNA)/(µg mt RNA)} x 100; *e.g.* (13 ÷ 55) x 100 = 24.

preparations was estimated. When expressed as a percent of the yield of mitochondrial RNA, cytosolic RNA appears to constitute 18-24% of the total mitochondrial RNA.

In an attempt to determine whether the cytosolic tRNA associated with the mitochondrial fraction represents a specific subset of the cytosolic tRNA population or is a random sampling, mitochondrial RNA containing radiolabelled cytosolic tRNA was electrophoresed in a 4 M urea, 10% polyacrylamide gel using the same conditions described for the first dimension of the two dimensional polyacrylamide gel electrophoresis protocol used to isolate individual tRNAs (section D, Material and Methods). No difference in pattern or relative intensities of the bands produced by the radiolabelled cytosolic tRNAs associated with the mitochondrial RNA fraction (Figure 1B) could be seen in comparison to cytosolic RNA similarly labelled with wheat mitochondrial tRNA nucleotidyltransferase (Figure 1C and 1D). Insufficient radiolabelled material was obtained to complete the second dimension of the electrophoresis.

(ii). Nuclease treatment of isolated mitochondria

In an attempt to decrease the amount of cytosolic RNA isolated with mitochondrial RNA, several experiments involving treatment of isolated mitochondria with micrococcal nuclease were conducted. The results of two of these experiments are presented in Tables 6A and 6B. In these experiments radiolabelled cytosolic RNA was added to wheat embryos and monitored through scintillation counting as described above, except 2 g of embryos and 80 μg of

Table 6A. Micrococcal nuclease treatment [2] of wheat embryo mitochondria: scintillation counting of subcellular fractions and cytosolic and mitochondrial RNA preparations following addition of radiolabelled cytosolic RNA to a wheat embryo mitochondrial RNA isolation procedure. [1]

	Total counts per minute [3]									
		Supernatant [5]								
	Initial [4]	1st or 2nd low-speed spin	1st high-speed spin	3rd high-speed spin			Cytosolic [6] RNA	Mitochondrial RNA [7]		
					Nuclease-treated [2]				Nuclease-treated	
Experiment				Control	1.0 mM $CaCl_2$	0.1 mM $CaCl_2$		Control	1.0 mM $CaCl_2$	0.1 mM $CaCl_2$
3	18,594,700	13,155,235	11,994,180	33,698	62,112	37,679	2,229,312	785	393	835
4	17,973,000	11,660,550	10,318,000	45,942	84,441	~[8]	5,099,213	1,565	545	~[8]

[1] The procedure used to isolate wheat embryo mitochondrial RNA is detailed in the Material and Methods, sections A and B. Radiolabelled cytosolic RNA was added to germinated wheat embryos as described in the text.

[2] The mitochondrial pellet of the 2nd high-speed spin (18,000*g*) was resuspended in homogenizing buffer containing either 0 (control), 0.1 or 1.0 mM $CaCl_2$ and incubated with micrococcal nuclease as described in the text.

[3] Total counts per minute were determined through liquid scintillation counting of ^{32}P-labelled cytosolic RNA present in the RNA preparations or supernatants as described below.

[4] Total counts per minute in the ^{32}P-labelled cytosolic RNA (added to the wheat embryos).

Table 6A. Continued.

[5] Total counts per minute in the supernatants of either the first or second low-speed spin (1,000 or 2,000*g*) and the first and third high-speed spins (18,000*g*). See Material and Methods, section A(iii) for details of the low-speed spins and the first high-speed spin. The third high-speed spin in these experiments differs from that of the experiments presented in Table 5. The third high-speed spin in these experiments follows the nuclease treatment and is prior to the sucrose gradient (see text for details).

[6]and [7] Total counts per minute in the final, isolated cytosolic and mitochondrial RNA preparations, respectively. See Material and Methods sections B(i) and (ii).

[8] Signifies that experiment 4 did not include a 0.1 mM $CaCl_2$ buffer treatment.

Table 6B. Micrococcal nuclease treatment of wheat embryo mitochondria: RNA yield and determination of percent cytosolic RNA content of mitochondrial RNA.

Experiment	Yield (µg)				Specific [4] activity (cpm/µg) cyt RNA	cyt RNA (µg) in mt RNA [5]			Percent cyt RNA in mt RNA [6]		
	cyt RNA[2]	mt RNA [3]									
		Control	Nuclease-treated [1]			Control	Nuclease-treated		Control	Nuclease-treated	
			1.0 mM $CaCl_2$	0.1 mM $CaCl_2$			1.0 mM $CaCl_2$	0.1 mM $CaCl_2$		1.0 mM $CaCl_2$	0.1 mM $CaCl_2$
3	10,336	16	11	18	216	4	2	4	25	18	22
4	10,032	18	10	~[7]	508	3	1	~[7]	17	10	~[7]

[1] Nuclease treatments are as described for experiments 3 and 4 of Table 6A.

[2] and [3] Yield (as determined from A_{260}) of the final, cytosolic and mitochondrial RNA preparations, respectively. See sections B(i) and (ii) of the Material and Methods.

[4] Specific activity of the cytosolic RNA was determined as described in Table 5B.

[5] Amount (µg) of cytosolic RNA in the mitochondrial RNA preparations was determined as described in Table 5B.

[6] Percent cytosolic RNA in the mitochondrial RNA preparations was determined as described in Table 5B.

[7] Signifies that experiment 4 did not include a 0.1 mM $CaCl_2$ buffer treatment.

Figure 1. Gel profiles of wheat cytosolic tRNA associated with mitochondrial RNA extracted from micrococcal nuclease-treated and untreated mitochondria. Cytosolic RNA was radiolabelled with [α-^{32}P]ATP using wheat mitochondrial tRNA-nucleotidyltransferase and added to wheat embryos from which mitochondria were isolated, as described in the text. Mitochondrial RNA (containing radiolabelled cytosolic tRNA) was extracted and electrophoresed in a 4 M urea, 10% polyacrylamide gel (see section D(i), Material and Methods). (A) Cytosolic tRNA associated with RNA extracted from micrococcal nuclease-treated mitochondria (see section A(ii), Results). (B) Cytosolic tRNA associated with RNA extracted from mitochondria not treated with micrococcal nuclease (see section A(i), Results). (C) and (D) Cytosolic RNA radiolabelled with [α-^{32}P]ATP using wheat mitochondrial tRNA nucleotidyltransferase.

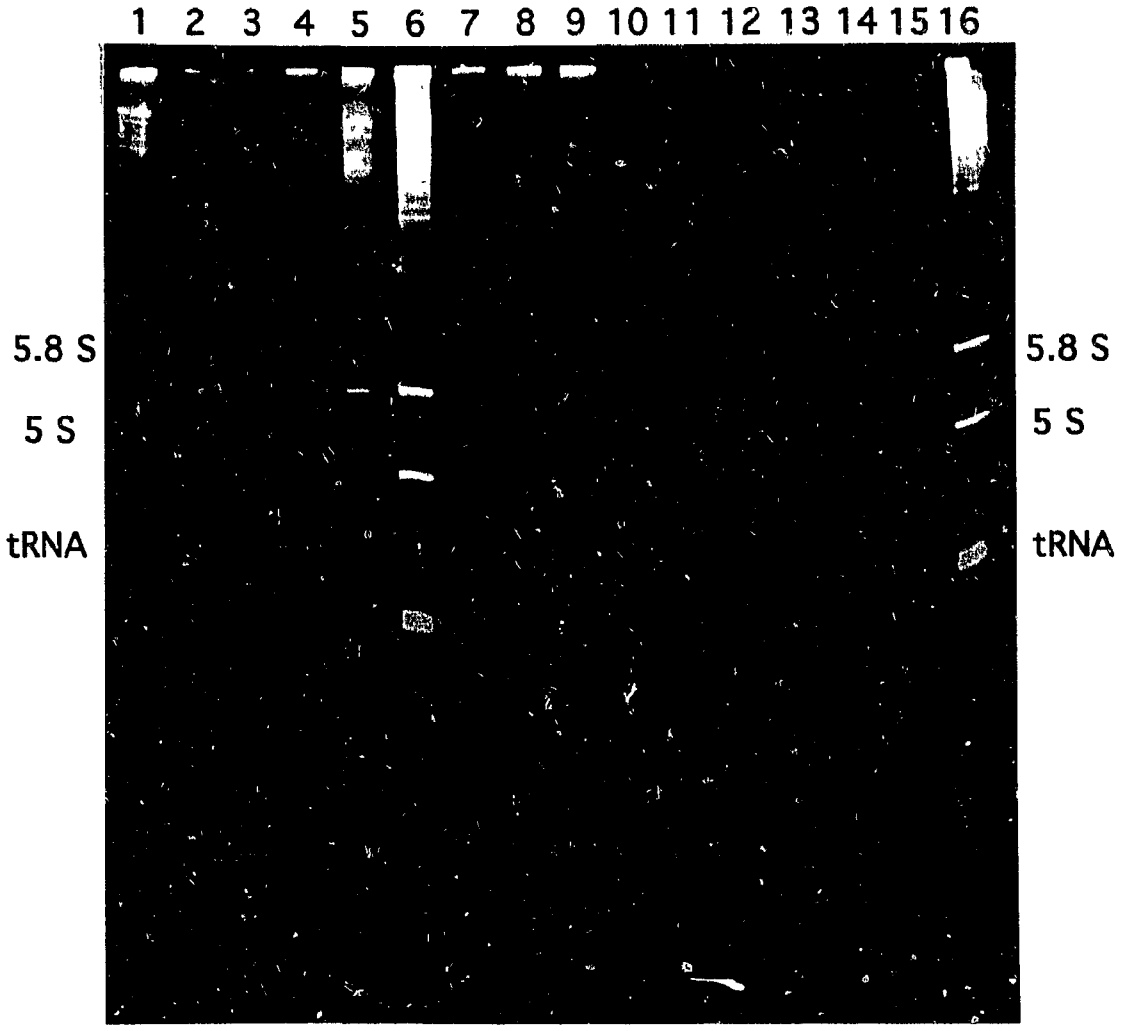


Figure 2. Continued

cytosolic RNA labelled with 2 mCi of [α-^{32}P]ATP and wheat mitochondrial tRNA nucleotidyltransferase were used. Following the second high-speed (18,000*g*) centrifugation of the mitochondria, mitochondrial pellets were resuspended in homogenizing medium (0.44 M sucrose, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM β-mercaptoethanol, 0.1% BSA) containing either 0, 0.1 or 1.0 mM $CaCl_2$ for experiment 3 and 0 or 1.0 mM $CaCl_2$ for experiment 4. Micrococcal nuclease (480 units/ml of homogenate) was added to mitochondria resuspended in homogenizing buffers containing $CaCl_2$, and the suspensions were then incubated for 1 hr at 4°C. The mitochondrial suspensions were diluted with 3 volumes of homogenizing medium containing 3 mM EDTA and centrifuged at 18,000*g* for 20 min. This is the third high-speed spin in the isolation of nuclease-treated mitochondria and occurs before the sucrose gradient. The third high-speed spin for mitochondria not treated with nuclease (Experiments 1 and 2 in Table 5) occurs after the sucrose gradient.

As in the experiments designed to estimate the amount of cytosolic RNA present in mitochondrial preparations, >90% of the radiolabelled cytosolic RNA added is removed following the first high-speed spin (Table 6A). For mitochondrial preparations incubated with micrococcal nuclease in 1.0 mM $CaCl_2$ buffer, total counts per minute in the supernatants of the third high-speed spin were approximately two-fold greater than for either the control (no nuclease) or the nuclease-treated mitochondria in 0.1 mM $CaCl_2$ buffer. In experiment 3, the total counts per minute in the mitochondrial RNA from the 1.0 mM $CaCl_2$ nuclease

treatment were approximately half that of either the control or the 0.1 mM $CaCl_2$ nuclease treatment. In experiment 4, the total counts per minute in the mitochondrial RNA from nuclease-treated mitochondria were approximately one-third of the control. However, as yields of mitochondrial RNA were reduced by the 1.0 mM $CaCl_2$ nuclease treatment in both experiments, the percentage of cytosolic RNA in the mitochondrial RNA was higher than anticipated from the scintillation count data (Table 6B).

To determine if the cytosolic tRNA remaining in the mitochondrial RNA of nuclease treated mitochondria was a specific subset of the cytosolic tRNA population, the mitochondrial RNA was electrophoresed in a 4 M urea, 10% polyacrylamide gel, as described above (Figure 1A). Although the signal is faint, no obvious selectivity is apparent (compare Figures 1A and 1B).

Because the yield of mitochondrial RNA following the 0.1 mM $CaCl_2$ nuclease treatment was maintained and the percentage of cytosolic RNA in the mitochondrial RNA was not significantly reduced, the effectiveness of this buffer for micrococcal nuclease activity was tested. Either 5 or 10 μg of cytosolic RNA were incubated with 250 units/ml of micrococcal nuclease for either 30 or 60 minutes in either 0.1, 0.5 or 1.0 mM $CaCl_2$ homogenization medium (see above). The reaction was stopped by the addition of 5 μl of 0.5 M EGTA. Effectiveness of the latter in halting the micrococcal nuclease activity was tested by adding cytosolic RNA to one of the treatments following EGTA addition. As can be seen in Figure 2, the 0.1 mM $CaCl_2$ buffer is the least effective one for supporting

Figure 2. Micrococcal nuclease degradation of cytosolic RNA

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Incubation time (min)	30	30	30	30	30	30	30	30	30	60	60	60	60	60	60	–
RNA (µg)	5	5	5	5	5	10	10	10	10	5	5	5	10	10	10	5
[$CaCl_2$] (mM)	1.0	1.0	0.5	0.1	1.0	1.0	1.0	0.5	0.1	1.0	0.5	0.1	1.0	0.5	0.1	-
Nuclease (250 units/ml)	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
RNA post EGTA (µg)	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-

Cytosolic RNA (5 or 10 µg) was incubated with micrococcal nuclease in homogenization medium (see text) containing either 0, 0.1, or 1.0 mM $CaCl_2$ for 30 or 60 min. The reaction was stopped by the addition of EGTA to 5 mM. Reaction mixtures were extracted with phenol, and RNA precipitated with ethanol and electrophoresed in a 6 % polyacrylamide gel. The gel was then stained with ethidium bromide (1 µg/ml) and bands were observed under ultraviolet light. Migration positions of the 5.8 S rRNA, 5 S rRNA and tRNAs are indicated.

micrococcal nuclease activity (compare lane 4 with lane 2). However, as judged by the intensity of the tRNA-sized or larger RNA bands remaining in the 5 μg RNA sample incubated for 30 min (lane 4) and in the 10 μg RNA sample incubated for 1 h (lane 15) the amount of residual RNA was low. No tRNA-sized or larger RNA bands were seen in the 5 μg RNA sample incubated for 1 h (lane 12). No tRNA-sized or larger RNA bands were observed when 10 μg of RNA sample were incubated with the 1.0 mM $CaCl_2$ buffer for 1 h (lane 13). The addition of EGTA appeared effective in halting nuclease activity as RNA added after the addition of EGTA was not digested (lane 5).

B. Southern hybridizations of total mitochondrial tRNA to either mtDNA or nDNA

Mitochondrial tRNAs eluted following hybridization with mtDNA and separated by 2-D gel electrophoresis (Figure 3B) were found to represent only a fraction of the total mitochondrial tRNA population (Figure 3A). All but two of these tRNAs correspond to mtDNA-encoded tRNAs identified by partial sequence analysis following a similar electrophoretic separation of total mitochondrial RNA (Joyce and Gray, 1989a). The two additional spots remain unidentified although they could correspond to mtDNA-encoded $tRNA^{His}$(GUG) and $tRNA^{Asp}$(GUC), as will be discussed in a later section. Spots corresponding to the three cytosolic-like mitochondrial tRNAs identified by Joyce and Gray (1989a) (*i.e.*, $tRNA^{Gly}$(GCC), $tRNA^{Leu}$(CAA) and $tRNA^{Val}$(GAC)) were not found.

When mitochondrial tRNAs hybridizing specifically to nDNA were

Figure 3. Two-dimensional electrophoretic separation of wheat tRNAs specifically end-labelled with [α-^{32}P]ATP using wheat mitochondrial tRNA nucleotidyltransferase. Conditions for electrophoresis were as described in section D(i), Material and Methods. For A, B and C the tRNA samples were electrophoresed on the same gel in the first dimension but due to gel size restrictions only A and B could be run on the same gel for the second dimension. D was electrophoresed independently under similar conditions as A, B and C. (A) total mt tRNA; (B) mt tRNAs recovered following hybridization to mtDNA; (C) mt tRNAs recovered following hybridization to nDNA; (D) total cytosolic tRNA. Closed arrows denote some of the tRNAs that appear in C but not B. Closed arrowheads denote tRNAs migrating to similar positions in C and B. Open arrowheads denote some of the tRNAs that appear in D but not C. Three cytosolic-like tRNAs previously identified by Joyce and Gray (1989) are indicated (Gly, Val and Leu) in A, C and D.

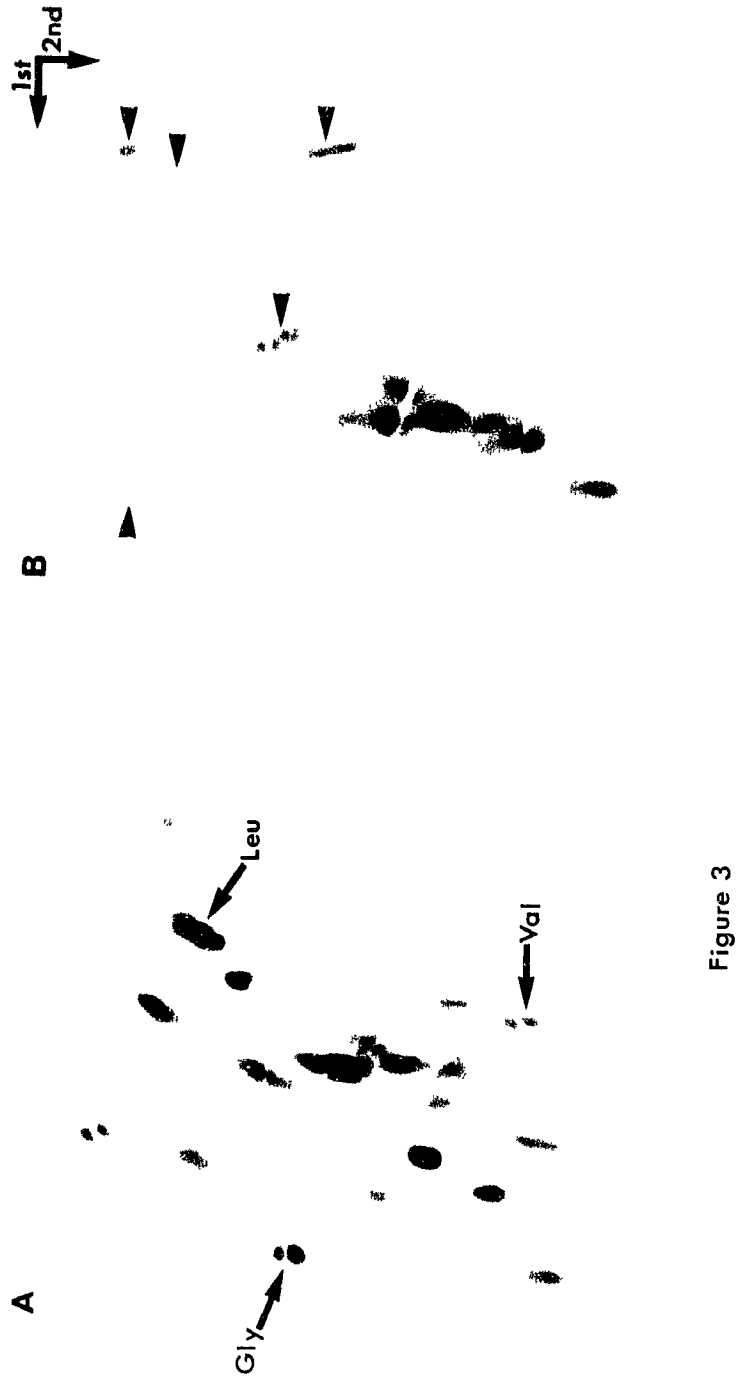


Figure 3

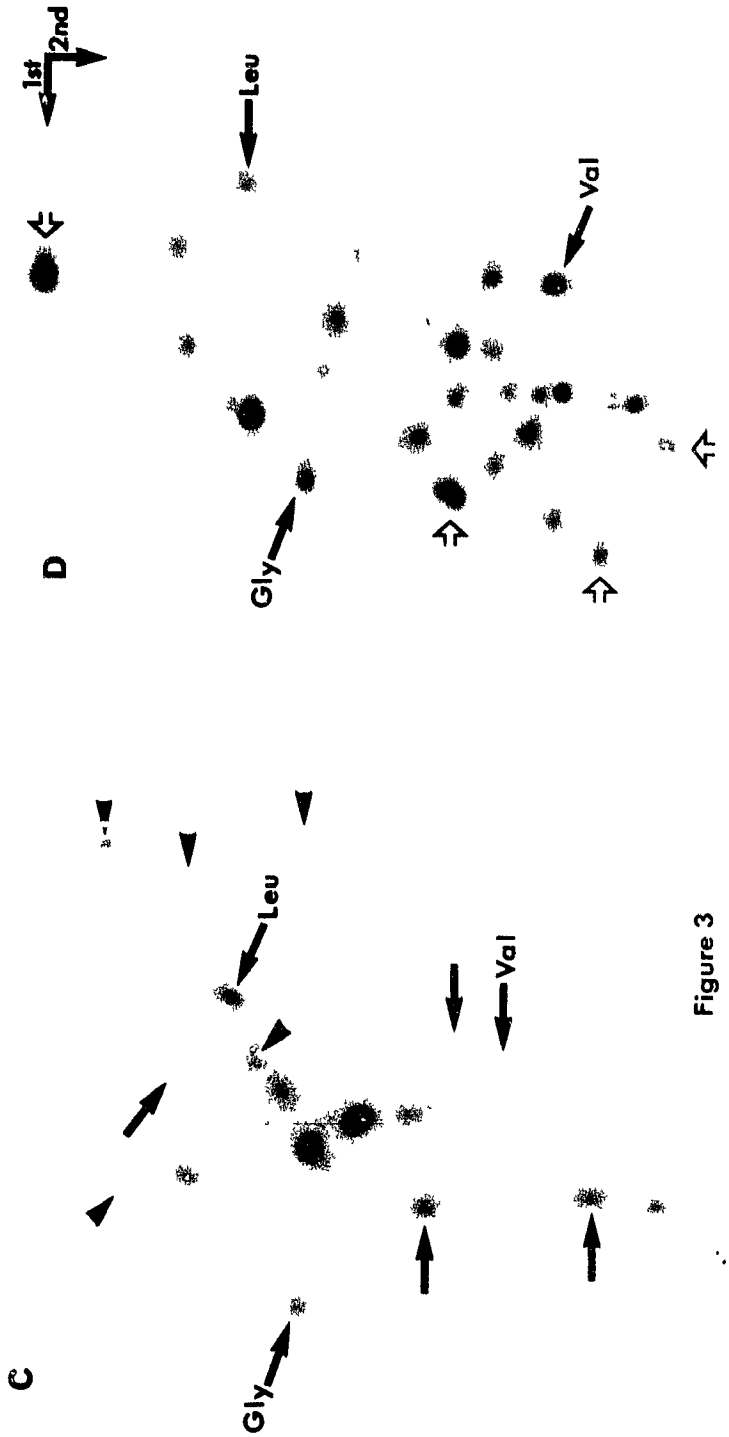


Figure 3

recovered and subjected to 2-D gel electrophoresis, a different pattern was obtained (Figure 3C). The three spots corresponding to cytosolic-like mitochondrial tRNAs were identified and many other spots (closed arrows) not seen in Figure 3B were observed.

Although relative spot intensities may vary, many of the spots obtained in the mitochondrial tRNA/nDNA hybridization pattern comigrate with spots obtained in the mitochondrial tRNA/mtDNA hybridization pattern (closed arrowheads; Figure B and C). Some of these spots may contain two or more tRNAs, specific for both mtDNA-encoded and nDNA-encoded mitochondrial tRNAs, that have similiar mobilities under the conditions used. They may also represent mtDNA-encoded tRNAs present as a result of mtDNA contamination of the nDNA preparation. As the mitochondrial genome is much smaller than the nuclear genome, gene copy number per unit mass of DNA is expected to be much larger and thus a small amount of mtDNA contaminating the nDNA may produce a signal.

In comparing the 2-D gel electrophoretic pattern of mitochondrial tRNAs obtained following hybridization to nDNA with that of the total cytosolic tRNA pattern, spots unique to the cytosolic tRNA pattern are seen (open arrowheads; Figure 3D). This observation reduces the likelihood that significant cytosolic tRNA contamination of the mitochondrial tRNA has occurred and that the hybridization to nDNA is due to cytosolic tRNA and not mitochondrial tRNA.

C. Isolation of tRNAs from wheat mitochondria

In an attempt to isolate nDNA-encoded mitochondrial tRNAs, mitochondrial RNA (3' end-labelled with [α-^{32}P]ATP and wheat mitochondrial tRNA nucleotidyltransferase) was hybridized to nDNA, and the bound RNA species eluted and separated by 2-D polyacrylamide gel electrophoresis as described in sections F(i) and D(i), Material and Methods. However, the recovery of labelled tRNAs was not sufficient for subsequent sequence analysis of individual tRNAs.

To obtain sufficient material for sequence analysis, 125 µg of unlabelled salt-soluble RNA from wheat mitochondria was separated by 2-D polyacrylamide gel electrophoresis (conditions described in section D(ii), Material and Methods). Figure 4 shows the RNA profile following ethidium bromide staining of the gel. Sixty-six spots were excised. Heterogeneity of the 3'-CCA terminus of the tRNAs was expected (Joyce, 1989) and is evident in Figure 4. A single tRNA species could be represented by as many as four spots, differing from each other in the degree of completion of the 3' end (*i.e.*, tRNA-, tRNA-C, tRNA-CC and tRNA-CCA). As is particularly evident at the periphery of the RNA profile, a single tRNA in general appears to be represented by three spots. This was confirmed by sequence analysis. The 3' end heterogeneity of the tRNAs, although expected, complicated the RNA separation and led to repeated sequencing of single tRNA species.

In labelling the RNA spots, the numbers 1 through 50 are intended to correspond to a 2-D gel electrophoretic pattern obtained by Joyce and Gray (1989a) in an expermient in which wheat mitochondrial tRNAs were 3' end-

Figure 4. Fractionation of wheat mitochondrial tRNAs by two-dimensional polyacrylamide gel electrophoresis. Conditions of electrophoresis are specified in section D(ii), Material and Methods. (A) Schematic representation of (B). (B) Photograph taken under ultraviolet light showing the tRNA pattern after ethidium bromide staining of the gel. Spots have been numbered from 1 to 51 and 70 to 79 as explained in the text. Lowercase letters denote potential separation of a single tRNA species due to 3' end heterogeneity. The arrows show the directions of migration in the first and second dimensions.

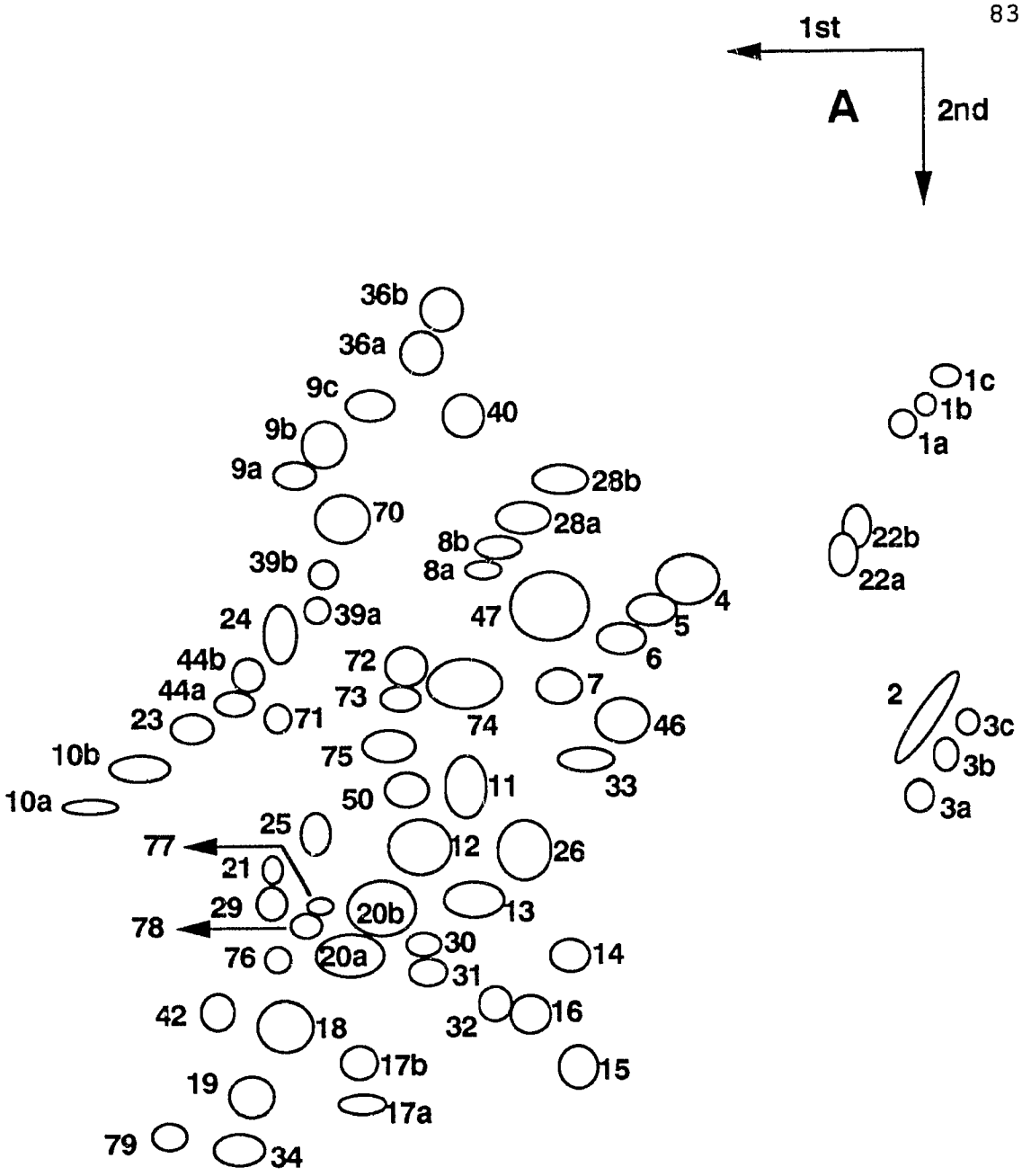


Figure 4. Continued

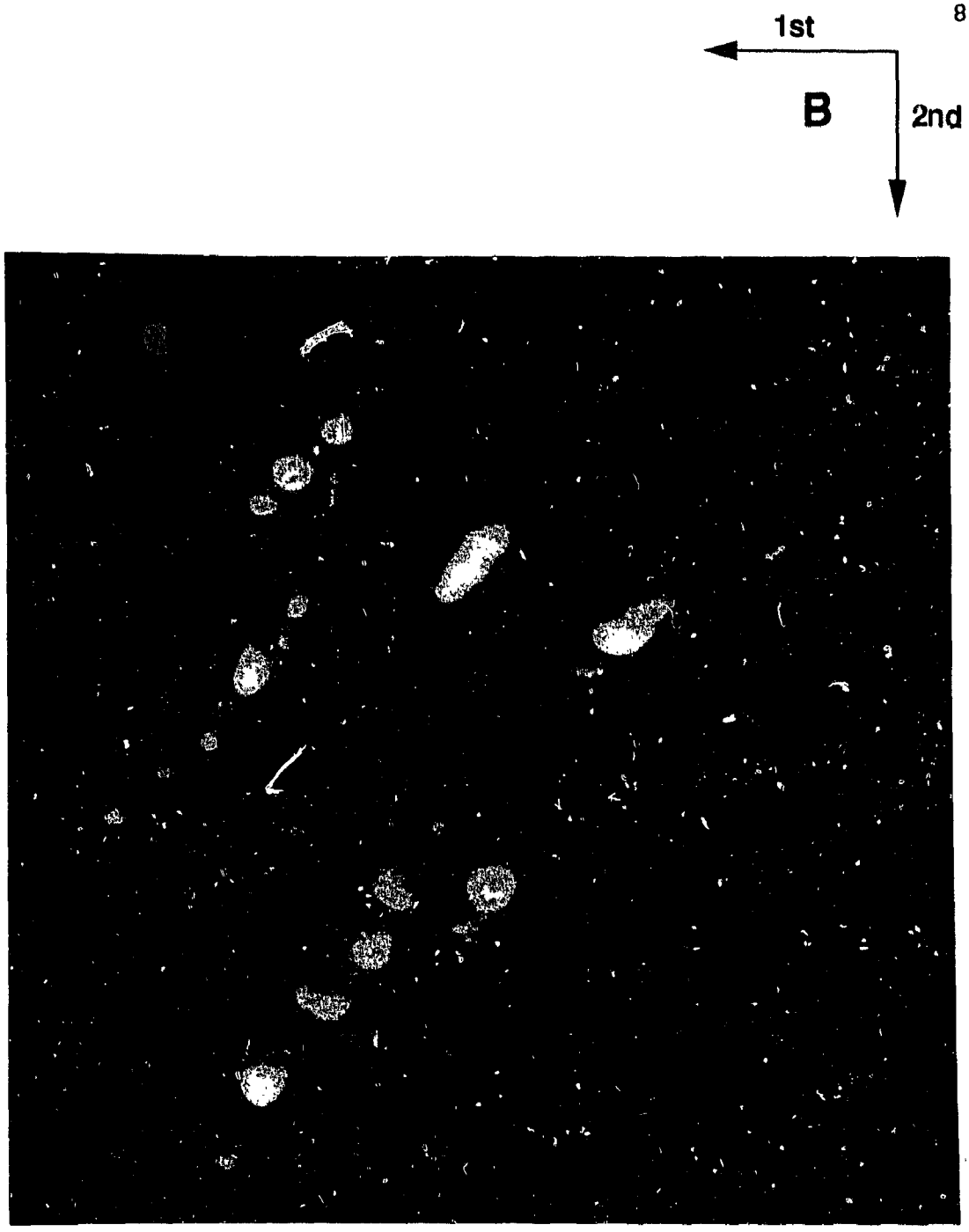


Figure 4. Continued

labelled with [α-^{32}P]ATP using wheat tRNA nucleotidyltransferase, thereby eliminating the 3'-end heterogeneity. The letters I use in association with the RNA number denote putative single tRNA species with varying degrees of completion of the 3' terminus. Transfer RNAs numbered 70 through 79 do not appear analogous to any spots obtained by Joyce and Gray (1989a).

Transfer RNAs isolated from the 2-D polyacrylamide gel were 3' end-labelled with [5'-^{32}P]pCp and electrophoresed in a fully denaturing polyacrylamide gel (Figure 5). Many of the tRNAs isolated from the 2-D polyacrylamide gel were pure (solitary species), producing single bands in the third dimension. However many of the tRNAs generated two, three and occasionally four bands in the third dimension. This again was primarily the result of spots overlapping in the 2-D gel due to 3' end heterogeneity, although it is known that tRNAs can exist in different forms due to the presence or absence of various post-transcriptional modifications (Maréchal *et al.*, 1985). Bands resolving in the third dimension are identified by the numbers 1-4 (in order of increasing mobility) following the decimal point.

D. Sequence analysis

(i). General

In an attempt to avoid sequencing of previously identified mtDNA-encoded tRNAs (Joyce and Gray, 1989a), spots obviously analogous to these tRNAs were not analyzed. This included primarily tRNAs on the periphery of the 2-D gel pattern (*e.g.* 1a, 1b and 1c) because in these areas Joyce and Gray (1989a) had found singular spots (*e.g.* 1) to be homogeneous, and thus no unidentified tRNA

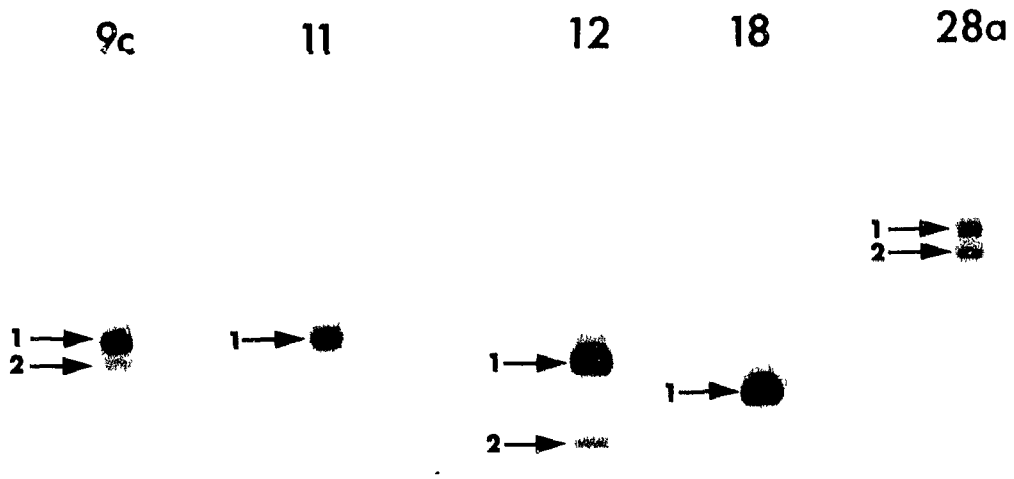


Figure 5. Autoradiogram showing the further resolution of some of the tRNAs recovered from a two-dimensional electrophoretic separation (see Figure 4), following [5'-^{32}P]pCp 3' end-labelling and electrophoresis in a fully denaturing polyacrylamide gel (the third dimension; see section D(ii), Material and Methods). Separated components are designated by the number following the decimal point (1 to 4), in order of increasing mobility.

would likely have been overlooked. However in more congested regions of the 2-D gel pattern, many previously identified mtDNA-encoded tRNAs were sequenced again in the course of the present study. In total, 44 spots from the 2-D gel were electrophoresed in the third dimension generating 78 bands. All of these bands were subjected to chemical sequence analysis. Table 7 lists the tRNAs tentatively identified as nDNA-encoded mitochondrial tRNAs. (Sequences corresponding to previously identified mtDNA-encoded tRNAs are not reported here.)

In general, when the products of chemical sequencing reactions were electrophoresed in polyacrylamide gels containing formamide, at 50^{0}C, relatively good sequence data were obtained (Figure 6). In some instances nucleosides could not be identified due to the presence of more than one band at a single position in the sequencing ladder. In other cases nucleosides could not be identified because the banding pattern was too faint or not apparent. The alkali ladder could then be used to verify the presence of a nucleoside at that position. Nucleosides that could not be identified are designated by the letter, X.

Nucleoside modifications can be tentatively identified with chemical sequencing (Lankat-Buttgereit *et al.*, 1987). However, as I was primarily concerned with identification of the tRNA and not complete sequence analysis, in only a few instances did I identify potential nucleoside modifications. For example, dihydrouridine has been shown to produce bands in both the C and U lanes of chemical sequencing reactions (Lankat-Buttgereit *et al.*, 1987) and can be tentatively identified on this basis. I have incorporated this in my sequence

Table 7. Wheat nDNA-encoded mitochondrial tRNAs[1] separated by 3-D polyacrylamide gel electrophoresis and identified by RNA sequence analysis.

Number[2]	Amino Acid	Anticodon		
		Chemical Sequencing	Reverse Transcriptase Sequencing	Thin Layer Chromatography
18, 19, 20a.1	Ala	GGc[4]		
25.2	Ala	ugX		
42.2	Ala	XGX		
24.1, 39a.1, 39b, 44a, 44b.1	Arg	GCG	XCG	ICG
42.1	Arg	XXU	CCU	
75.3	Asp	GUC		
10a, 10b, 23	Gly	GCC	[3]	
24.2, 39a.2	Gly	UCC	UCC	
21, 25.1, 29.1, 29.2	His	GUG		
74.1	Ile	GAU	GAU	
8b.1, 28a.1	Leu	GAG		
8b.2, 28a.2	Leu	UAG		
4.1, 5.1, 6.1	Leu1	XAA		
4.2, 4.3, 5.2, 5.3, 6.2, 6.3	Leu4	XAA		
14	Val	UAX	XAC	
15.1, 15.2	Val	GAC	GAC	

Table 7 continued:

[1] The tRNAs in this table have been tentatively identified as nDNA-encoded mt tRNAs based on sequence analysis. Their cellular localization and identity are discussed further in following sections.

[2] Numbers and letters preceding the decimal point correspond to those in Figure 4 and numbers following the decimal point refer to mobility in the third dimension of electrophoresis (see Figure 5).

[3] No reverse transcriptase sequencing of this tRNA was conducted.

[4] Lowercase letters denote nucleosides that could not be positively identified and "X" denotes nucleosides that could not be identified.

Figure 6. Chemical sequencing of wheat mitochondrial tRNAs. Autoradiograms show the results of chemical sequencing reactions of three tRNAs recovered from 3-D polyacrylamide gel electrophoresis (section D(ii), Material and Methods). All tRNAs were 3' end-labelled with [5'-^{32}P]pCp before separation in the third dimension. (A) $tRNA^{Leu1}$(XAA); (B) $tRNA^{Arg}$(GCG); (C) $tRNA^{Ile}$(GAU). Sequence of each tRNA is depicted to the left of the sequencing gel autoradiogram: G = guanosine; C = cytidine; A = adenosine; U = uridine; X = unassigned nucleoside; d = potential dihydrouridine; and N = alkali ladder. Anticodon sequences are bracketed. The tRNAs are aligned at position 71, which is indicated. The arrow in (C) indicates the modified nucleoside whose enhanced reactivity in the C-specific reaction results in loss of C bands 5' to this position.

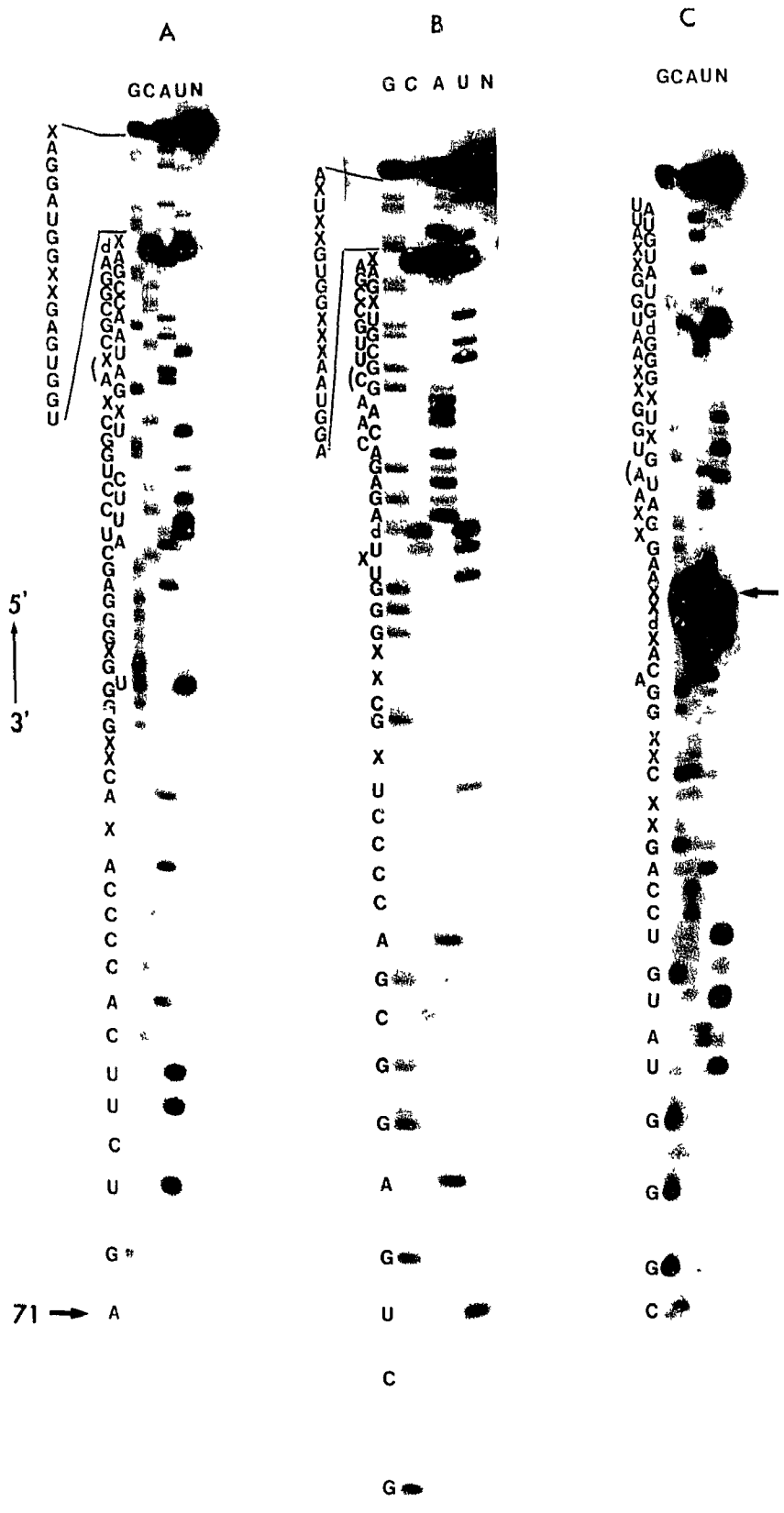


Figure 6 Continued.

analysis, designating a potential dihydrouridine by the lowercase letter, d (Figure 6A and B).

One problem that occurred with unfortunate frequency was the failure of the C reaction due to the presence of a modified nucleoside with enhanced reactivity, leading to virtual complete scission of the polynucleotide chain at these positions. This resulted in very faint or no bands in the C reaction ladder following the modification (Figure 6C). An anomalous C-specific cleavage that resembles the one observed here, occurs at a 3-methyluridine (m^3U) residue found in the small subunit rRNAs of *E. coli* and wheat mitochondria (Schnare, 1984). However, no m^3U has been identified in tRNA and in reverse transcriptase sequencing of $tRNA^{Ile}$(GAU), the modified nucleoside was recognized as a G. In cases where this anomalous C-specific cleavage occurred and very faint bands were seen in the C reaction lane following the modification, the bands were designated by the lowercase letter, c.

Reverse transcriptase sequencing of all the tRNAs listed in Table 7 was completed except for $tRNA^{Gly}$(GCC). In general, oligonucleotides used as primers for reverse transcriptase sequencing were 20 nucleotides long and complementary to sequence at the 3' end of the tRNA. (Table 4 lists primer oligonucleotide sequences.) Therefore depending on the 3' end sequence available following chemical sequence analysis, the primer usually terminated in the T loop or 5' half of the T stem (see Table 4 and Figure 8 for location of primer 3'-termini). In addition to generally confirming chemical sequence data, reverse transcriptase

analysis was particularly useful in establishing sequence in the variable loop (a region containing many unassigned nucleosides in the chemical sequence analysis) and in allowing identification of C residues missing from the chemical sequence analysis for the reason discussed above. In Figure 7, reverse transcriptase sequence for three tRNAs is shown. Although the entire sequence determined from reverse transcriptase sequencing is presented in Figure 7 many different lengths of autoradiogram exposure were required to clearly discern nucleosides in specific regions of a given tRNA.

Only the three alanine tRNAs produced no signficant reverse transcriptase sequence. This may be due to modifications in the variable loop; however, no plant $tRNA^{Ala}$ sequence is available for comparison with the chemical sequencing results obtained here. Reverse transcriptase sequence often terminated at position 26 (the unpaired position connecting the D and the anticodon stems) or position 20 of the D loop (see Figure 8 for nucleoside positions in the tRNA). Both of these nucleoside positions in the tRNA are frequently modified in plants (*e.g.*, N^2, N^2-dimethylguanosine (m_2^2G) is often found at position 26). Although sequence of the 5' half of the anticodon stem and in many cases the remainder of the tRNA was clearly discernable, the anticodon loop was often difficult to read. Again nucleosides in this region of the tRNA are frequently modified, with so-called, hypermodified nucleosides often found at the first ("wobble") position of the anticodon and/or immediately 3' of it. Nevertheless, reverse transcriptase sequencing confirmed or expanded anticodon sequence information for six of the

Figure 7. Reverse transcriptase sequencing of wheat mitochondrial tRNAs. Oligonucleotides specific for nDNA-encoded wheat mitochondrial tRNAs (see Table 4, Material and Methods) were used as primers for extension by reverse transcriptase with wheat mitochondrial RNA as template. Autoradiograms depict reverse transcriptase sequencing reactions for (A) $tRNA^{Val}$(GAC); (B) $tRNA^{Gly}$(UCC); and (C) $tRNA^{Arg}$(CCU); and chemical sequencing reactions for (D) $tRNA^{Arg}$(CCU). Sequence of each tRNA is depicted to the left of the sequencing gel autoradiogram: G = guanosine; C = cytidine; A = adenosine; U = uridine; X = unassigned nucleoside; N = alkali ladder; and E = primer extension lane. Anticodon sequence is bracketed. (Note that the lanes of the reverse transcriptase sequencing autoradiogram are labelled with the nucleoside complementary to the actual sequence reaction, so that the tRNA sequence can be read directly.)

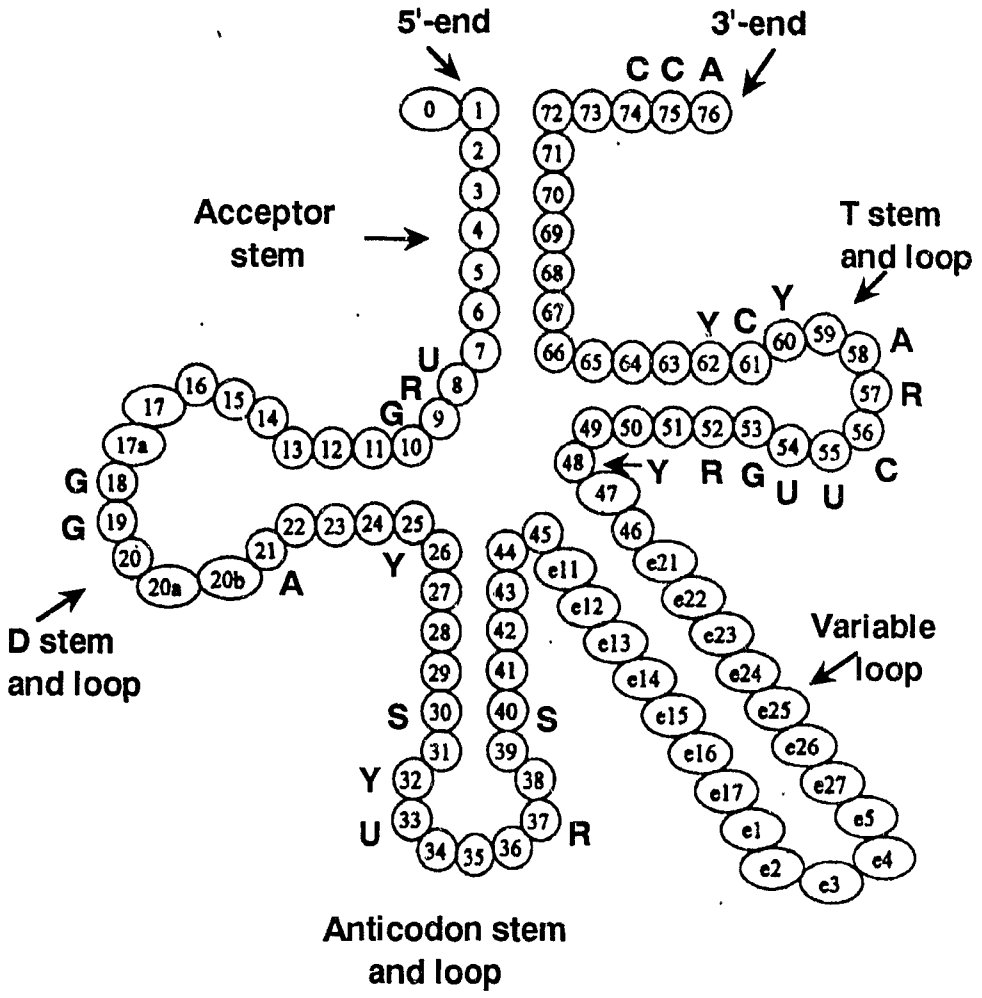


Figure 8. Invariant and semi-invariant nucleosides in eubacterial, archaebacterial, eukaryotic cytoplasmic and chloroplast tRNAs. Circles represent nucleotides that are always present; ovals represent nucleotides that are not present in every structure (Steinberg *et al.*, 1993). Letters on the figure indicate the identities of highly conserved nucleosides (Nicoghosian *et al.*,1987): A = adenosine; G = guanosine; C = cytidine; U = uridine; R = A or G; Y = C or U; and S = G or C.

tRNAs (Table 7).

In chemical sequencing reactions the modified nucleoside, inosine (I), is cleaved in the G reaction and thus appears as a band in the G lane of the sequencing gel. Inosine is known to be present at the first position of the anticodons of several plant tRNAs (the only position that inosine has ever been localized in tRNA). For example, wheat germ $tRNA^{Ile}$(IAU) (Kusama-Eguchi *et al.*, 1991) and $tRNA^{Arg}$(ICG) (Barciszewska *et al.*, 1986) have been characterized, and in potato mitochondria, a nDNA-encoded $tRNA^{Ala}$(IGC) has been identified (Maréchal-Drouard *et al.*, 1990). Also, a lupin cytosol $tRNA^{Val}$(IAC) has been sequenced (listed in Steinberg *et al.*, 1993). In the present study, terminal nucleotide analysis of the anticodon loop region of $tRNA^{Arg}$(GCG) was conducted through 5'-^{32}P end-labelling the tRNA partial alkaline hydrolysis products, followed by P1 nuclease treatment and thin layer chromatography of the resulting 5' terminal ^{32}P-labelled nucleotides (pN). This analysis showed that the first position of the anticodon of this tRNA is inosine (Figure 9).

The presence of inosine in the anticodons of $tRNA^{Ala}$(GGX), $tRNA^{Ile}$(GAU) and $tRNA^{Val}$(GAC) remains to be determined, however reverse transcriptase sequence data provide some indication of whether a modified base is present. The nucleotide in the first position of the anticodon for $tRNA^{Arg}$(ICG) could not be determined in the sequence generated by reverse transcriptase. There were bands in every lane, which could be due to a modified nucleoside at that position but the pausing could also be generated by modifications at adjacent nucleoside positions

Figure 9. Determination of the anticodon sequence of wheat mitochondrial $tRNA^{Arg}$(GCG) by thin layer chromatography (tlc). Partial hydrolysis products of $tRNA^{Arg}$(GCG) were 5' end-labelled with [γ-^{32}P]ATP and electrophoresed in a sequencing gel containing formamide (see sections E(iii) and (iv), Material and Methods). Bands corresponding to the anticodon loop were excised, treated with P1 nuclease and subjected to tlc in two different solvents: A, 95% ethanol:H_2O, 4:1 (Lane, 1963); B, saturated ammonium sulfate:propan-2-ol, 40:1 (Singh and Lane, 1964). Migration positions of marker nucleotides visualized under ultraviolet light are indicated. The anticodon sequence is depicted and additional nucleosides of the anticodon loop marked by dots at the sample application origin.

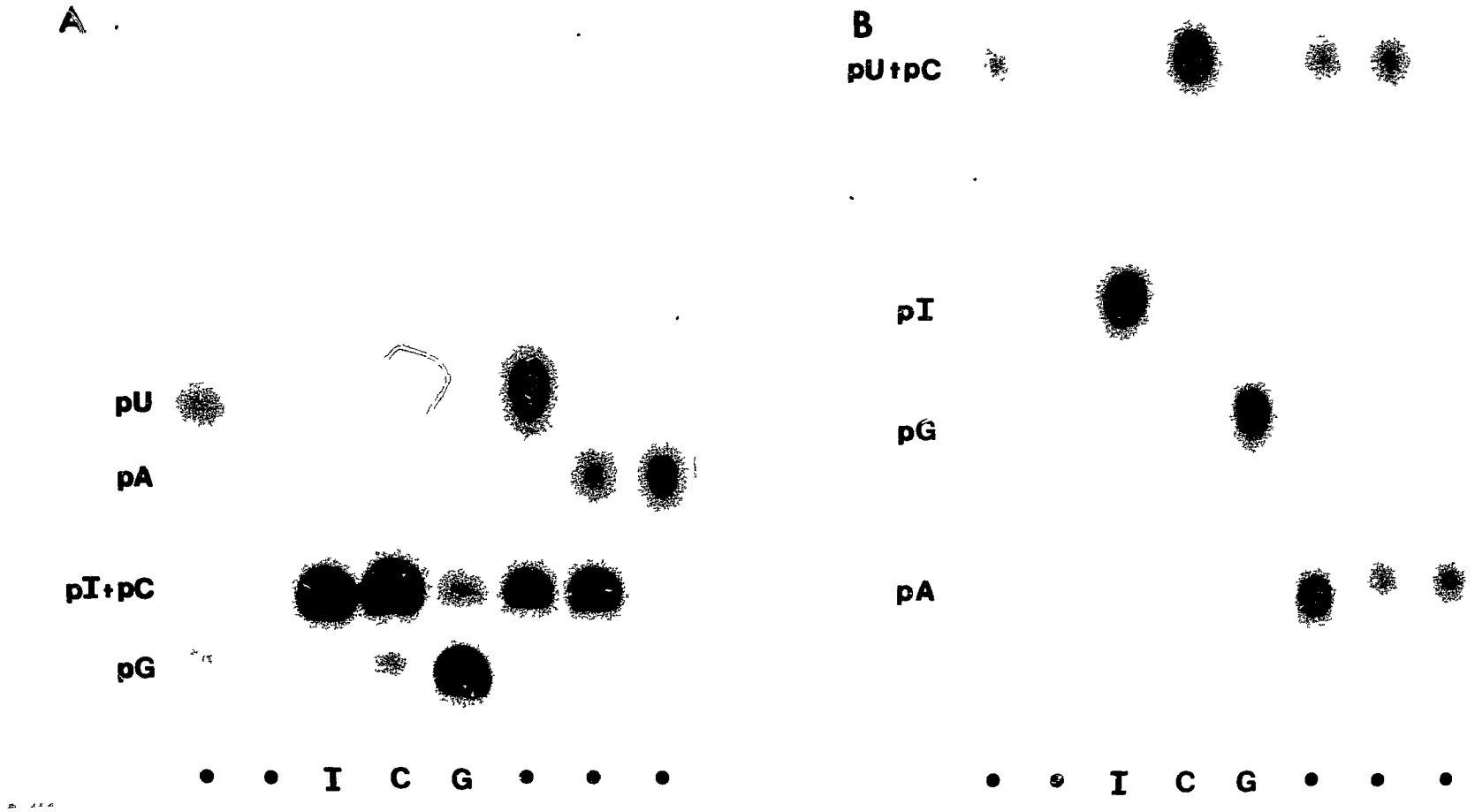


Figure 9 Continued.

or possibly the result of secondary or tertiary structural interactions if the tRNA was not completely denatured. Reverse transcriptase sequence of $tRNA^{Ile}$(GAU) also showed pausing in every lane, however a stronger band was seen in the C reaction. Thus, this position was identified as G although it is probably a modified nucleoside and possibly an inosine residue. Reverse transcriptase sequence of $tRNA^{Val}$(GAC) showed no anomalous bands in the first position of the anticodon and a strong band in the G lane (see Figure 7A), and thus this nucleoside was identified as guanosine.

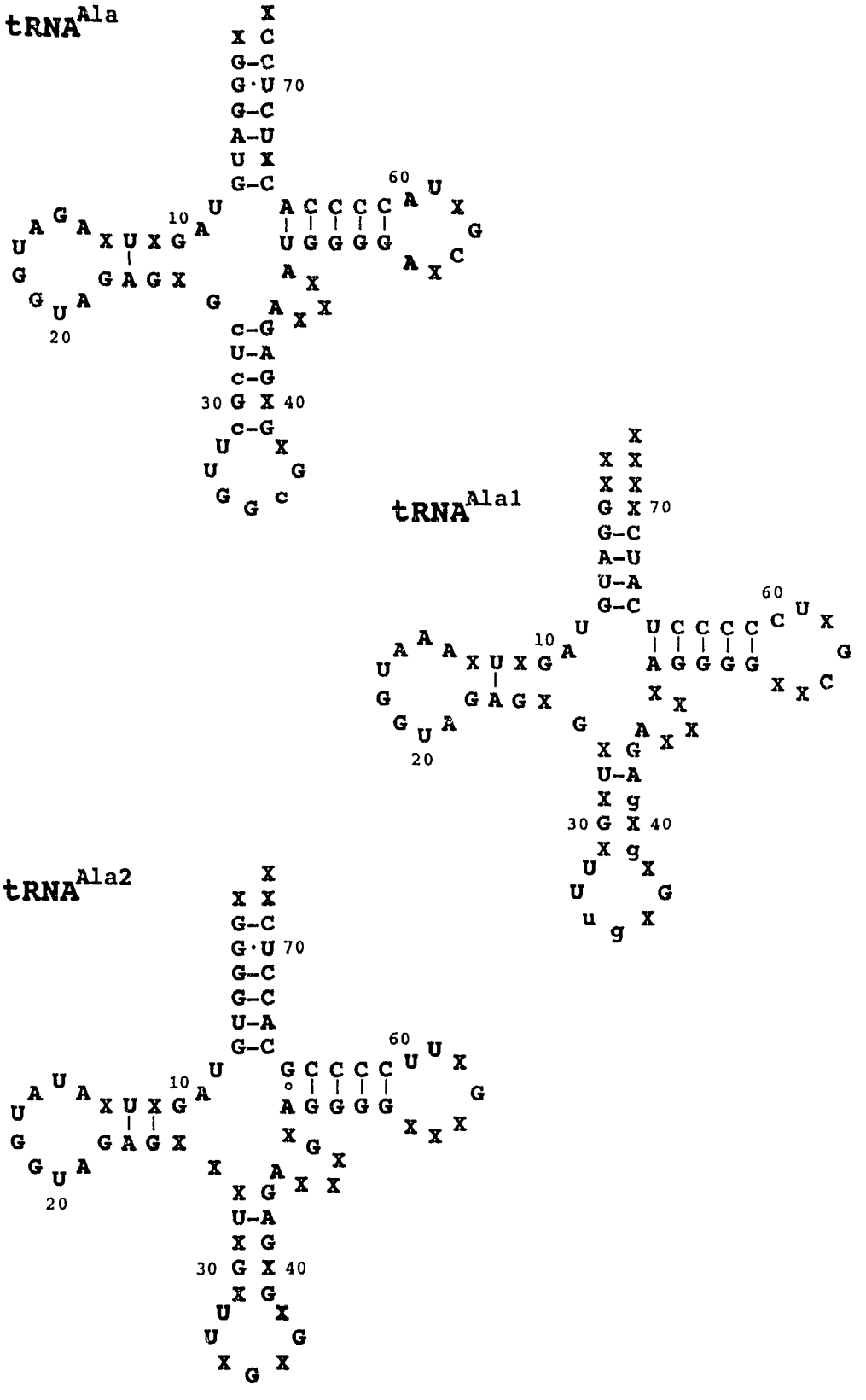
(ii). Primary sequence and secondary structure of tRNAs

The primary sequences and potential secondary structures for the tRNAs listed in Table 7 are presented in Figure 10. All of these plant tRNAs can be folded into the standard cloverleaf secondary structure with a typical acceptor stem, D stem and loop, anticodon stem and loop, and T stem and loop. Most of these tRNAs also contain the characteristic nucleosides of the generalized tRNA model (Figure 8). However there are some exceptions.

All three alanine tRNAs contain A_{16} which is more typically C or U and $tRNA^{Ala}$(GGc) contains A_{54} which is generally 5-methyluridine (T). Interestingly, adenosine is found at these positions in *Drosophila melanogaster* $tRNA^{Ala}$(AGC) (listed in Steinberg *et al.*, 1993). The $tRNA^{Ala2}$(XGX) has a U residue at position 15, which typically is G or A. The only plant $tRNA^{Ala}$ sequence available, that of *Arabidopsis thaliana* (Akama and Tanifuji, 1990), also has a U at this position.

The $tRNA^{Ile}$(GAU) has a U_{30}:G_{40} base pair rather than the more typical

Figure 10. Primary sequences and potential secondary structures of the wheat mitochondrial tRNAs listed in Table 7, derived from partial chemical reaction and reverse transcriptase sequence data. Nucleosides that could not be identified are indicated by the letter "X". Nucleosides that are tentatively identified are indicated by lowercase letters. Sequence of the 5' terminal region of $tRNA^{Val}$(GAC) was not determined (*).



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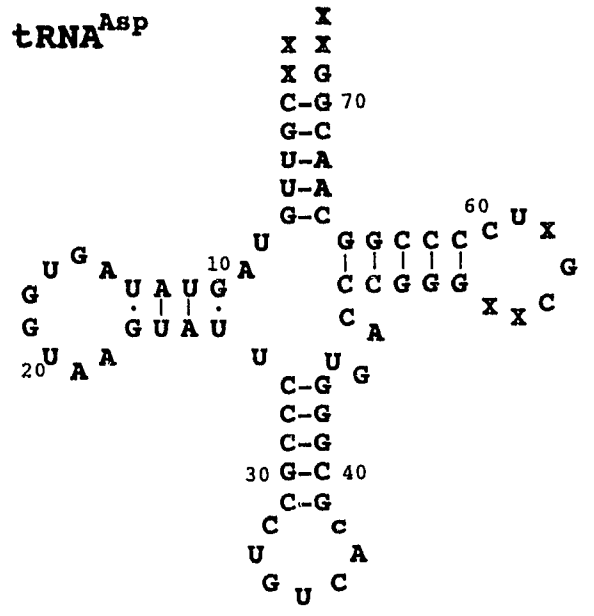

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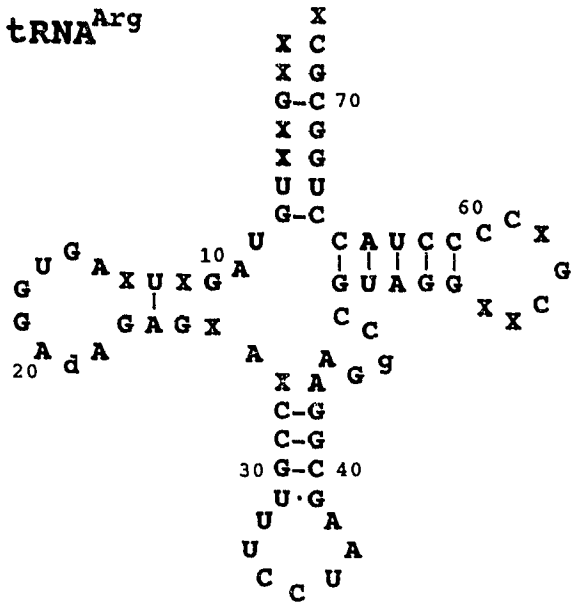

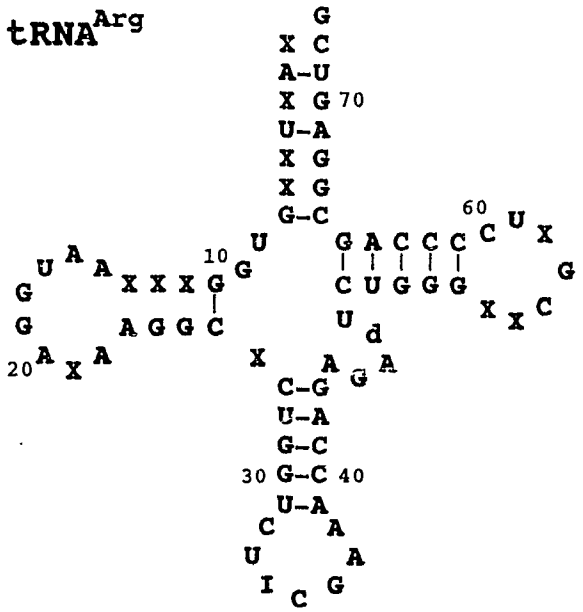

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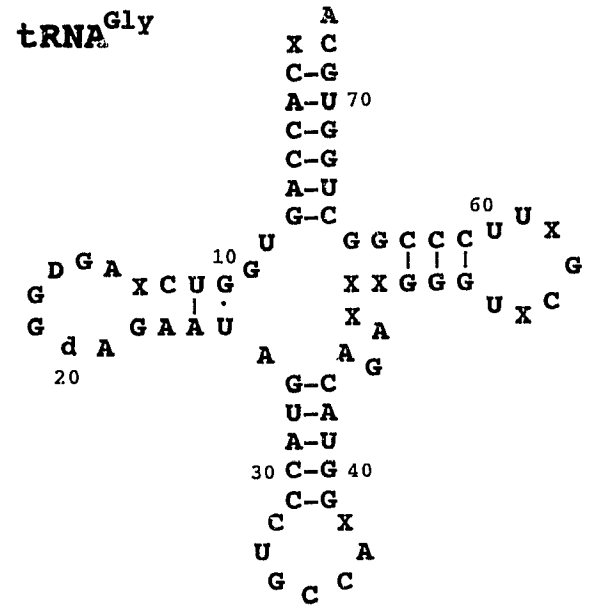

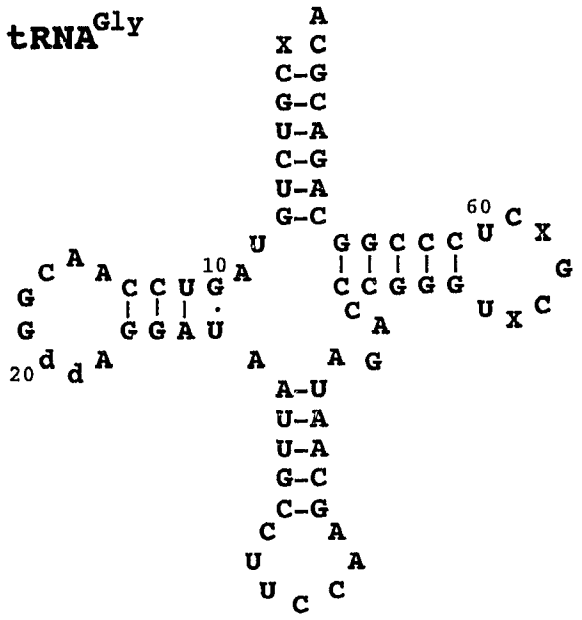

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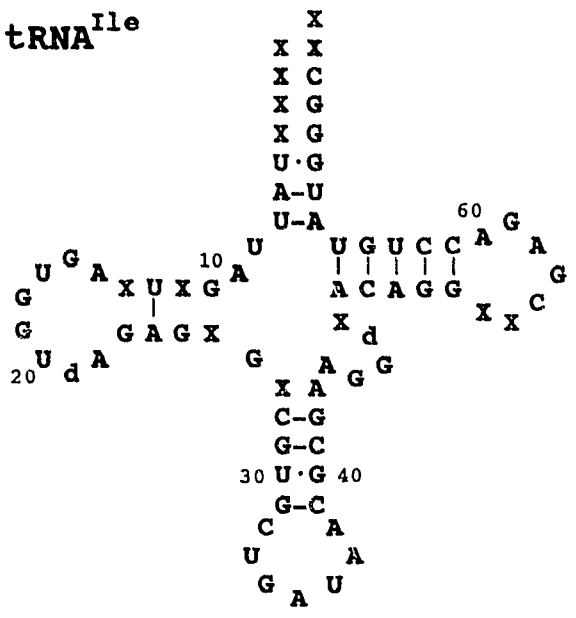

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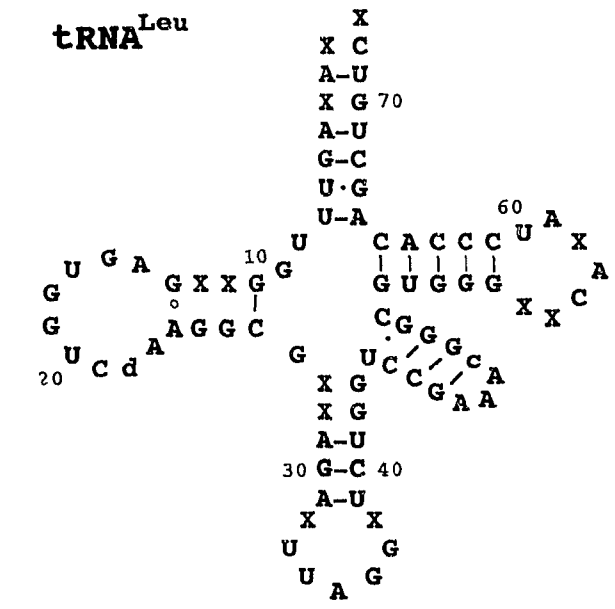

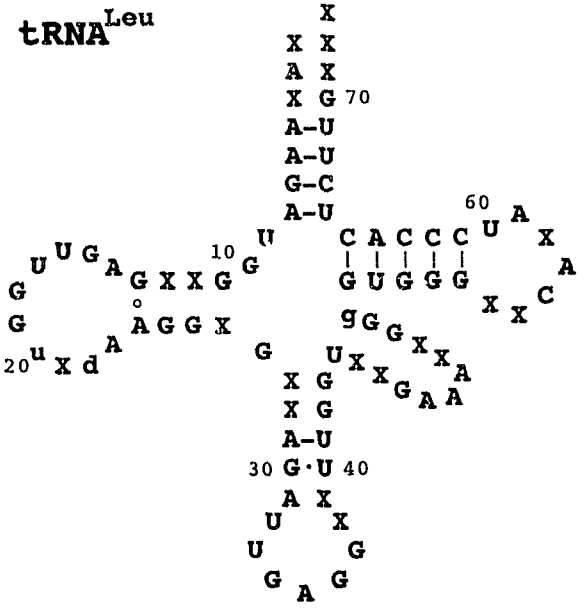

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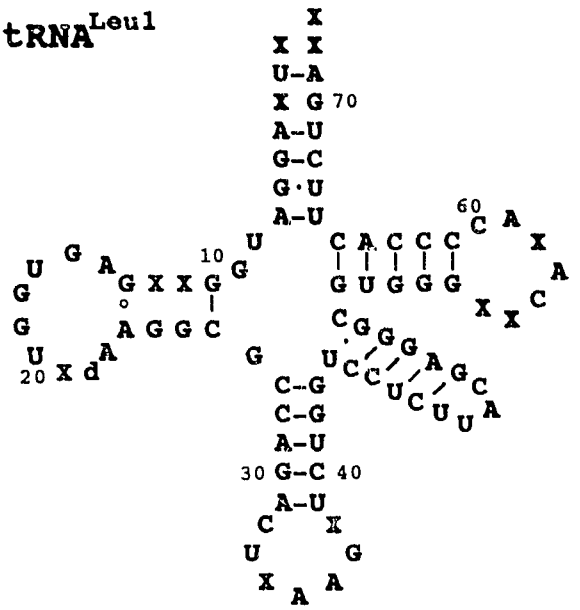

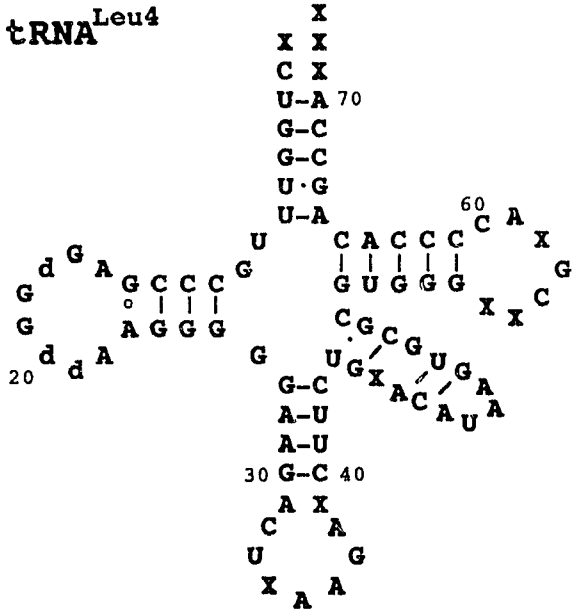

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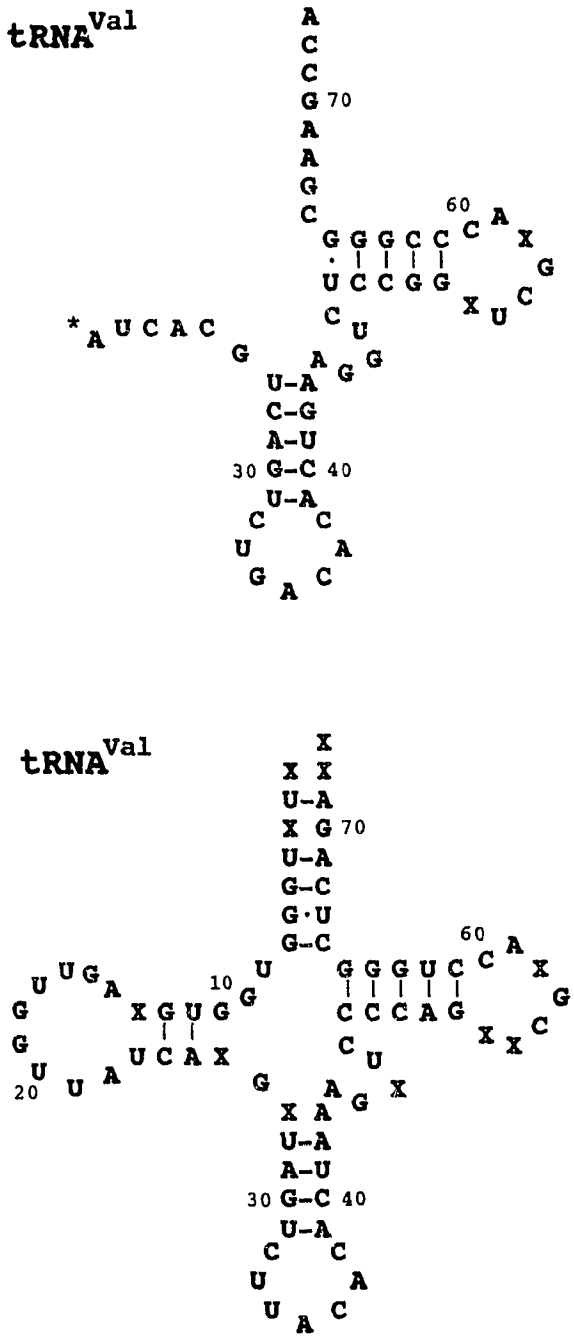


Figure 10. Continued

C/G_{30}:G/C_{40} base pair, although U is also found at position 30 in both lupin and wheat germ cytosolic $tRNA^{Ile}$(IAU) (Barciszewska *et al.*, 1988; Kusama-Eguchi *et al.*, 1991).

The $tRNA^{Leu}$(GAG) has a U_{40} while all other plant leucine tRNAs identified have the more typical G or C residue. However, this wheat $tRNA^{Leu}$ is the first plant leucine tRNA in which a GAG anticodon has been identified.

The $tRNA^{Leu4}$(XAA) has a C_{10} which is typically a G residue. This tRNA also has the appropriate nucleoside at position 25 (G_{25}) to permit normal base pairing. This C_{10} residue is not found in any other plant leucine tRNAs nor in any of the other tRNAs with which it has been compared (see Table 8).

The $tRNA^{Arg}$(CCU) has a U_6:U_{67} mismatch. Wheat mtDNA-encoded mitochondrial $tRNA^{Ser}$(GCT) also has a U:U mismatch at this position (Joyce *et al.*, 1988).

(iii). Primary sequence comparisons

The primary nucleotide sequences of the wheat tRNAs presented in Figure 10 were compared with the homologous sequences from various organisms and organelles (Tables 8 and 9). The degree of primary sequence identity was calculated from pairwise nucleotide comparison of aligned sequences and expressed as a percentage of the total number of nucleosides identified in each wheat tRNA. In some cases an identical or synonomous anticodon sequence was not available and the sequence of another isoaccepting tRNA species was used for comparison. In a few cases no related sequence was available or, in the case of

Table 8. Percent identity[+] between wheat nDNA-encoded mitochondrial tRNA sequences and their homologues* in other organisms.

	Ala GGc	Ala1 uGX	Ala2 XGX	Arg ICG	Arg CCU	Asp GUC	Gly GCC	Gly UCC	His GUG	Ile GAU	Leu GAG	Leu UAG	Leu1 XAA	Leu4 XAA	Val GAC	Val UAC
Chloroplast:																
Rice	67.8[a]	70.2[a]	69.2[a]	54.1[d]	58.1[f]	64.1	65.1	62.3	47.6	73.8	47.5[k]	45.6	54.2[m] 52.7[n]	53.4[n] 44.9[m]	48.0	44.4
Tobacco	66.1[a]	70.2[a]	67.3[a]	52.4[d]	59.7[f]	60.9	65.1	63.8	47.6	73.8	52.4[k]	47.0	51.4[m]	63.0[n]	48.0	42.9
Marchantia	69.6[a]	70.2[a]	69.2[a]	54.1[d]	64.5[f]	65.6	61.9	63.7	47.6	72.1	52.4[k]	47.0	51.4[m]	54.8[n]	48.0	39.7
Mitochondria:																
Human	39.3[a]	42.6[a]	48.1[a]	32.8[e]	----	32.8	39.7[g]	40.6	44.4	37.7	32.8[k]	30.9	----	----	48.0[o]	33.3
Yeast	53.6[a]	59.6[a]	61.5[a]	45.9[d]	50.0[f]	42.2	47.6[g]	50.7	61.9	60.6	----	----	52.8[n]	49.3[n]	54.0[o]	52.4
Marchantia	64.3[a]	68.1[a]	61.5[a]	47.5[d]	62.9[f]	51.6	58.7[g]	65.2	63.5	54.1[i]	63.9[k]	54.4	54.2[m] 54.8[n]	38.3[n] 48.6[m]	36.0[o]	42.9
Eubacteria:																
E. coli	69.6[b]	72.3[a]	71.1[b]	62.3	62.9	67.2	63.5	63.7	68.2	70.5	62.3	57.3	40.3[m] 52.7[n]	42.5[n] 50.7[m]	52.0	46.0
Nuclear:																
Yeast	69.6[c]	59.6[a]	67.3[c]	60.7[d]	53.2[f]	65.6	73.0	75.4[h]	57.1	67.2[j]	62.3[k]	75.0	62.5[m]	71.2[n]	68.0[p]	60.3
Drosophila	91.1[c]	89.4[c]	86.5[c]	65.6[d]	----	76.6	77.8	71.0[h]	65.1	82.0[j]	63.9[l]	69.1[l]	91.7[m]	65.3[m]	82.0[p]	63.5[q]

[+] Sequences were aligned based on highly conserved nucleosides and secondary structure characteristics. Percent identity was calculated from pairwise comparisons and expressed as a percent of the total number of nucleotides in the wheat mitochondrial tRNA.
* tRNA sequences containing identical or synonymous anticodons were available for comparison in most instances. In a few cases a related sequence was used for comparison. When no related sequences were available, no comparison was made. Sequence anticodons differing from the anticodons listed at the top of the table can be identified by the superscripted letter:
[a] UGC; [b] GGC; [c] AGC; [d] ACG; [e] UCG; [f] UCU; [g] UCC; [h] GCC; [i] CAT; [j] AAT; [k] UAG; [l] CAG; [m] CAA; [n] UAA; [o] UAC; [p] IAC; [q] NAC.
All sequences are listed in Steinberg *et al.* (1993).

Table 9. Percent identity[+] between wheat nDNA-encoded mitochondrial tRNA sequences and their mitochondrial and/or cytosolic homologues* in various plants

	Ala GGc	Ala1 ugX	Ala2 XGX	Arg ICG	Arg CCU	Asp GUC	Gly GCC	Gly UCC	His GUG	Ile GAU	Leu GAG	Leu UAG	Leu1 XAA	Leu4 XAA	Val GAC	Val UAC
Mitochondria:[#]																
Wheat						57.8										
Maize						57.8			47.6	57.4[l]						
Lupin							60.3	53.6[a]								
Oenothera							57.1		47.6							
Sunflower									46.0		85.2[e]	86.8[e]				
Potato										57.4[l]						
Cytosol:																
Wheat				100.0			100.0	72.5[a]		91.8						
Rice							100.0									
Arabidopsis	83.9[b]	85.1[b]	84.6[b]												98.0[m]	81.0[m]
Soybean						100.0										
Lupin							100.0		92.1	70.5	91.8[d] 82.0[k]	77.9[k]	90.4[i]		94.0[n]	77.8[n]
Sorghum							100.0									
Bean											85.2[c]	100.0[c]	91.7[f] 93.1[g]	87.7[h]		
Cucumber													95.8[g]			
Potato													94.4[g]			

Table 9. Continued:

[+] For percent identity determination, see Table 8.

[*] Only tRNA sequences containing synonymous or closely related anticodons were compared. Anticodon sequences differing from the anticodons listed at the top of the table can be identified by the superscripted letter:[a] GCC; [b] TGC; [c] NAG; [d] IAG; [e] AAG; [f] CAA; [g] NAA; [h] NAA (note that this sequence is different from the bean NAA[g] sequence); [i] UAA; [k] CAG; [l] CAU (the C is modified and the tRNA has been shown to have Ile-accepting capacity; see Table 2.); [m]AAC; [n] IAC.

[#] Except for the sunflower mitochondrial $tRNA^{Leu}$(AAG), the mitochondrial tRNAs listed in this table were identified through DNA sequencing and are thus mtDNA-encoded tRNAs. The sunflower mitochondrial $tRNA^{Leu}$(AAG), identified through RNA sequence analysis, was shown to be nDNA-encoded (Ceci *et al.*, 1992).

Sequence for the majority of these tRNAs and/or their genes can be found in Steinberg *et al.* (1993) except for the following: wheat $tRNA^{Ile}$ (Kusama-Eguchi *et al.*, 1991); lupin $tRNA^{Leu}$ (UAA), (IAG) and (CAG) (Barciszewska *et al.*, 1990); cucumber $tRNA^{Leu}$ (Jayabaskaran and Puttaraju, 1993) and sunflower $tRNA^{Leu}$ (AAG) (Ceci *et al.*, 1992).

certain mtDNA-encoded tRNA sequences, the tRNA is aminoacylated with a different amino acid than predicted by its anticodon sequence. For example, the GAG and UAG anticodons of yeast mitochondrial tRNAs specify threonine, not leucine (Dirheimer and Martin, 1990). As I only have partial sequence data for each wheat tRNA sequenced here, the percent identities calculated must be regarded as estimates only. The number of comparable nucleotide positions differs for each tRNA and ranges from 47 to 73.

In comparing the wheat tRNA sequences with non-angiosperm tRNA sequences, greatest similarity was generally with the *Drosophila* nuclear tRNA sequences. With $tRNA^{Gly}$(UCC), percent identity is slightly greater with the yeast than with the *Drosophila* species; however this comparison is with yeast and *Drosophila* tRNAs containing a GCC anticodon. Similarly, with $tRNA^{Leu}$(UAG) and $tRNA^{Leu4}$(XAA) the percent identity is slightly greater with the yeast nuclear tRNA sequence than with the *Drosophila* sequence. Again, sequences of tRNAs having the same anticodon as the wheat $tRNA^{Leu}$(UAG) have not been determined for either *Drosophila* or yeast. The only *Drosophila* tRNA sequence available for comparison with the wheat $tRNA^{Leu4}$(XAA) contains a CAA anticodon. Although the percent identity is marginally greater for *Drosophila* than for yeast in the case of $tRNA^{Leu}$(GAG) there is probably no significant difference between the two values, nor any significant difference between these and the percent identities in the *Marchantia* mitochondrial or *E. coli* comparisons. Again, a *Drosophila* cytosolic tRNA sequence containing the GAG anticodon was not available.

Where tRNAs having homologous anticodon sequences were available for comparison, the only case in which a wheat tRNA sequence did not show greatest similarity with a nuclear sequence was for $tRNA^{His}$(GUG). Here the *E. coli* sequence had the greatest percent identity. However differences between values obtained with the *E. coli, Drosophila, Marchantia* mitochondrial and yeast mitochondrial tRNA sequences in this comparison are probably not significant.

Barring the $tRNA^{His}$(GUG) comparisons, the tRNA sequences of human and yeast mitochondria generally showed the least similarity to the wheat tRNA sequences characterized here. Also, for a given tRNA species, the percent identities were very comparable with *E. coli*, *Marchantia* mitochondrial, and all chloroplast species. As plant mtDNA-encoded mt tRNAs and chloroplastic tRNAs have been shown to have approximately 65-80% sequence identity with eubacterial tRNAs (Joyce and Gray, 1989a), this relationship is not unexpected.

I have referred to the wheat mitochondrial tRNAs that I have sequenced and identified as "nDNA-encoded". This has been partially based on the sequence comparison analysis. Most plant cytosolic tRNAs identified have shown low sequence similarity with eubacterial tRNAs and greatest sequence similarity with the corresponding cytosolic tRNAs of animals. For example, $tRNA^{Trp}$ and $tRNA^{iMet}$ of wheat germ show 84% and 87% sequence identity with their respective vertebrate tRNA homologues, whereas the $tRNA^{Trp}$ shows only 68% sequence identity with $tRNA^{Trp}$ species of yeast and only 54% sequence identity with bacterial species (Ghosh and Ghosh, 1984).

One can also compare the tRNA sequences I have identified with homologous mitochondrial tRNA or tRNA gene sequences from other plants and with plant cytosolic tRNAs or their gene sequences. Not all the tRNAs I have sequenced have been identified in other plants but a subset of these sequences is available and can be used for sequence comparison (Table 9). With the exception of the $tRNA^{Leu}$(GAG) and $tRNA^{Leu}$(UAG) sequences compared with the sunflower mitochondrial $tRNA^{Leu}$(AAG) sequence, greatest percent identity is with cytosolic tRNA or nuclear gene sequences. However, the sunflower mitochondrial $tRNA^{Leu}$ was shown by Southern hybridization to be nDNA-encoded (Ceci *et al.*, 1992); thus a wheat nDNA-encoded mt $tRNA^{Leu}$ would be expected to show greater similarity with this sunflower species than other wheat nDNA-encoded mt species would with their mtDNA-encoded counterparts. All comparisons made with the remaining plant mitochondrial tRNA sequences listed in Table 9 (which were identified through mtDNA sequencing) generated sequence identity values of <60%.

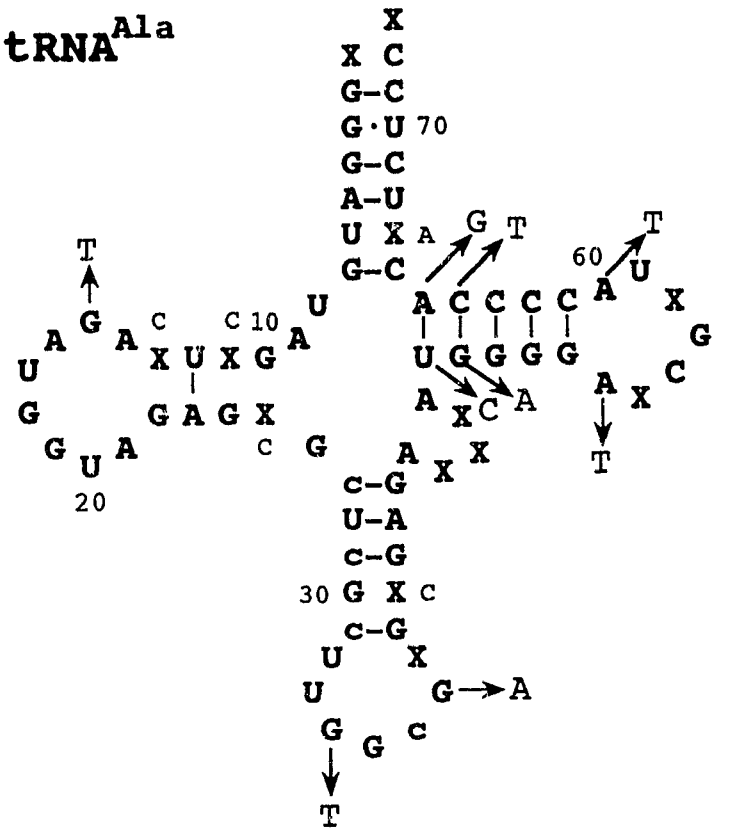
The $tRNA^{Arg}$(ICG), $tRNA^{Asp}$(GUC) and $tRNA^{Gly}$(GCC) sequences are identical (within the regions of assigned nucleotide sequence) to the other plant cytosolic tRNAs for which sequence is available. The $tRNA^{Leu}$(UAG) sequence is identical to that of bean $tRNA^{Leu}$(NAG) (Maréchal-Drouard *et al.*, 1988) but has only 80% identity with a lupin $tRNA^{Leu}$(CAG) sequence (Barciszewska *et al.*, 1990). The $tRNA^{Val}$(GAC) sequence is virtually identical with a $tRNA^{Val}$(AAC) gene sequence of *Arabidopsis* (Gokhman and Zamir, 1990). The only difference

is the adenosine nucleoside in the anticodon of the *Arabidopsis* tRNA. A lupin $tRNA^{Val}$ has been sequenced and shown to have an IAC anticodon (Barciszewska and Jones, 1987). The gene sequence for this anticodon would be AAC because inosine is the post-transcriptional conversion product of adenosine. As discussed earlier inosine is recognized in the chemical sequencing reactions as guanosine and therefore the presence of the modified nucleoside would not be detected. However reverse transcriptase sequence showed no anomalies at the first position of the anticodon sequence (Figure 7A) and the enzyme recognized this nucleoside as guanosine. Therefore it remains to be determined whether the wheat $tRNA^{Val}$(GAU) contains inosine in the anticodon.

For the most part the remaining tRNAs showed 80% or greater sequence identity when compared with synonomous plant cytosolic tRNA sequences. One notable difference is the $tRNA^{Ile}$ of lupin (Barciszewska *et al.*, 1988) which has only 70.5% sequence identity with the wheat mitochondrial $tRNA^{Ile}$.

Differences between the wheat mitochondrial tRNA sequences and synonymous or related plant cytosolic tRNA sequences are depicted in Figure 11. The alanine tRNAs are compared to the only other plant $tRNA^{Ala}$ sequence available, that of the *Arabidopsis* $tRNA^{Ala}$(TGC) gene sequence. The $tRNA^{Gly}$(UCC) sequence is compared to a related wheat $tRNA^{Gly}$(GCC) sequence and differs from that tRNA at 19 positions. The tRNAs for leucine (GAG) and valine (UAC) are also compared to related sequences, those of bean $tRNA^{Leu}$(UAG) and *Arabidopsis* $tRNA^{Val}$(AAC), respectively. The leucine tRNAs

Figure 11. Comparison of the primary sequences and potential secondary structures of the wheat mitochondrial tRNAs listed in Table 7 with cytosolic isoaccepting tRNAs in various plants. Wheat mitochondrial tRNA sequences identical to those of homologous tRNAs in other plants are not shown (see text). Also, no synonomous plant cytosolic $tRNA^{Arg}$(CCU) is available for comparison with the wheat mt $tRNA^{Arg}$(CCU). Differences between the wheat mitochondrial and plant cytosolic tRNA sequences to which they are compared, are indicated by the arrows pointing toward the cytosolic residue. Nucleosides missing in cytosolic tRNA sequence are indicated by the open square (□). Additional nucleosides in the cytosolic tRNA sequences are boxed. Lowercase letters denote tentative identification of nucleosides and unassigned nucleosides are indicated by "X". Cytosolic tRNA nucleosides corresponding to "X"s in helical regions of the wheat mitochondrial tRNA sequences are indicated. The following comparisons of wheat nDNA-encoded mt tRNAs to cytosolic tRNA sequences were made: $tRNA^{Ala}$(GGc), $tRNA^{Ala}$(ugX) and $tRNA^{Ala}$(XGX) to *Arabidopsis* $tRNA^{Ala}$(TGC); $tRNA^{Gly}$(UCC) to wheat $tRNA^{Gly}$(GCC); $tRNA^{His}$(GUG) to lupin^{His}(GUG); $tRNA^{Ile}$(GAU) to wheat $tRNA^{Ile}$(IAU); $tRNA^{Leu}$(GAG) to bean $tRNA^{Leu}$(UAG); $tRNA^{Leu1}$(XAA) to potato^{Leu}(XAA); $tRNA^{Leu4}$(XAA) to bean $tRNA^{Leu4}$(XAA); and $tRNA^{Val}$(UAC) to *Arabidopsis* $tRNA^{Val}$(AAC). Cytosolic tRNAs sequences are listed in Steinberg *et al.*(1993) except for wheat $tRNA^{Ile}$(IAU) which can be found in Kusama-Eguchi *et al.* (1991).



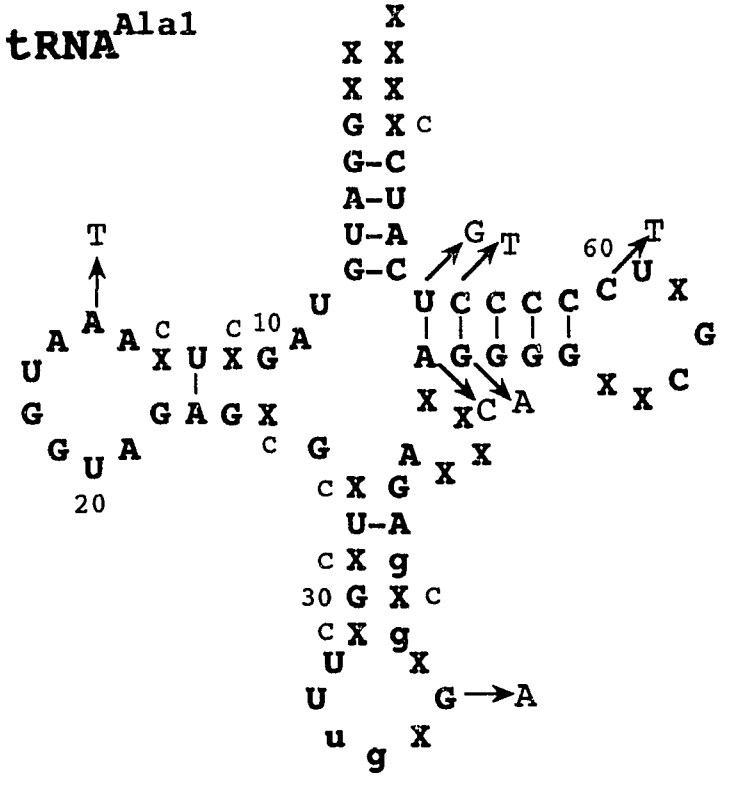


Figure 11. Continues

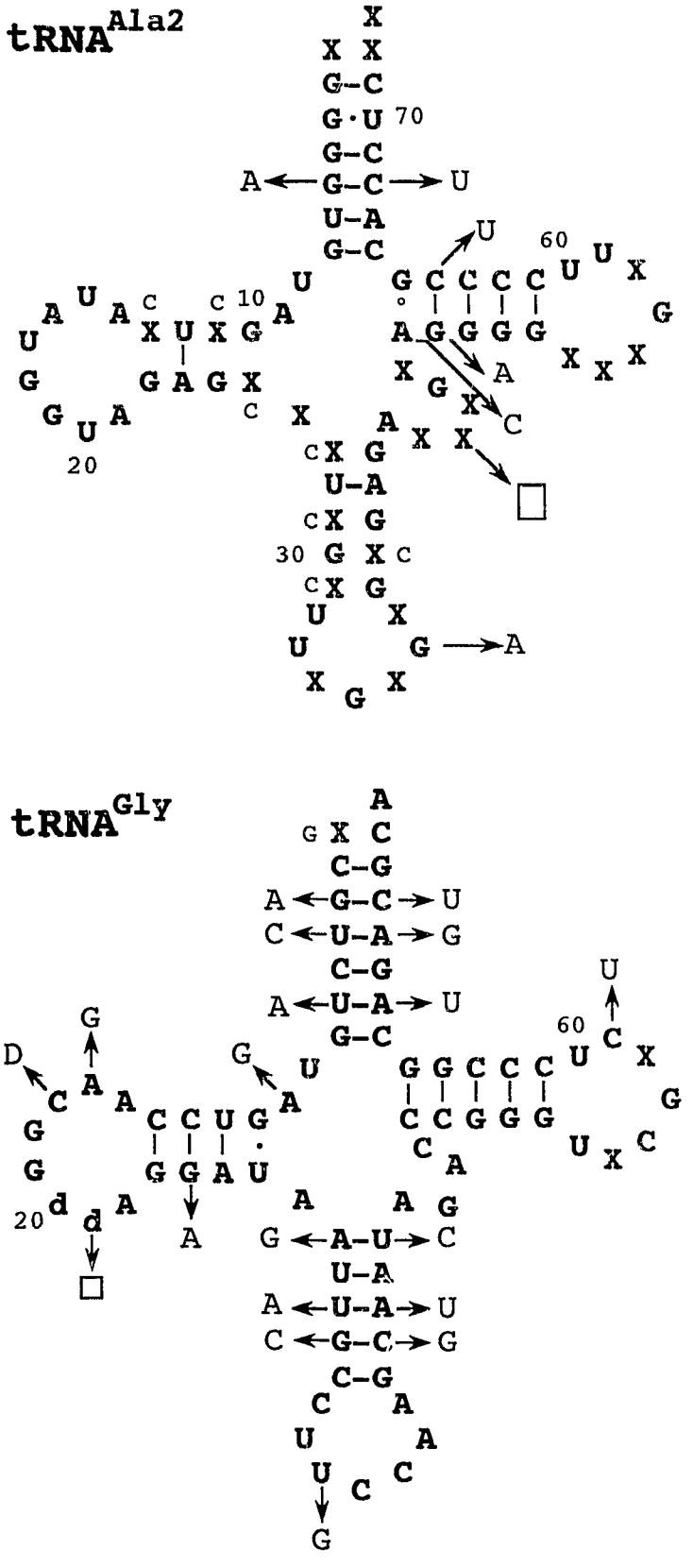
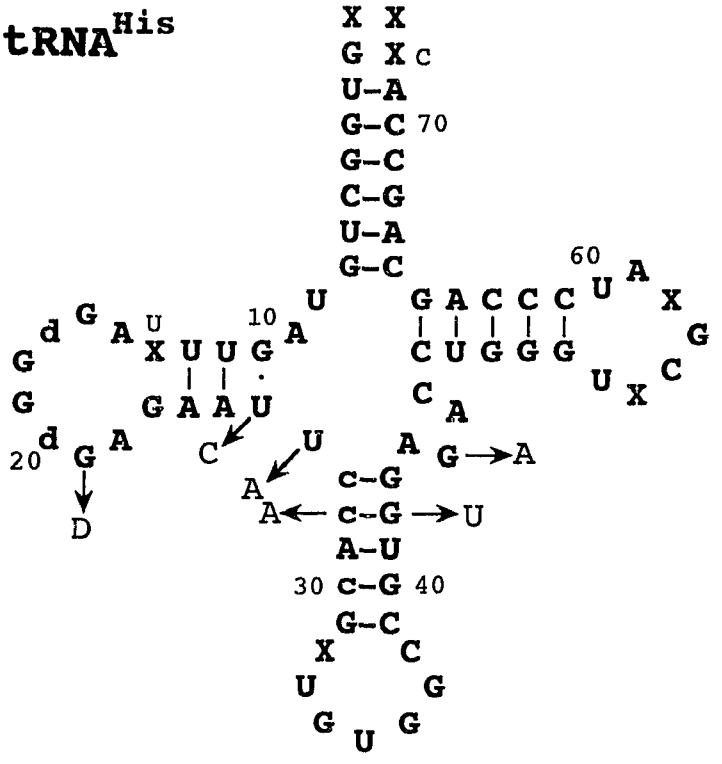


Figure 11. Continues



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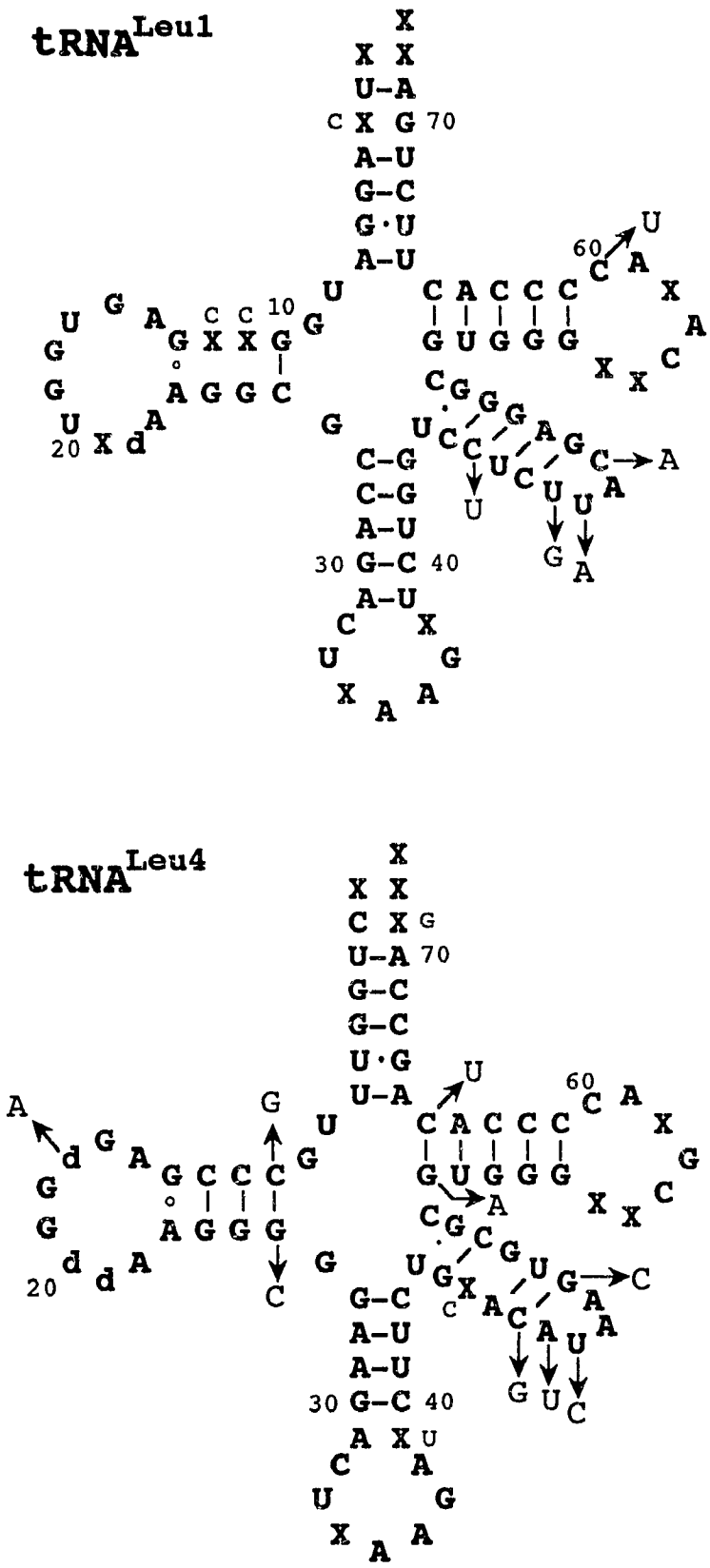


Figure 11. Continues

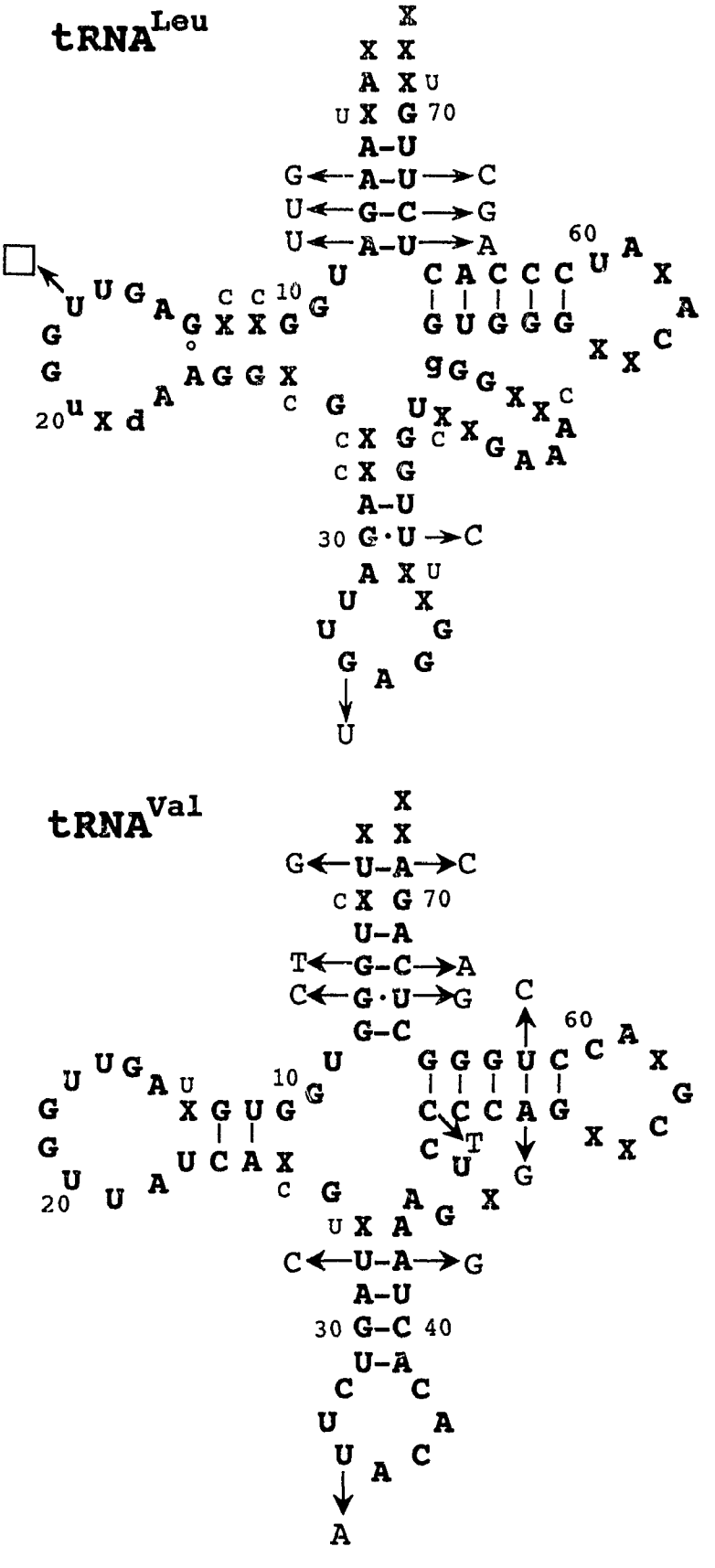


Figure 11. Continued

differ at only nine positions while the valine tRNAs differ at 12. The $tRNA^{His}$, $tRNA^{Ile}$, $tRNA^{Leu1}$ and $tRNA^{Leu4}$ differ from synonomous tRNAs in lupin, wheat, potato and bean by six, seven, four and nine positions, respectively.

E. Subcellular localization of tRNAs

In an effort to distinguish nDNA-encoded mitochondrial tRNAs actively used by the mitochondria from potential cytosolic tRNA contaminants of the mitochondrial preparation, slot blots containing mitochondrial RNA and cytosolic RNA were prepared and hybridized with oligonucleotides specific for each of the tRNAs listed in Table 7. The oligonucleotides were the same as those used for reverse transcriptase sequencing and in general were complementary to the 3' end of the tRNA (see Table 4, Material and Methods). One exception was the oligonucleotide specific for $tRNA^{Gly}$(GCC) (which was not sequenced by the reverse transcriptase method); this oligonucleotide was complementary to the anticodon loop region of this tRNA. Two additional oligonucleotides were used in this experiment. One was specific for a mtDNA-encoded mt $tRNA^{Pro}$(UGG) and the other was specific for a nDNA-encoded cytosolic $tRNA^{Phe}$(GAA). The latter is not required in wheat mitochondria because the mtDNA encodes a synonymous $tRNA^{Phe}$(GAA).

The hybridization pattern for a mtDNA-encoded mt tRNA was expected to be different from that of a nDNA-encoded mt tRNA and both of these patterns were expected to be different from the hybridization pattern for a nDNA-encoded cytosolic tRNA not required for use in the mitochondria. In fact, three distinct

hybridization patterns were obtained. For hybridization with the $tRNA^{Pro}$(UGG) oligonucleotide a strong signal was obtained with mitochondrial RNA whereas a much weaker signal was seen with cytosolic RNA (Figure 12A). For hybridization with the $tRNA^{Phe}$(GAA) oligonucleotide a strong signal was obtained with cytosolic RNA and a much weaker signal was obtained with mitochondrial RNA (Figure 12B). In the case of a putative nDNA-encoded mt tRNA such as $tRNA^{Gly}$(GCC), the oligonucleotide hybridization pattern with mitochondrial RNA was apparently equivalent to that obtained with cytosolic RNA (Figure 12C).

The relative strengths of the various hybridization signals obtained can be estimated by optical scanning of the relevant autoradiograms. The density of each spot can then be related to the concentration of RNA in each slot. In order to compare the strengths of a signal obtained with mitochondrial RNA and cytosolic RNA, the densities of the bands on the blot must be related linearly to the concentration of RNA on the blot. When this condition is met, signal strength is directly proportional to RNA concentration. The slope of the lines, or regression coefficients, obtained when a oligonucleotide is hybridized to both mitochondrial RNA and cytosolic RNA can then be compared, thus giving an estimate of the relative stengths of the two signals. Figure 12B shows the linear regression plots for the $tRNA^{Pro}$(UGG)-, $tRNA^{Phe}$(GAA)- and $tRNA^{Gly}$(GCC)-specific oligonucleotides whose hybridization patterns are presented in Figure 12A.

Regression coefficients from slot blot hybridization analyses of presumed nDNA-encoded mt tRNAs and the control tRNAs are presented in Table 10. The

Figure 12A. Slot blot analyses of three wheat tRNA-specific oligonucleotide probes hybridized to wheat mitochondrial RNA and cytosolic RNA. Autoradiograms show the hybridization of 5'-^{32}P end-labelled oligonucleotides specific for: (A) mtDNA-encoded mt $tRNA^{Pro}$(UGG); (B) nDNA-encoded cyt $tRNA^{Phe}$(GGA); and (C) nDNA-encoded mt $tRNA^{Gly}$(GCC), to: wheat mitochondrial RNA (M); cytosolic RNA (C); in the case of $tRNA^{Pro}$(UGG) and $tRNA^{Phe}$(GGA) total cellular RNA that had been treated with DNase (T^1); and in the case of $tRNA^{Pro}$(UGG) cytosolic RNA that had been treated with DNase (C^1). RNA samples were serially diluted 2-fold to produce amounts ranging from 6 μg to 0.047 μg and applied to wells of the slot blot apparatus as described in section G, Materials and Methods.

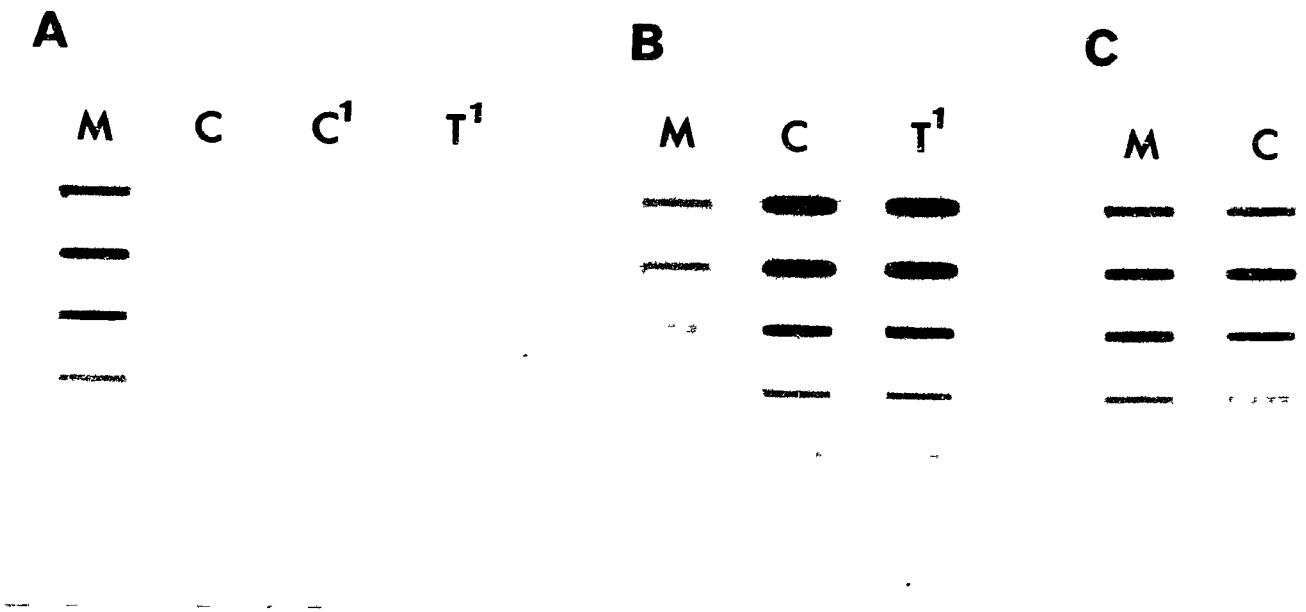
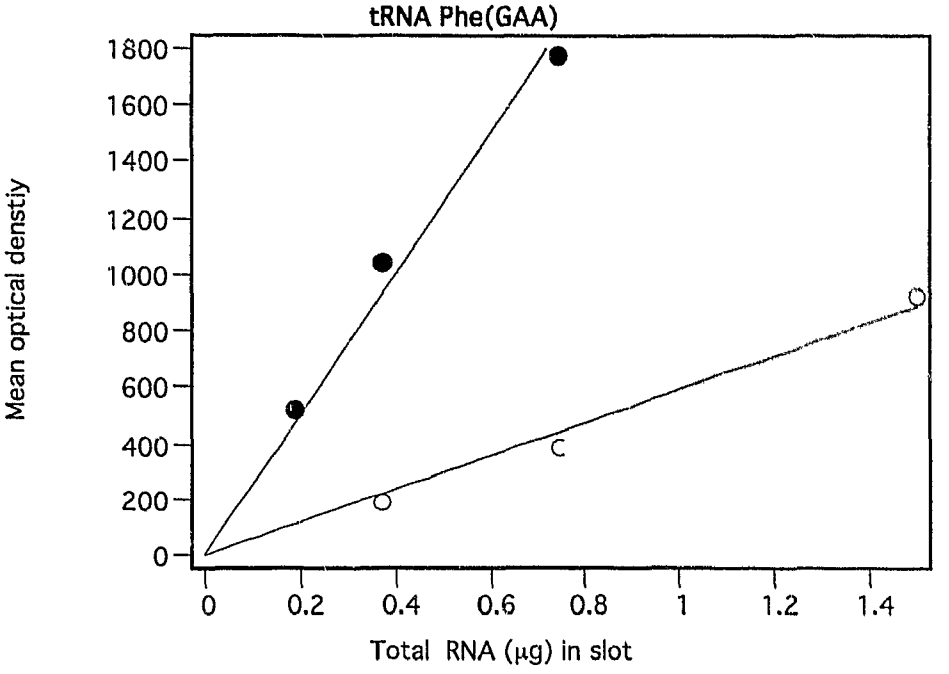


Figure 12A Continued.



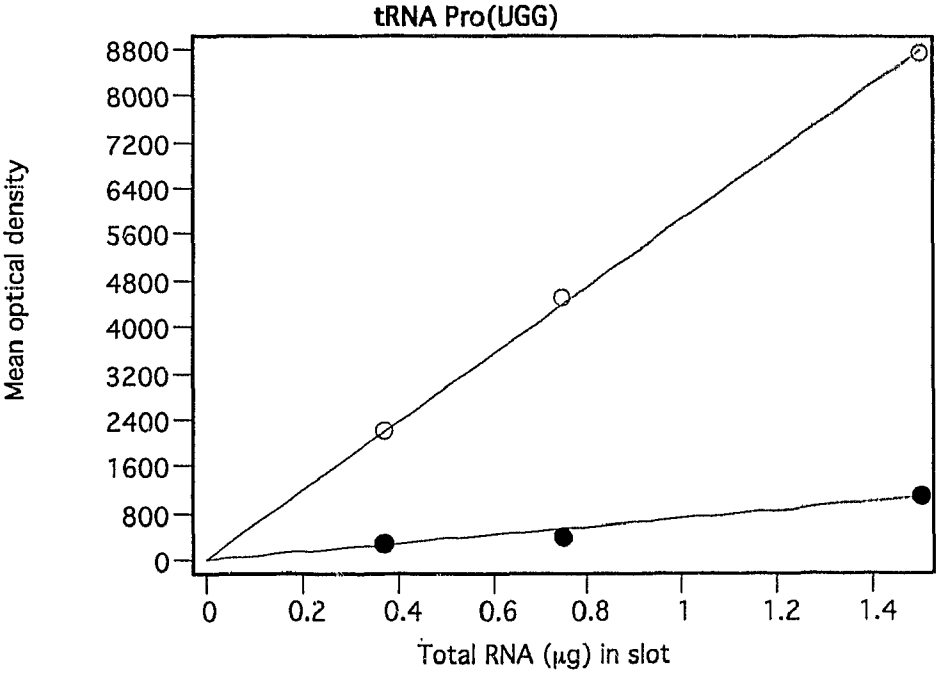


Figure 12B. Linear regression plots for the three slot blot analyses depicted in Figure12A.
• cytosolic RNA
o mitochondrial RNA

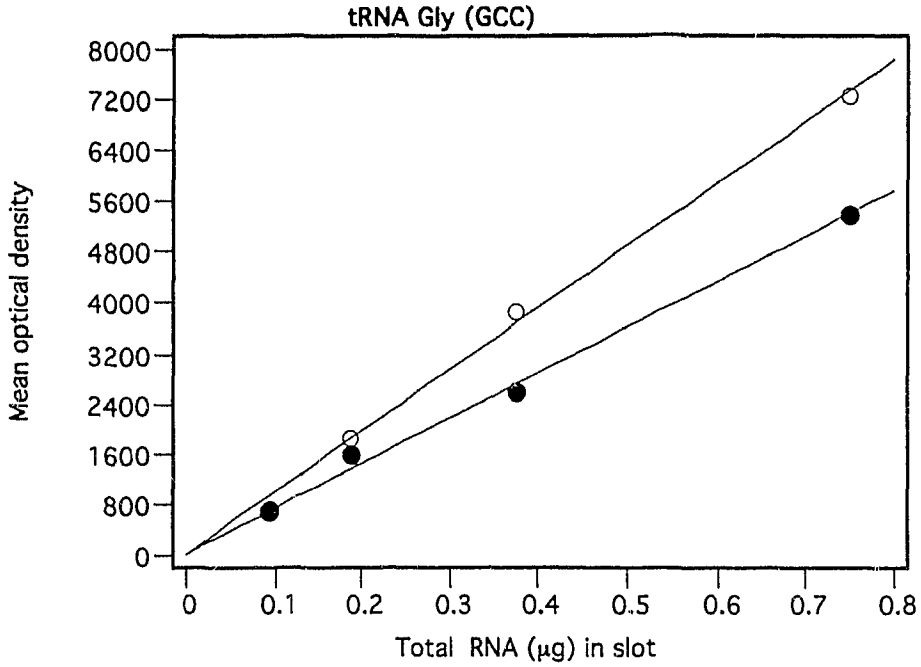


Figure 12B. Continued

Table 10. Linear regression coefficients from slot blot hybridization analyses[1] of wheat nDNA-encoded mitochondrial tRNAs using tRNA-specific oligonucleotide probes[2].

	Linear Regression Coefficients[3]		Ratio of regression coefficients
tRNA	mt RNA	cyt RNA	mt RNA/cyt RNA
Phe(GAA)[4]	591	2,474	0.24
Pro(UGG)[5]	5,868	707	8.30
Ala(GGc)	2,214	1,235	1.72
Ala1(uGX)	7,043	6,680	1.05
Ala2(XGX)	6,807	6,952	0.98
Arg(ICG)	3,290	3,509	0.94
Arg(CCU)	8,640	9,483	0.91
Asp(GUC)	3,121	10,102	0.31
Gly(GCC)	9,786	7,216	1.35
Gly(UCC)	6,246	6,001	1.04
His(GUG)	2,754	2,727	1.01
Ile(GAU)	25,266	14,426	1.75
Leu(GAG)	2,010	4,297	0.47
Leu(UAG)	5,963	3,985	1.50
Leu1(XAA)	3,031	2,670	1.14
Leu4(XAA)	17,733	3,633	4.88
Val(GAC)	13,292	13,291	1.00
Val(UAC)	14,125	13,328	1.06

[1] Slot blots containing mitochondrial (mt) RNA and cytosolic (cyt) RNA were prepared as described in the Material and Methods.
[2] Sequence for the oligonucleotide probes is given in Table 4 of the Material and Methods.
[3] Correlation coefficients (R^2) for the regression coefficients ranged from 0.96 to 0.99.
[4] This is a nDNA-encoded cytosolic tRNA thought not to be used in the wheat mitochondrion because a mtDNA-encoded $tRNA^{Phe}$(GAA) has been identified.
[5] This tRNA is mtDNA-encoded.

ratio of the regression coefficients (mt RNA/cyt RNA) for $tRNA^{Phe}$(GAA) was only 0.24 as compared to the ratio of the regression coefficients for $tRNA^{Pro}$(UGG) which was 8.3. Not surprisingly the ratio of the regression coefficients for $tRNA^{Gly}$(GCC) was close to 1 (1.35). The majority of the nDNA-encoded mt tRNAs characterized here had regression coefficient ratios of 1.0 or greater. Thus, the majority of these tRNAs are present in the mitochondria at levels greater than are found for a nDNA-encoded cytosolic tRNA that is not required by the mitochondrion for protein synthesis. In one instance ($tRNA^{Leu4}$ (XAA)) the ratio of the regression coefficients was significantly greater than 1.0. This may indicate that this tRNA is not used or is not required in large amounts in the cytosol, and/or is required in the mitochondria in greater amounts than in the cytosol.

Two of the nDNA-encoded tRNAs identified as potential wheat mitochondrial tRNAs had regression coefficient ratios significantly less than 1.0. The $tRNA^{Asp}$(GUC) had a regression coefficient ratio of only 0.31 and the $tRNA^{Leu}$(GAG) had a regression coefficient ratio of 0.47. The $tRNA^{Asp}$ is not present in mitochondria at a level substantially different from that of the nDNA-encoded $tRNA^{Phe}$, which is not required by the mitochondrial translation system. On the basis of this criterion, the nDNA-encoded $tRNA^{Asp}$ cannot be distinguished from a cytosolic tRNA that may be present in the mitochondrial RNA preparation solely as the result of contamination. The $tRNA^{Asp}$ was isolated from spot 75 on the 2-D polyacrylamide gel and when further electrophoresed in the third dimension, it only appeared as a minor band. Therefore it is not unexpected that

this tRNA is present in the mitochondria at levels that possibly reflect contamination.

On the other hand, the $tRNA^{Leu}$(GAG) was isolated from spots 28a and 8b on the 2-D polyacrylamide gel, both of which were major spots (see Figure 4). When electrophoresed in the third dimension the bands containing this tRNA were equal in intensity to the $tRNA^{Leu}$(UAG) bands (compare 28a.1 and 28a.2, Figure 5); although the specific activities of these tRNAs were not determined. There are several possible explanations for this result.

The first possibility is that the oligonucleotide used in this hybridization is not specific for the $tRNA^{Leu}$(GAG). Except in the case of the $tRNA^{Gly}$(GCC) oligonucleotide, all the oligonucleotides used for the slot blot hybridization experiments were also used as primers for reverse transcriptase sequencing. As mentioned previously, reverse transcriptase sequence was successfully generated for every tRNA except the alanine tRNAs. This allows at least partial confirmation of the hybridizing target in the mitochondrial RNA fraction. In only one instance was there evidence that an oligonucleotide recognized more than the specific tRNA for which it was designed. The oligonucleotide specific for the $tRNA^{His}$(GUG) generated the sequence for this tRNA and also a mtDNA-encoded $tRNA^{Ser}$ sequence. However the stringency of the washes in the slot blot hybridization make it unlikely that the $tRNA^{His}$ oligonucleotide remains bound to $tRNA^{Ser}$ on the blot. In the case of a $tRNA^{Ile}$(GAU)-specific oligonucleotide having a single mismatch with its target sequence, accurate reverse transcriptase sequence could

be generated but no signal was produced following slot blot hybridization. With the fully complementary oligonucleotide, a normal slot blot hybridization pattern was obtained. The $tRNA^{His}$-specific oligonucleotide has three positions of mismatch with the $tRNA^{Ser}$ sequence. Therefore, it is highly unlikely the $tRNA^{His}$ oligonucleotide remains bound to $tRNA^{Ser}$ throughout the washes in the slot blot hybridization procedure, although this cannot be ruled out.

A second possible explanation for the result obtained with the $tRNA^{Leu}$(GAG)-specific oligonucleotide is non-specific binding of this probe to RNA, particularily cytosol RNA. It is possible the probe is binding generally to RNA; that is not in a manner that would generate a readable sequence during reverse transcriptase sequencing, but that would produce a general background signal. This was a potential problem for all of the slot blot hybridizations and was addressed in a slot blot hybridization experiment using an oligonucleotide having sequence identical to a maize mtDNA-encoded $tRNA^{His}$(GUG). This oligonucleotide is complementary to the antisense strand of the tRNA gene and will be referred to as the antisense oligonucleotide. Results of this hybridization (Figure 13) showed very faint bands in the mitochondrial RNA, cytosolic RNA and total cellular RNA lanes, with the signal in the mitochondrial RNA lane being the strongest. The latter signal most likely represents hybridization to mtDNA whereas the faint signals in the other lanes presumably represent non-specific background. It should be noted that this blot had to be exposed to autoradiography film for nine days in order to see any signal. Generally the length of exposure used for the slot

Figure 13. Slot blot hybridization of an antisense $tRNA^{His}$(GUG)-specific oligonucleotide probe to wheat mitochondrial, cytosolic and total cellular RNA. The autoradiogram shows the hybridization of the 5' ^{32}P end-labelled oligonucleotide, identical in sequence to the 3'-end of the maize mtDNA-encoded $tRNA^{His}$(GUG), to: wheat mitochondrial RNA (M); cytosolic RNA (C); cytosolic RNA that had been treated with DNase (C^1); total cellular RNA (T); and total cellular RNA that had been treated with DNase (T^1). The slot blot was prepared as described in Figure 12A.

blot hybridizations was in the order of one to three days, the longest exposure being five days. It is therefore unlikely that specific hybridization to mtDNA or non-specific hybridization to either mitochondrial or cytosolic RNA is contributing significantly to the signals observed.

A third possible explanation for the hybridization pattern obtained with the $tRNA^{Leu}$(GAG)-specific oligonucleotide is that this particular tRNA actually constitutes a greater proportion of the cytosolic RNA than it does of the mitochondrial RNA. Thus although the amount of this tRNA observed in the third dimension of the electrophoretic separation of a mitochondrial tRNA preparation is comparable to other tRNAs that have regression coefficient ratios close to 1.0, this tRNA cannot be distinguished from cytosolic tRNAs non-specifically localized in the mitochondria.

F. Reverse transcriptase sequencing of mtDNA-encoded $tRNA^{Asp}$(GUC) and $tRNA^{His}$(GUG)

Both a $tRNA^{Asp}$(GUC) and a $tRNA^{His}$(GUG) were isolated from a mitochondrial RNA preparation in this study. They were identified as nDNA-encoded based on their high sequence similarity with synonymous plant cytosolic tRNAs (see Table 9). However, genes (distinct in sequence) for both of these tRNAs have been identified in the mtDNA of monocotyledonous plants. The $tRNA^{Asp}$ gene was identified in wheat mitochondria (Joyce *et al.*, 1988) and the $tRNA^{His}$ gene was identified in maize mitochondria (Iams *et al.*, 1985). However, tRNAs corresponding to these genes were not found by Joyce and Gray (1989a)

nor in my analysis of wheat mitochondrial tRNAs. In an attempt to determine if these tRNAs exist in wheat mitochondria the oligonucleotides used for the slot blot hybridization experiments were used for reverse transcriptase sequencing. Sequences for both of these tRNAs were obtained (Figure 14). Although there is evidence that mtDNA is present in trace amounts in the mitochondrial RNA preparation (Figure 13), it is unlikely that these sequences were generated from mtDNA because there is strong termination of the sequence ladder corresponding to the mature 5' end of the tRNA and many pauses within the sequence itself, diagnostic of modified nucleosides. The predicted secondary structures for these tRNAs are presented in Figure 15. The sequences are identical to the previously published gene sequences at all positions of identified nucleosides.

On the basis of the slot blot analysis the nDNA-encoded mt $tRNA^{Asp}$ could not be distinguished from a cytosolic tRNA that is potentially non-specifically associated with mitochondria. Relative to other nDNA-encoded tRNAs, this tRNA was also not present in large amounts in the 3-D polyacrylamide gel electrophoretic separation. The mtDNA-encoded mt $tRNA^{Asp}$ is also apparently present in small amounts in wheat mitochondria, as it eluded detection in this work and in the work of Joyce and Gray (1989a). Similar results were obtained in potato mitochondria (Maréchal-Drouard *et al.*, 1990). They located the same $tRNA^{Asp}$ by oligonucleotide hybridization only, using a blot containing mitochondrial tRNAs separated by 2-D polyacrylamide gel electrophoresis. Maréchal-Drouard *et al.* (1990) stated that the signal for this tRNA was very weak in their mitochondrial

Figure 14. Reverse transcriptase sequencing of wheat mtDNA-encoded $tRNA^{Asp}$(GUC) and $tRNA^{His}$(GUG). Oligonucleotides specific for wheat mtDNA-encoded $tRNA^{Asp}$(GUC) and $tRNA^{His}$(GUG) were used as primers for extension by reverse transcriptase with wheat mitochondrial RNA as template. Autoradiograms depict reverse transcriptase sequencing reactions for (A) $tRNA^{His}$(GUG); (B) and (C) $tRNA^{Asp}$(GUC). Figure (C) is a longer autoradiogram exposure of the same sequencing gel shown in (B). Sequence for the $tRNA^{Asp}$(GUC) begins in (B) and continues in (C) where the 5' terminal nucleosides can be seen. Sequence of each tRNA is depicted to the left of the sequencing gel autoradiogram: G = guanosine; C = cytidine; A = adenosine; U = uridine; X = unassigned nucleoside; and E = primer extension lane. Anticodon sequence is bracketed. (Note that the lanes of the reverse transcriptase sequencing autoradiogram are labelled with the nucleoside complementary to the actual sequence reaction so that the tRNA sequence can be read directly.)

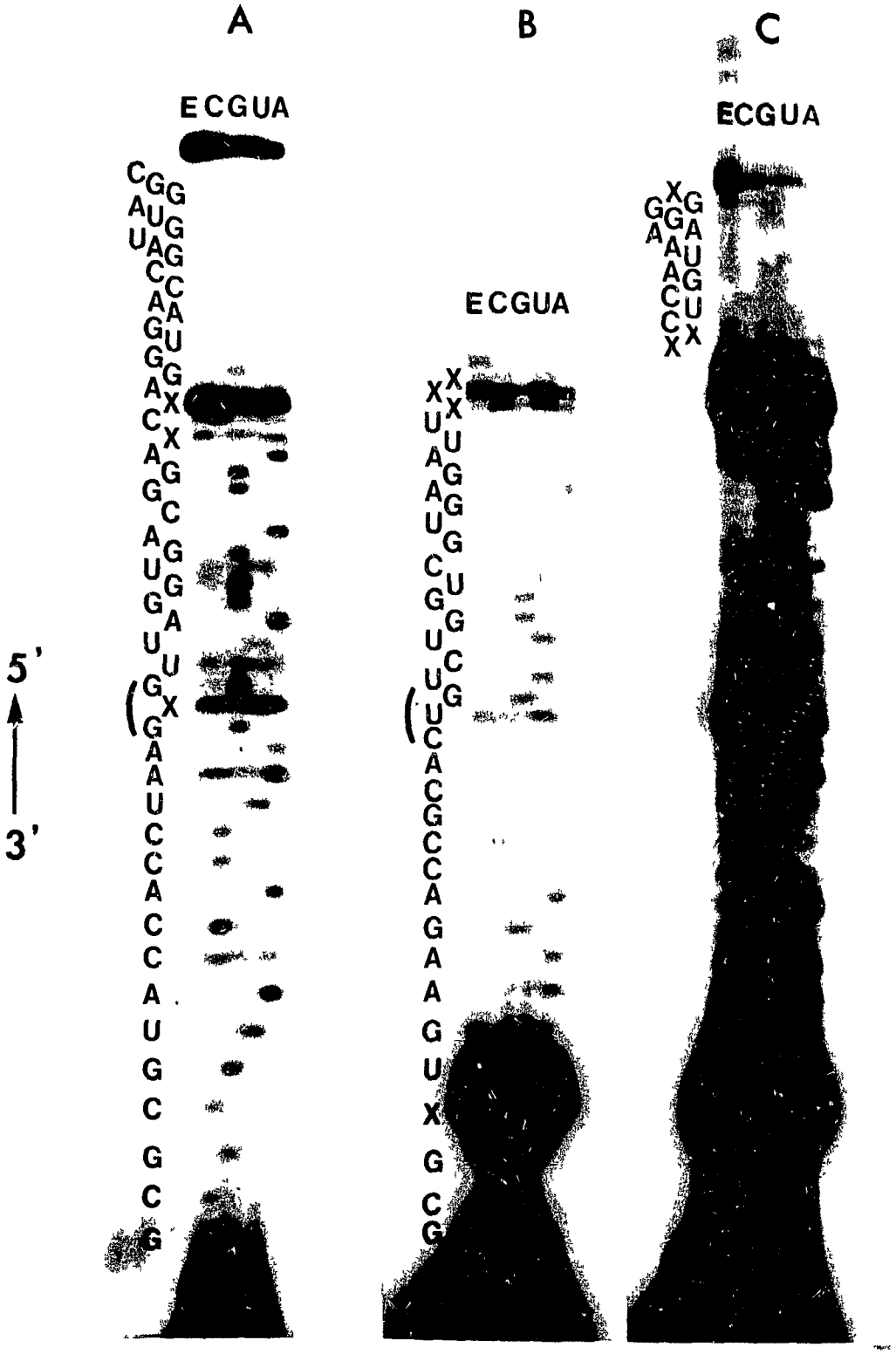
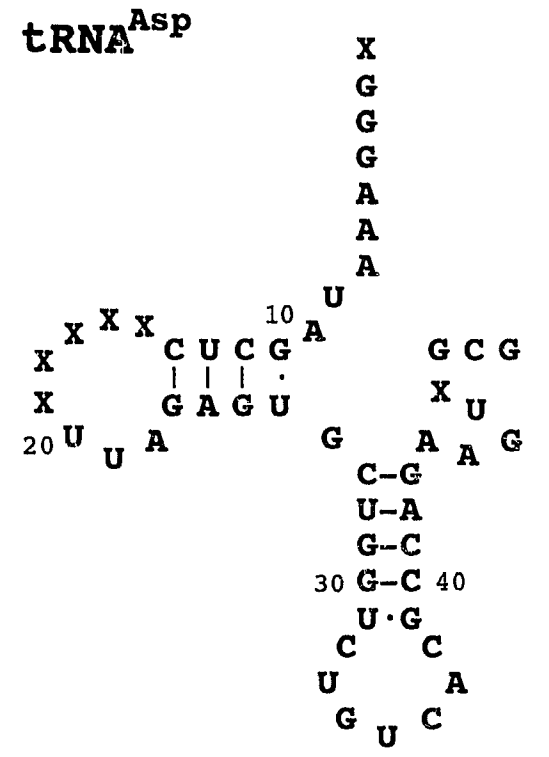


Figure 14 Continued.

Figure 15. Primary sequences and potential secondary structures of the wheat mtDNA-encoded mt $tRNA^{Asp}$(GUC) and $tRNA^{His}$(GUG) derived from reverse transcriptase sequencing data. Nucleosides that could not be identified are indicated by the letter "X".



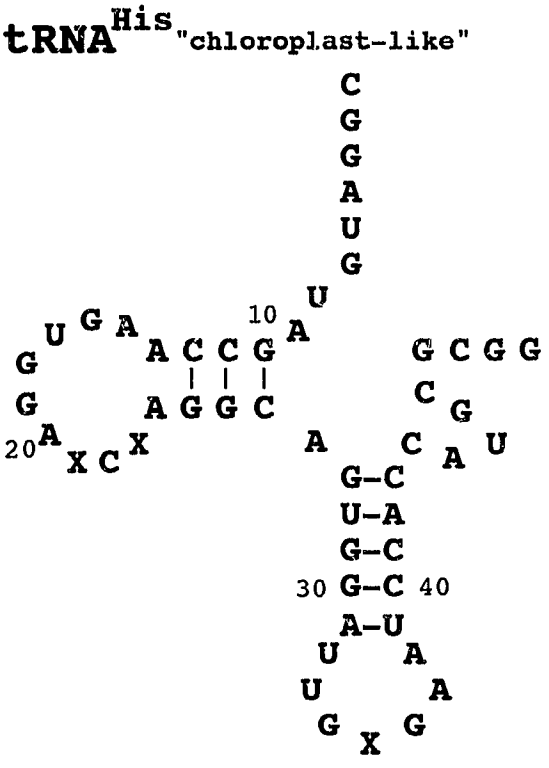


Figure 15. Continued

tRNA preparations.

Results of the slot blot analysis for the nDNA-encoded $tRNA^{His}$ suggest that this tRNA is present in the mitochondria. Again, the mtDNA-encoded $tRNA^{His}$ is apparently present in small amounts in the mitochondria or is not easily identified through chemical sequencing. This tRNA was partially sequenced in potato mitochondria (Maréchal-Drouard *et al.*, 1990) and labelled mitochondrial RNA has been used as a probe to isolate the gene in maize (Iams *et al.*, 1985).

As discussed in Section B of Results, two spots in a 2-D gel electrophoretic separation of wheat mitochondrial tRNAs that were eluted following hybridization to mtDNA did not correspond to previously identified mtDNA-encoded tRNAs. These two spots may be the mtDNA-encoded $tRNA^{Asp}$ and $tRNA^{His}$ characterized here.

G. Southern hybridization analysis

In an attempt to confirm the nuclear origin of the mitochondrial tRNAs identified as nDNA-encoded in this study, Southern hybridizations of the $tRNA^{Ile}$(GAU)-, $tRNA^{Gly}$(GCC)- and $tRNA^{Leu}$(UAG)-specific oligonucleotide probes with mtDNA and nDNA were conducted. No hybridization was evident with any of these probes in either the mtDNA or the nDNA lanes. As positive controls, hybridizations were conducted using oligonucleotide probes specific for the mtDNA-encoded $tRNA^{fMet}$ and the nDNA-encoded $tRNA^{Phe}$. The $tRNA^{fMet}$ probe produced a signal with mtDNA (Figure 16A) while the $tRNA^{Phe}$ probe produced no signal (Figure 16B). Even when the specific activity of the

Figure 16. Southern hybridization analysis of wheat mtDNA-encoded $tRNA^{fMet}$ and wheat nDNA-encoded $tRNA^{Phe}$. A Southern blot containing electrophoretically separated *Eco*RI (E), *Hin*dIII (H) and *Sal*I (S) digests of wheat mtDNA and nDNA was probed sequentially with 5' end-labelled oligonucleotides specific for either the mtDNA-encoded $tRNA^{fMet}$ (A) or the nDNA-encoded $tRNA^{Phe}$ (B). The migration positions of selected *Hin*dIII marker fragments of lambda DNA are indicated.

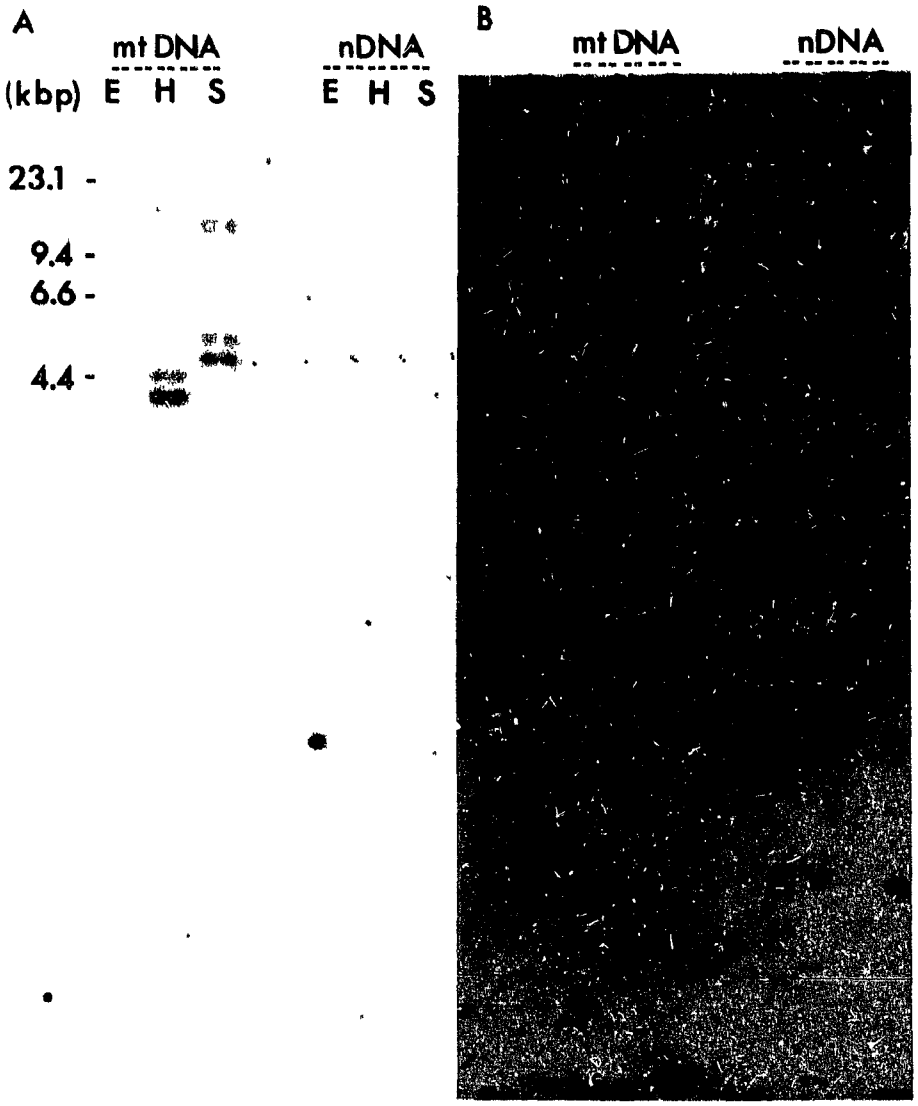


Figure 16 Continued.

oligonucleotide probe was increased through 3' end-labelling with terminal transferase and [α-^{32}P]dATP, no hybridization with nDNA could be detected. For this reason, further Southern hybridizations with the remaining nDNA-encoded mitochondrial tRNAs were not conducted.

Southern hybridization was also conducted using oligonucleotide probes specific for wheat mtDNA-encoded tRNA^{Asp} and maize mtDNA-encoded tRNA^{His}. Both probes produced bands in the mtDNA lanes whereas the tRNA^{His} probe generated bands in the nDNA lane, as well (Figure 17). The latter bands are most likely due to chloroplast DNA contaminating the nDNA preparation (Joyce and Gray, 1989b). However, a control experiment with chloroplast DNA would be necessary to prove this. The magnitude of the signal obtained is probably due to the amount of nDNA loaded on the gel in an attempt to detect bands with probes specific for nDNA-encoded mitochondrial tRNAs. Thus, the mtDNA lanes contained 0.25 μg of DNA whereas the nDNA lanes contained 25 μg.

Figure 17. Southern hybridization analysis of wheat mtDNA-encoded $tRNA^{His}$(GUG) and $tRNA^{Asp}$(GUC). Southern blots containing electrophoretically separated *Eco*RI (E) and *Hin*dIII (H) digests of wheat mtDNA (mt) and nDNA (n) were probed with 5' end-labelled oligonucleotides specific for either the wheat mtDNA-encoded $tRNA^{Asp}$(GUC) (A) or the maize mtDNA-encoded $tRNA^{His}$(GUG) (B). The migration positions of selected *Hin*dIII marker fragments of lambda DNA are indicated. Arrows indicate bands in the mtDNA lanes.

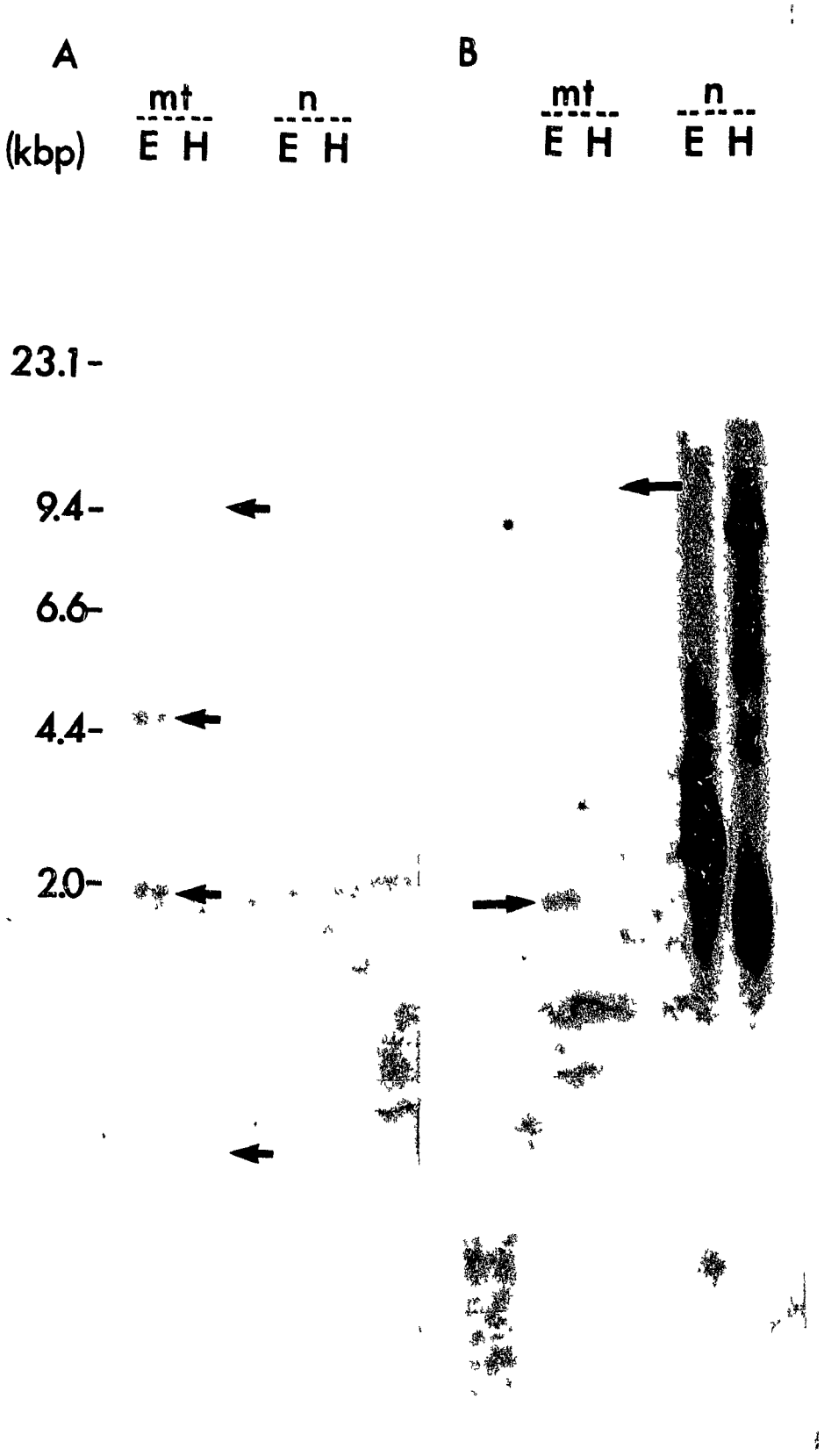


Figure 17 Continued.

IV. Discussion

A. Purity of wheat mitochondrial tRNA

In determining the subcellular localization of a cell constituent, the possibility of artifactual localization or localization as a result of preparative technique must be considered. In the literature reporting RNA import from the cytosol into mitochondria, several lines of evidence are generally given to exclude the possibility that the RNA of interest is a contaminant. One type of evidence is the absence of other characteristic cytosolic RNAs, particularly the rRNAs, in the mitochondrial RNA fraction (Lye *et al.*, 1993; Hancock and Hajduk, 1990). A second commonly presented type of evidence is the resistance of the "imported" RNA to nuclease treatment of the mitochondria prior to mitochondrial RNA extraction (Maréchal-Drouard *et al.*, 1988; Hancock and Hajduk, 1990; Schneider *et al.*, 1994). A third type of evidence pertains specifically to the importation of cytosolic tRNAs into mitochondria. Two-dimensional electrophoretic separations of cytosolic tRNAs and mitochondrial tRNAs have been shown to produce unique patterns (Maréchal-Drouard *et al.*, 1988; Suyama, 1986). It can usually be demonstrated that certain cytosolic tRNAs are not found in the mitochondrial tRNA profile, or conversely, that the cytosolic tRNAs present in the mitochondria are a subset of the total cytosolic tRNA population. In very few instances have attempts been made to quantify the amount of cytosolic tRNA present in the mitochondria

or to show that the nuclease treatment of the mitochondria was effective in eliminating contaminating cytosol tRNA.

Mitochondrial tRNAs isolated from etiolated shoots of *Phaseolus vulgaris* were estimated to contain 30% cytosolic tRNAs (Guillemaut and Weil, 1990). This estimate was obtained through specific aminoacylation using cytosolic aminoacyl-tRNA synthetases, but no additional information was given. Hancock and Hajduk (1990) were also concerned about the amount of cytosolic tRNA that co-isolates with mitochondrial tRNA, particularly cytosolic tRNA that may become entrapped within mitochondria during isolation, possibly as a result of breakage and resealing of the mitochondrial membrane. To determine the degree to which entrapped cytosolic tRNAs were contaminating mitochondrial vesicles of *Trypanosoma brucei*, Hancock and Hajduk (1990) added radiolabelled tRNA and rRNA to cell suspensions before cell lysis and subsequently determined the amount of radiolabel recovered in isolated mitochondrial vesicles. They found that the amount of radiolabelled cytosolic tRNA and rRNA recovered with the mitochondrial vesicles was 0.001% and 0.002%, respectively, of the total non-mitochondrial radiolabel recovered. Based on the amount of tRNA recovered in the cytosol and in the mitochondrial vesicles following cell fractionation, they estimated that approximately 4.7% of total cellular tRNA is found within the mitochondrial vesicles. Considering the three orders of magnitude difference between 4.7% of total RNA and 0.001% of total radiolabel recovered in the mitochondria, Hancock and Hajduk (1990) concluded that the amount of entrapped

cytosolic RNA in their mitochondrial vesicle preparations was insignificant. If cytosolic RNA represents approximately 95% of the total cellular RNA and 0.001% of this is associated with the purified mitochondrial fractions after subcellular fractionation, then approximately 0.02% of the mitochondrial RNA is cytosolic RNA.

In a similar type of experimentation, estimates of the amount of cytosolic RNA in wheat mitochondrial RNA preparations were determined in the research reported here. Radiolabelled cytosolic tRNA, added to homogenizing medium containing imbibed wheat embryos before initiation of mitochondria isolation, was found to represent 18-24% of the mitochondrial tRNA (Table 5B). One important difference between the estimates of contamination determined for the *T. brucei* experiment and my experimentation was the determination of the specific activity of the cytosolic RNA following addition of the radiolabelled tRNA and subsequent determination of the amount of radiolabelled RNA in the mitochondrial RNA preparation. This allows determination of the percent of cytosolic RNA present in the mitochondrial RNA preparation; *i.e.*, the value obtained is not simply an expression of the amount of radiolabelled RNA remaining in the mitochondria relative to the amount of radiolabelled RNA added initially or present in the non-mitochondrial fractions. In my experiments, the amount of radiolabelled cytosolic tRNA in the wheat mitochondrial RNA as a percent of that found in the cytosolic RNA fraction (the supernatant of the first 18,000 x g centifiguation; see Table 5A) was on average 0.05%.

Determination of the specific activity of the radiolabelled cytosolic tRNA fraction and the amount of radiolabelled cytosolic tRNA present in isolated mitochondrial RNA becomes particularly important when trying to determine the effectiveness of a nuclease treatment in eliminating non-mitochondrial RNA from isolated mitochondria. In the two experiments presented in this thesis involving micrococcal nuclease treatment of wheat mitochondria, the amount of radiolabelled cytosolic tRNA found in mitochondrial RNA was reduced by at least 50% over that of untreated mitochondria (see Table 6A). However, the amount of mitochondrial RNA obtained was also reduced. This meant that, on average, the micrococcal nuclease treatment reduced contamination by approximately 35%, not 50% as might be concluded from the observed reduction in radiolabelled cytosolic tRNA. I could not find any literature giving a quantitative or semi-quantitative estimate of the effectiveness of nuclease treatment of mitochondria in reducing cytosolic RNA contamination. Interestingly, I did find several reports of a reduction in mitochondrial RNA yield following nuclease treatment. Micrococcal nuclease treatment of *T. brucei* mitochondrial vesicles reduced the amount of tRNA recovered 2.5-fold compared with untreated vesicles (Hancock and Hajduk, 1990). This reduction was consistent with electron microscopic observations that some of the mitochondrial membranes in vesicle preparations appeared to be disrupted. In *P. vulgaris*, 12 mg of enriched mitochondrial RNA were obtained from 35 kg of hypocotyls whereas only 25 μg of mitochondrial RNA were obtained from 1.3 kg of hypocotyls following treatment of the mitochondria with pancreatic RNase,

RNase T1 and snake venom phosphodiesterase (Guillemaut and Weil, 1990). This represents approximately an 18-fold reduction in yield.

B. Slot blot hybridization

Micrococcal nuclease treatment of the wheat mitochondria did not reduce the amount of cytosolic tRNA co-isolating with mitochondrial tRNA to what could be considered a negligible amount (<5%). It therefore became necessary to develop another method of distinguishing cytosolic tRNAs imported into the mitochondria for use there, from cytosolic tRNAs non-specifically associated with mitochondrial RNA extracts, perhaps as a result of preparative contamination. As mentioned at the beginning of the Discussion, lack of significant cytosolic RNA contamination of a mitochondrial RNA preparation is often established by the apparent absence of characteristic cytosolic RNAs within the mitochondrial RNA extracted. This is usually demonstrated by comparison of mitochondrial and cytosolic RNA migration patterns following polyacrylamide gel electrophoresis. The absence of characteristic cytosolic RNAs has also been shown through northern hybridization using oligonucleotide probes specific for certain cytosolic RNAs (Tarassov and Entelis, 1992). Use of oligonucleotide probes in the identification and quantification of individual tRNAs is complicated by the extensive conservation of tRNA primary and secondary structure and the prevalence of nucleotides that are modified post-transcriptionally. However, antisense DNA oligonucleotides complementary to the T stem and acceptor stem region of six human tRNAs were used successfully to detect individual tRNA

isoacceptors in slot blots, northern blots and dot blots of human tRNA (Utz *et al.*, 1994). The acceptor stem of the tRNA has been shown to contain sequences important for recognition by specific aminoacyl-tRNA synthetases and is believed to be the major region determining tRNA identity (McClain, 1993). Typically this region does not contain many modified nucleotides and in comparing various regions of the tRNA molecule for hybridization efficiency using complementary oligonucleotide probes, Kumazawa *et al.* (1992) considered this region to be excellent.

For the majority of the wheat mitochondrial tRNAs identified in this work reverse transcriptase sequencing confirmed that the oligonucleotides used for the slot blot hybridization were indeed recognizing the tRNA of interest. However, it was still necessary to establish that the probes were not also hybridizing to other irrelevant RNAs or contaminating DNA. As discussed in Section E. of Results, hybridization of an oligonucleotide probe complementary to the antisense strand of the mtDNA-encoded $tRNA^{His}$ showed that there was probably a small amount of mtDNA in the mitochondrial RNA and in the cytosolic RNA fractions a very weak signal that could be considered non-specific background. However, due to the length of exposure required to observe these signals they were not expected to contribute significantly to the slot blot analyses.

One other aspect of the slot blot hybridization analysis requires comment. When the mtDNA-encoded $tRNA^{Pro}$ olignucleotide probe was hybridized to the slot blots containing mitochondrial and cytosolic RNAs, the resulting pattern was

that expected for a mtDNA-encoded mitochondrial tRNA. However, the strength of the signal obtained with cytosolic RNA was greater than anticipated based on the perception that plant mitochondrial DNA does not constitute more than 1% of the total cellular DNA and presumably the proportion of mitochondrial RNA in total cellular RNA should be correspondingly low. To address this issue, the $tRNA^{Pro}$ oligonucleotide was hybridized to a slot blot containing mitochondrial RNA, cytosolic RNA and total cellular RNA. The signal obtained with total cellular RNA was only marginally greater than the signal obtained with cytosolic RNA. This suggests that the majority of the mitochondria are lost during preparation of the mitochondrial RNA fraction. This is not unexpected as isolation conditions in this study were optimized for mitochondrial purity, not yield. There are very few published estimates of the mtDNA content of plant cells or the number of mitochondria per cell. Estimates of the number of mitochondria per cell in root apical meristem of maize ranged from 50 to 1000 depending on the location of the cell with respect to distance from the root cap (Juniper and Clowes, 1965). Other estimates ranged from 216 to 9,619 mitochondria/cell for tapetal tissue of maize, and from 6,677 to 179,053 mitochondria/cell for sporogenous tissue, depending on the developmental stage of the anther (Lee and Warmke, 1979). However, the range in estimates of mtDNA per cell do not parallel the range in numbers of mitochondria per cell, although this may simply reflect the differences in types of tissue studied. In pea, for example, mtDNA content per cell varied from 0.3% to over 1.5% of total cellular DNA depending on the tissue from which

the mtDNA was isolated (Lamppa and Bendich, 1984). Although an exhaustive search of the literature was not conducted, I found no studies estimating the number of mitochondria per cell of plant embryonic tissue.

One final point needs to be made with regard to interpretation of the slot blot hybridization analysis and subsequent determination of the subcellular localization of the tRNAs studied in this thesis. It is possible to distinguish tRNAs present in wheat mitochondrial RNA at concentrations greater than that of a cytosolic tRNA not required for use by the mitochondria, from tRNAs that may be present solely as a result of contamination. Unless contamination of mitochondrial RNA is from specific cytosolic tRNAs and is not a random sampling of the cytosolic tRNA population, this method of identifying a tRNA as imported should be accurate. However, the slot blot hybridization analysis does not determine the level of cytosolic tRNA contamination of the mitochondrial RNA preparation. Without knowing the level of contamination, it is not possible to determine the importation "status" of tRNAs that do not accumulate at concentrations significantly greater than that of a cytosolic tRNA not required by mitochondria. It is probable that the presence of cytosolic tRNAs not required by mitochondria is the result of contamination only. However, it is possible that a portion of those tRNAs are legitimately imported.

As discussed in the Introduction, the legitimacy of a mitochondrial location for MRP RNA was questioned when the amounts detected in the mitochondria following nuclease treatment of mitochondria were considered too low for

biological function and were not distinguishable from the levels of other small nuclear RNA transcripts used as controls (Kiss and Filipowicz, 1992). Recently the localization of MRP RNA was assessed by ultrastructural *in situ* hybridization and was determined to be preferentially localized in nucleoli and mitochondria rather than in the nucleoplasm and cytoplasm (Li *et al.*, 1994). Furthermore, deletion of the midportion (57 nucleotides) of the coding region of the MRP RNA gene produced transcripts that failed to localize to the mitochondria.

C. tRNA identity and subcellular localization

The primary objective of this thesis was to identify the nDNA-encoded tRNAs of wheat mitochondria, imported from the cytosol for use in translation of mitochondrial mRNAs. The existence of nDNA-encoded mitochondrial tRNAs was suspected when studies to identity wheat mitochondrial tRNA genes established the presence of only 16 such genes specific for only 13 amino acids (Joyce and Gray, 1989a). Although it was considered possible that some tRNA genes were not detected, it seemed unlikely that the remaining 17 tRNA genes required to decode the genetic code according to standard wobble rules had been missed. The apparent limited number of wheat mtDNA-encoded tRNAs prompted a preliminary investigation of the wheat mitochondrial tRNA population, during which three "cytosolic-like" tRNAs were identified (Joyce and Gray, 1989a). However, in this study, no precautions were taken to avoid or account for possible cytosolic tRNA contamination of the wheat mitochondrial tRNA preparation.

With the realization that tRNAs can be encoded in one genome of a plant

cell and potentially used in more than one compartment of that cell, identification of a tRNA involves more than determination of its anticodon sequence and amino acid carrying capacity. Full characterization requires determination of the location of the gene for the tRNA and the subcellular localization of the tRNA itself.

In the research conducted for this thesis, 16 cytosolic-like tRNAs were identified in a wheat mitochondrial tRNA preparation. Identification of these tRNAs as cytosolic-like was based on an analysis of similarity with homologous tRNAs from other sources. These tRNAs show low sequence identity (an average of 49.7%) with mitochondrial tRNAs from other organisms and considerably higher sequence identity (an average of 76.0%) with *Drosophila* nuclear tRNA sequences. As has been mentioned in both the Introduction and Results sections, high sequence similarity with animal nDNA-encoded tRNA sequences and low sequence similarity with mtDNA-encoded tRNA sequences is characteristic of plant cytosolic tRNAs.

Where isoaccepting tRNA sequences of plant mtDNA-encoded tRNAs were available for comparison with the wheat cytosolic tRNAs identified in this study, percent identities were found to range from 46.0 to 57.8%. However when these cytosolic-like tRNAs were compared with plant cytosolic tRNA sequences, percent identities ranged from 70.5-100%. Therefore, based on sequence similarity these wheat mitochondrial tRNAs appear to be like their cytosolic counterparts and not like their mtDNA-encoded counterparts and have thus been referred to as "nDNA-encoded mt tRNAs" in the text of this thesis.

The next obvious step in unequivocally establishing the identity of these tRNAs would be to demonstrate directly that they are nDNA-encoded. Unfortunately, the origin of the genes for these tRNAs could not be positively established. In Southern hybridization analysis with blots containing both mtDNA and nDNA, only hybridizations of the oligonucleotide probes specific for mtDNA-encoded tRNAs with mtDNA were detected. Therefore the $tRNA^{Asp}$(GUC) corresponding to the wheat mitochondrial $tRNA^{Asp}$ gene identified previously (Joyce and Gray, 1989a) and the chloroplast-like $tRNA^{His}$(GUG) identified in the present work through reverse transcriptase sequencing, could be shown to be mtDNA-encoded. In the case of three of the nDNA-encoded mt tRNAs identified in this work ($tRNA^{Ile}$(GAU), $tRNA^{Gly}$(GGC), and $tRNA^{Leu}$(UAG)), hybridization of complementary oligonucleotide probes to nDNA could not be detected. In accounting for this negative result, one should consider that the estimated size of the unreplicated haploid nuclear genome of wheat is 16.55 pg where 1 pg equals 965 million base pairs (Shields, 1993). The size of the wheat mitochondrial genome has been estimated to be 430 kbp (Quetier *et al.*, 1985). This means the haploid wheat nuclear genome is approximately 40,000-fold larger than the mitochondrial genome. Therefore it is not unexpected that even though the nDNA:mtDNA ratio was 100:1 in the Southern blot experiments, no signal was obtained. In an attempt to circumvent this problem, the specific activity of the oligonucleotide probe was increased. This was attempted through 3'-end labelling using terminal transferase and [α-^{32}P]dATP. However, no hybridization was

observed. Improvement of the terminal transferase 3' end-labelling reaction to increase further the specific activity of the oligonucleotide may be possible, and increased amounts of nDNA could be used to prepare the Southern blot. It is important to note that although the three cytosolic-like tRNAs tested did not hybridize to nDNA they also did not hybridize to mtDNA. This establishes that the cytosolic-like $tRNA^{Ile}$(GAU), $tRNA^{Gly}$(GCC) and $tRNA^{Leu}$(UAG) are not mtDNA-encoded.

Hybridization of wheat mitochondrial tRNAs to nDNA was demonstrated when wheat mitochondrial tRNAs, 3' end-labelled using wheat tRNA nucleotidyl-transferase and [α-^{32}P]ATP, were hybridized to a dot blot containing nDNA. The hybridizing tRNAs were then eluted from the blot and separated by 2-D gel electrophoresis (Figure 3). There are two possible sources of contamination in this hybridization experiment. The first is mtDNA contamination of the nDNA preparation and is possibly seen where tRNAs apparently co-migrate to the same position as tRNAs eluted from a mt tRNA/mtDNA hybridization. The second is cytosolic tRNA contamination of the mitochondrial tRNA. Given that both types of contamination have probably occurred, it is still possible to deduce that some of the mitochondrial tRNAs have hybridized to nDNA and not mtDNA and that these mitochondrial tRNAs are not all simply the result of cytosolic tRNA contaminating the mitochondrial RNA preparation. First, the mt tRNA/nDNA hybridization pattern was distinct from that of the mt tRNA/mtDNA pattern. Therefore hybridization of mitochondrial tRNAs to nDNA was not simply targeting

mtDNA in the nDNA preparation. Second, if cytosolic tRNAs contaminating the mitochondrial RNA preparation were solely responsible for the unique spots obtained in the mt tRNA/nDNA hybridization, then the pattern obtained would be expected to contain all the spots of the cytosolic 2-D gel electrophoretic profile, or at least the major cytosolic species as the latter would be expected to be the major contaminating tRNAs. However, comparison of Figures 3C and 3D clearly shows that some of the major cytosolic tRNAs are not present in the mt tRNA/nDNA electrophoretic profile. Thus, although not all of the tRNA spots appearing in the 2-D gel electrophoretic profile of the mt tRNA/nDNA hybridization can be said to be nDNA-encoded mt tRNAs, a portion of the mt tRNAs did hybridize to nDNA and not mtDNA.

Differences between the 2-D gel electrophoretic profiles of mitochondrial tRNAs that had hybridized to nDNA or mtDNA were observed in bean (*Phaseolus vulgaris*) (Maréchal-Drouard *et al.*, 1988). Bean mitochondrial tRNAs eluted following hybridization to mtDNA represented only a fraction of the total mitochondrial tRNA population. Similarly, the mitochondrial tRNAs eluted following hybridization to nDNA represented only a fraction of the total mitochondrial tRNA population. In this case, there were no co-migrating spots in the two, 2-D gel electrophoretic profiles. Maréchal-Drouard *et al.* (1988) argued that if the mitochondrial tRNAs hybridizing to nDNA were cytosolic tRNA contaminants of the mitochondrial RNA preparation, then the complete set of cytosolic tRNAs should be observed in the mitochondrial tRNA/nDNA

hybridization. However, only a fraction of the cytosolic tRNA population was actually observed in the resulting 2-D gel electrophoretic profile.

Given that the majority of the 16 cytosolic-like mitochondrial tRNAs listed in Table 7 have significant sequence similarity to tRNAs isolated from the cytosol (in some cases as much as 100% sequence identity), that at least three of these tRNAs do not hybridize to mtDNA, that a portion of the mitochondrial tRNA population hybridizes to nDNA and that in an extensive search of the wheat mtDNA for tRNA genes these tRNAs sequences were not found (Joyce and Gray, 1989a), it can be concluded that these tRNAs are nDNA-encoded.

Another aspect in the characterization of the nDNA-encoded mt tRNAs is their subcellular localization. Considering the limitations of the slot blot analysis as discussed above, 14 of the 16 tRNAs listed in Table 7 can be confidently identified as nDNA-encoded mt tRNAs. The nDNA-encoded $tRNA^{Asp}$(GUC) was not present in the mitochondria at levels that are distinguishable from a nDNA-encoded tRNA not required for mitochondrial translation. Furthermore, a mtDNA-encoded $tRNA^{Asp}$(GUC) was identified by reverse transcriptase sequencing, which may suggest that the nDNA-encoded $tRNA^{Asp}$ might not be used in mitochondrial translation.

The other tRNA that can only be tentatively identified as a nDNA-encoded mt tRNA is the $tRNA^{Leu}$(GAG). In slot blot hybridization experiments with an oligonucleotide specific for the nDNA-encoded $tRNA^{Leu}$(GAG), the ratio of the regression coefficients clearly illustrates the limitations in interpreting the data from

the slot blot hybridization analyses. This tRNA had a ratio of regression coefficients (mt RNA/cyt RNA) of 0.47. This value is greater than that of the control nDNA-encoded $tRNA^{Phe}$, which had a ratio of 0.24, but is significantly lower than the ratios obtained for the majority of nDNA-encoded mt tRNAs, which were ≥ 1. In Section E of the Results three possible reasons are suggested to account for these observations: 1) The oligonucleotide is not specific for the $tRNA^{Leu}$(GAG); 2) The oligonucleotide is binding non-specifically to RNA; and 3) the $tRNA^{Leu}$(GAG) constitutes a greater proportion of the cytosolic RNA than it does of the mitochondrial RNA. In light of the limitations of the slot blot hybridization analysis in discriminating cytosolic tRNA contamination from legitimate import and given that the $tRNA^{Leu}$(GAG) was found in the mitochondria at levels that approximate those of $tRNA^{Leu}$(UAG), it is probable that the $tRNA^{Leu}$(GAG) is imported into the mitochondria for use there and will therefore be considered a mitochondrial tRNA in further discussion.

The final aspect considered in identification of the nDNA-encoded mt tRNAs was the anticodon sequence of the tRNA. The first (wobble) position of the anticodon sequence and the nucleoside 3' to the anticodon are often highly modified. These modifications can alter the reactivity of a nucleoside in the chemical sequencing reactions (Lankat-Buttgereit *et al.*, 1987), potentially leading to inaccurate identification of a nucleoside. However, more usually the modifications result in an inability to identity the nucleoside due to band compression and multiple band formation at a single position of the sequencing

gel. This often makes identification of a tRNA impossible, but the information obtained with RNA sequencing also reduces the chance of inaccurate identification as occurs when DNA sequence alone is used. For example, in potato, partial RNA sequence analysis revealed a tRNA with an NAU anticodon, whose amino acid specificity was determined following aminoacylation assays to be isoleucine and not methionine (Maréchal-Drouard *et al.*, 1990). The gene corresponding to this tRNA has a CAT anticodon, which normally specificies methionine (Weber *et al.*, 1990). In both wheat and maize, mitochondrial tRNAs were identified as $tRNA^{Met}$ on the basis of the CAT anticodon sequence of the gene. However, these tRNAs are nearly identical in sequence to the potato $tRNA^{Ile}$(NAU). Subsequent work has shown that the unidentified nucleoside in the wobble position of the potato $tRNA^{Ile}$ is a derivative of lysidine and, as in *E. coli*, this anticodon sequence specificies isoleucine (Muramatsu *et al.*, 1988). Clearly the assignment of the amino acid accepting capacity of a tRNA based only on anticodon sequence of the gene, must be considered tentative. Provided the universal genetic code is followed and modification of anticodon nucleosides does not change anticodon/codon recognition, the amino acid specificity of a tRNA can be predicted from RNA anticodon sequence. However, given the difficulties in nucleoside determination in the RNA chemical sequencing reactions of this study, aminoacylation experiments are needed to confirm tRNA identities.

The anticodon sequence of $tRNA^{Arg}$(CCU) was determined primarily through reverse transcriptase sequence analysis. Only the U residue in the third

position of the anticodon was confirmed by chemical sequence analysis. The only other angiosperm mitochondrial $tRNA^{Arg}$ identified with the capacity to decode the two codon family AGA and AGG was found in potato (Maréchal-Drouard *et al.*, 1990). Its sequence was partially determined and NCU was reported as the anticodon; however, sequence for the remainder of the tRNA has not been published. In many organisms the AGA and AGG codons are recognized by a $tRNA^{Arg}$ (UCU), where the U residue in the wobble position of the anticodon is modified to restrict base pairing of the tRNA to A and G residues. The reverse transcriptase sequence ladder shows incorporation of only one nucleoside (G) at the first (wobble) position of the anticodon and therefore the anticodon was designated CCU. I have found no discrepancies between my RNA chemical reaction sequence and reverse transcriptase sequence where data are available for both. Nevertheless, it is possible that this position is a modified U residue and that reverse transcriptase inserts guanosine only at this position and not adenosine or both guanosine and adenosine.

D. The wheat mitochondrial tRNA population

In this thesis, I report the identification of 16 nDNA-encoded tRNAs isolated from a wheat mitochondrial RNA preparation. As previously discussed, the cytosolic-like $tRNA^{Asp}$(GUC) cannot be unambiguously identified as a functioning tRNA of the mitochondrial population. However, the remaining 15 tRNAs are present at levels that suggest they could function in mitochondrial translation.

Of these 15 nDNA-encoded mt tRNAs only one (the $tRNA^{His}(GUG)$) is redundant with respect to the tRNAs encoded by the mitochondrial genome. Neither the mtDNA-encoded $tRNA^{His}$ nor its gene were identified in a previous study of the wheat mitochondrial genome (Joyce and Gray, 1989a), and I was not able to find this tRNA in my 2-D gel electrophoretic separation of wheat mitochondrial tRNAs. However, a chloroplast-like mtDNA-encoded $tRNA^{His}$ gene has been identified in maize (Iams *et al.*, 1985), and when an oligonucleotide probe based on the maize tRNA sequence was used for reverse transcriptase sequencing, this tRNA was found in wheat mitochondria. The probes used by Joyce and Gray (1989a) to detect wheat mitochondrial tRNA genes consisted primarily of radioactively labelled mitochondrial tRNAs. Perhaps failure to detect either the gene or the tRNA is indicative of the presence of only small amounts of this tRNA in the wheat mitochondria. One could speculate that both the nDNA-encoded and mtDNA-encoded tRNAs are, in fact, used in the mitochondria and that the nDNA-encoded $tRNA^{His}$ is required because levels of the mtDNA-encoded tRNA are insufficient. This is the first instance in which a nDNA-encoded mt tRNA and a mtDNA-encoded tRNA with the same anticodon have been identified in plant mitochondria. At this point it should be noted that the oligonucleotide probe specific for the nDNA-encoded $tRNA^{His}$ also hybridized to a mtDNA-encoded $tRNA^{Ser}$ during reverse transcriptase sequencing and although stringency of hybridization is greater in the slot blot analysis than in the reverse transcriptase sequencing protocol, it is possible that this oligonucleotide is recognizing the

mitochondrial $tRNA^{Ser}$ in the slot blot experiments and that the nDNA-encoded $tRNA^{His}$ is not present in mitochondria at levels expected for a functioning tRNA.

Assuming conservatively that wheat mitochondria have only one $tRNA^{His}$ (the chloroplast-like one) and only two tRNAs specific for alanine, the tRNA population as characterized to date includes 10 native, 7 chloroplast-like and 13 nDNA-encoded tRNAs for a total of 30 distinct species. The potential codon recognition pattern of these tRNAs is presented in Table 11. All 61 sense codons have been identified in the genes of five wheat mitochondrial respiratory chain proteins (Joyce, 1989) and there is no evidence for deviation from the universal genetic code (Jukes and Osawa, 1990). For example, unlike animal and fungal mitochondria, plant mitochondria use UGA as a termination codon and, unlike mitochondria of yeast and many animals, plant mitochondria use AUA for isoleucine. Given the use of the universal genetic code by plant mitochondria it becomes apparent that all the wheat mitochondrial tRNAs have not yet been identified. Clearly a tRNA(s) specific for the amino acid threonine remain(s) to be identified. However it is unclear how many other wheat mitochondrial tRNAs are yet to be found.

In Table 11, I have used the standard wobble rules for codon/anticodon recognition in wheat mitochondria. However, this has not been established. In mammalian and yeast mitochondrial translation systems, all of the codons for a single amino acid in a four codon family are read by one species of tRNA using

Table 11. Potential codon recognition pattern of wheat mitochondrial tRNAs

UUU	Phe	*gaa*	UCU	Ser	*gga*	UAU	Tyr	gua	UGU	Cys	*gca*
UUC	Phe	"	UCC	Ser	"	UAC	Tyr	"	UGC	Cys	"
UUA	Leu	**xaa**	UCA	Ser	uga	UAA	ter		UGA	ter	
UUG	Leu	**xaa**	UCG	Ser	"	UAG	ter		UGG	Trp	*cca*
CUU	Leu	**gag**	CCU	Pro	~	CAU	His	*gug*	CGU	Arg	**icg**
CUC	Leu	"	CCC	Pro	~	CAC	His	"	CGC	Arg	"
CUA	Leu	**uag**	CCA	Pro	ugg	CAA	Gln	uug	CGA	Arg	"
CUG	Leu	"	CCG	Pro	"	CAG	Gln	"	CGG	Arg	~
AUU	Ile	**gau**	ACU	Thr	~	AAU	Asn	*guu*	AGU	Ser	gcu
AUC	Ile	"	ACC	Thr	~	AAC	Asn	"	AGC	Ser	"
AUA	Ile	lau	ACA	Thr	~	AAA	Lys	uuu	AGA	Arg	~
AUG	Met fMet	*cau* cau	ACG	Thr	~	AAG	Lys	"	AGG	Arg	**ccu**
GUU	Val	**gac**	GCU	Ala	**ggc**	GAU	Asp	guc	GGU	Gly	**gcc**
GUC	Val	"	GCC	Ala	"	GAC	Asp	"	GGC	Gly	"
GUA	Val	**uac**	GCA	Ala	**ugx**	GAA	Glu	uuc	GGA	Gly	**ucc**
GUG	Val	"	GCG	Ala	"	GAG	Glu	"	GGG	Gly	"

Codons are in uppercase letters and anticodons of the corresponding tRNAs are in lowercase letters: standard font denotes a native mtDNA-encoded tRNA; *italicized font denotes a chloroplast-like mtDNA-encoded tRNA*; **bold font denotes a nDNA-encoded mt tRNA**.

The codon recognition pattern is based on standard wobble rules.

The mtDNA-encoded tRNAs were identified by Joyce and Gray (1989) except for $tRNA^{Asp}$ and $tRNA^{His}$, which along with the nDNA-encoded tRNAs were identified in the study reported here.

~ appears where a tRNA corresponding to the codon has not been identified.
x denotes an unidentified nucleoside.

U:N wobbling between the first base of the anticodon and the third base of the codon. In vertebrate mitochondria only 22 tRNAs are required to conduct translation. In angiosperm chloroplasts, 30 tRNAs have been identified. Because chloroplasts also use the universal genetic code a minimum of 32 tRNAs is required if standard wobble base pairing occurs (Pfitzinger *et al.*, 1990). The angiosperm chloroplast genomes are apparently lacking tRNAs that would decode the GCU/C (Ala), CGC/A/G (Arg), CUU/C (Leu) and CCU/C (Pro) codons. However, in an *in vitro* tRNA-dependent translation system from wheat germ, Pfitzinger *et al.* (1990) were able to demonstrate that the chloroplast $tRNA^{Ala}$(UGC), $tRNA^{Pro}$(UGG), $tRNA^{Arg}$(ICG) and $tRNA^{Leu}$(UAG) are able to read all four codons of the respective amino acid families. As discussed in the Introduction, the presence of a modified U in the wobble position of the anticodon of both the $tRNA^{Ala}$ and the $tRNA^{Pro}$ and inosine in the wobble position of $tRNA^{Arg}$ led to the conclusion that a "two out of three" base pair recognition mechanism is used in angiosperm chloroplasts (Pfitzinger *et al.*, 1990). The wobble position of the chloroplast $tRNA^{Leu}$(UAG) is unmodified and therefore U:U and U:C base pairing is possible, in this case.

There are eight, four codon amino acid families in the universal genetic code. It is therefore possible that these 32 codons could be decipered by 8 tRNAs with UNN anticodons. Examination of Table 11 shows that UNN anticodons have been identified in tRNAs corresponding to at least 5 and possibly 6 (in $tRNA^{Ala}$(ugX), the wobble position residue was only tentaively identified) of these

8 families. However, in 5 of these families tRNAs bearing a GNN anticodon have also been identified, making U:N wobble unnecessary. This might suggest that expanded wobble does not occur and therefore a $tRNA^{Pro}$(GGG) remains to be found.

In addition to the "missing" tRNAs for threonine and proline, there are possibly two others: $tRNA^{Arg}$(UCU) and $tRNA^{Arg}$(CCG). As already discussed the $tRNA^{Arg}$(CCU) anticodon was identified by reverse transcriptase sequencing only. It is possible that the C residue in the first position of the anticodon is a modified U and thus an additional tRNA would not be required. Thin layer chromatography of the anticodon sequence of this tRNA is necessary to establish whether or not the first nucleoside of the anticodon is an unmodified C.

The remaining arginine codon (CGG) for which a corresponding tRNA has not been identified may be recognized by $tRNA^{Arg}$(ICG). This would involve a "two out of three" base pair recognition mechanism as proposed for the $tRNA^{Arg}$(ICG) of angiosperm chloroplasts (Pfitzinger *et al.*, 1990).

E. Comparison of the monocot, dicot and liverwort mitochondrial tRNA populations

Although wheat and potato represent, respectively, the most extensively studied monocot and dicot mitochondrial tRNA populations, the limited information from other angiosperm mitochondrial genomes concurs with what has been determined for wheat and potato (see Table 2). In Table 12 the moncot, dicot and liverwort (*M. polymorpha*) mitochondrial tRNA populations are

Table 12. Comparison of the potential codon recognition pattern in the mitochondrial genomes of monocotyledonous and dicotyledonous plants and liverwort (*Marchantia polymorpha*).

	M	D	L		M	D	L		M	D	L		M	D	L
UUU Phe	*gaa*	gaa	gaa	UCU Ser	*gga*	*gga*	~	UAU Tyr	gua	gua	gua	UGU Cys	*gca*	gca	gca
UUC Phe	"	"	"	UCC Ser	"	"	~	UAC Tyr	"	"	"	UGC Cys	"	"	"
UUA Leu	**xaa**	**xaa**	uaa	UCA Ser	uga	uga	uga	UAA ter				UGA ter			
UUG Leu	**xaa**	**caa**	caa	UCG Ser	"	"	"	UAG ter				UGG Trp	*cca*	*cca*	cca
CUU Leu	**gag**	**nd**	~	CCU Pro	~	~	~	CAU His	*gug*	*gug*	gug	CGU Arg	**icg**	**icg**	acg
CUC Leu	"	"	~	CCC Pro	~	~	~	CAC His	"	"	"	CGC Arg	"	"	~
CUA Leu	**uag**	**xag**	uag	CCA Pro	ugg	ugg	ugg	CAA Gln	uug	uug	uug	CGA Arg	"	"	ucg
CUG Leu	"	"	"	CCG Pro	"	"	"	CAG Gln	"	"	"	CGG Arg	~	~	"
AUU Ile	**gau**	**nd**	~	ACU Thr	~	**nd**	ggu	AAU Asn	*guu*	*guu*	guu	AGU Ser	gcu	gcu	gcu
AUC Ile	"	"	~	ACC Thr	~	"	"	AAC Asn	"	"	"	AGC Ser	"	"	"
AUA Ile	lau	lau	cau	ACA Thr	~	**nd**	~	AAA Lys	uuu	uuu	uuu	AGA Arg	~	**xcu**	ucu
AUG Met	*cau*	*cau*	cau	ACG Thr	~	"	~	AAG Lys	"	"	"	AGG Arg	**ccu**	"	"
fMet	cau	cau	cau												
GUU Val	**gac**	nd	~	GCU Ala	**ggc**	**igc**	~	GAU Asp	guc	guc	guc	GGU Gly	**gcc**	gcc	gcc
GUC Val	"	"	~	GCC Ala	"	"	~	GAC Asp	"	"	"	GGC Gly	"	"	"
GUA Val	**uac**	nd	uac	GCA Ala	**ugx**	"	ugc	GAA Glu	uuc	uuc	uuc	GGA Gly	**ucc**	~	ucc
GUG Val	"	"	"	GCG Ala	"	~	"	GAG Glu	"	"	"	GGG Gly	"	~	"

Table 12 Continued:

Codons are in uppercase letters and anticodons of the corresponding tRNAs are in lowercase letters: standard font denotes a native mtDNA-encoded tRNA; *italicized font denotes a chloroplast-like mtDNA-encoded tRNA*; **bold font denotes a nDNA-encoded mt tRNA**. The codon recognition pattern is based on standard wobble rules. References identifying the angiosperm mitochondrial tRNAs can be found in Table 2 except for the monocot nDNA-encoded mt tRNAs that were identified in the study reported here. The liverwort mitochondrial tRNA sequences can be found in Oda *et al.* (1992b).

M - moncotyledonous plants
D - dicotyledonous plants
L - liverwort
~ appears where a tRNA corresponding to the codon has not been identified.
x denotes an unidentified nucleoside.
nd indicates the anticodon was not determined and the tRNA was identified by aminoacylation and/or oligonucleotide hybridization.

compared with respect to the genetic origin of the tRNAs and their anticodon sequences, where available.

In liverwort, mitochondrial tRNA sequences were inferred following complete sequencing of the mitochondrial genome (Oda *et al.*, 1990). Twenty-nine genes for 27 distinct species of tRNAs were identified. By sequence similarity analysis these tRNAs were characterized as native, leading to the conclusion that the liverwort genome contains no chloroplast-like tRNAs. The potential codon recognition pattern of these tRNAs, as presented by the Oda *et al.* (1990), allows for both G:U and U:N wobble base pairing (see Table 3). However as this has not been demonstrated I have used the standard wobble rules for codon/anticodon recognition in Table 12. Allowing only G:U wobble, 8 additional tRNAs are required to decode the 61 sense codons found in liverwort mitochondrial protein genes. One of the "missing" tRNAs corresponds to the CGC codon of arginine. The adenosine nucleoside in the first position of the anticodon of the $tRNA^{Arg}$(ACG) identified may be post-transcriptionally modified to inosine which could then recognize the CGC codon.

Of the remaining 7 additional tRNAs that would be required to decode the liverwort mitochondrial genetic code according to standard wobble base pairing, 5 correspond to nDNA-encoded mitochondrial tRNAs found in both moncots and dicots: $tRNA^{Leu}$(UAG), $tRNA^{Ile}$(GAU), $tRNA^{Val}$(GAC), $tRNA^{Thr}$(nd) and $tRNA^{Ala}$(IGC). Even with U:N wobble, tRNAs specific for the isoleucine codons, AUU and AUC, and the threonine codons, ACA and ACG, are not encoded in the

liverwort mitochondrial genome. It has been proposed that these particular tRNAs are imported from the cytosol (Oda *et al.*, 1990). Indeed, evidence for import of a nDNA-encoded $tRNA^{Ile}$ species into liverwort mitochondria has recently been obtained (K. Ohyama, personal communication). In this context, it is not radical to propose import of additional tRNAs. The fact that 5 of the 7 tRNAs required (assuming no G:N wobble) correspond to imported tRNAs in angiosperms perhaps lends credence to the suggestion that these tRNAs are also imported in liverwort.

There are 10 native mtDNA-encoded tRNAs common to the liverwort, monocot and dicot mitochondrial genomes: $tRNA^{Ile}$(LAU), $tRNA^{fMet}$(CAU), $tRNA^{Ser}$(UGA), $tRNA^{Pro}$(UGG), $tRNA^{Tyr}$(GUA), $tRNA^{Gln}$(UGG), $tRNA^{Lys}$(UUU), $tRNA^{Asp}$(GUC), $tRNA^{Glu}$(UUC) and $tRNA^{Ser}$(GCU). The final similarity between the liverwort and angiosperm mitochondrial genomes is the apparent absence of a $tRNA^{Pro}$(GGG) gene. As the liverwort mitochondrial genome has been entirely sequenced, this tRNA is either not required or is also imported into the mitochondria.

Before I begin comparing the tRNA populations of moncots and dicots, I will briefly comment on the analysis of the potato mitochondrial tRNA population published by Maréchal-Drouard *et al.* (1990), as this is the study in which the majority of the nDNA-encoded tRNAs of dicots have been identified. These authors identified 31 individual tRNAs in potato mitochondria: 15 native mtDNA-encoded, 5 chloroplast-like mtDNA-encoded and 11 nDNA-encoded. Maréchal-Drouard *et al.* (1990) identified these tRNAs by a variety of techniques including

aminoacylation, sequencing or hybridization to specific oligonucleotides. Anticodon sequences for the majority of the nDNA-encoded mitochondrial tRNAs were not determined. However Maréchal-Drouard *et al.* (1990) state that the 31 tRNAs identified are sufficient to read all sense codons. Given that the anticodon sequences that have not been identified do indeed correspond to either a GNN or UNN codon, this still required postulating U:N wobble or a "two out of three" base pair recognition mechanism for $tRNA^{Pro}$ (UUG), $tRNA^{Ala}$(IGC) and $tRNA^{Arg}$(ICG). Moreover, the only glycine tRNA found has a GCC anticodon. The authors do not comment on this point, or propose how the $tRNA^{Gly}$(GCC) recognizes all four codons in that family.

As many of the anticodon sequences of nDNA-encoded mitochondrial tRNAs of dicots were not available or the wobble positions not identified, I have liberally assigned the tRNAs to either the appropriate GNN or UNN codons in Table 12. The only contentious assignment with respect to the available anticodon sequence of monocot mitochondrial tRNAs is the XCU anticodon of the dicot $tRNA^{Arg}$. It is possible that this tRNA corresponds to the $tRNA^{Arg}$(CCU) that I have identified, although as previously discussed the nucleoside in the first position of the anticodon remains to be confirmed in my sequence as well.

Monocotyledonous and dicotyledonous plants have 10 native mtDNA-encoded, 5 chloroplast-like and at least 8 (if the $tRNA^{Arg}$(CCU) and (XCU) species are assumed to be the same) nDNA-encoded mt tRNAs in common. Further, if monocots import two threonine tRNAs as expected, then moncots and dicots have

10 nDNA-encoded mt tRNAs in common.

In both moncots and dicots, native mtDNA-encoded tRNAs corresponding to codons for initiator methionine, proline, tyrosine, glutamine, lysine, aspartic acid, glutaminc acid, isoleucine (AUA codon) and serine (AGY and UCR codons) have been identified. Chloroplast-like mtDNA-encoded tRNAs corresponding to codons specifying elongator methionine, histidine, asparagine, tryptophan and serinc (UCY codons) have been found in both monocots and dicots. In the case of angiosperm nDNA-encoded mt tRNAs, tRNAs specific for leucine, isoleucine (AUY), alanine and arginine codons have been identified in both monocots and dicots. Wheat and other monocots probably also import tRNAs corresponding to the threonine codons, as has been found for dicotyledonous plants.

Although moncots and dicots have many tRNAs in common, it is also evident from a comparison of the genetic origins of their tRNAs that there are also significant differences. Dicotyledonous plants have 5 additional native mtDNA-encoded tRNAs: $tRNA^{Phe}$(GAA), $tRNA^{Cys}$(GCA), $tRNA^{Gly}$(GCC) and two $tRNAs^{Val}$ (anticodons unknown). In monocots, these tRNAs correspond to chloroplast-like mtDNA-encoded $tRNA^{Phe}$(GGA) and $tRNA^{Cys}$(GCA) and nDNA-encoded mt $tRNA^{Gly}$(GCC), $tRNA^{Val}$(GAC) and $tRNA^{Val}$(UAC).

It is interesting to note that although liverwort maintains the largest number of native mtDNA-encoded tRNA species, dicotyledonous plants may have a native mtDNA-encoded $tRNA^{Val}$, which is not present in the liverwort mitochondrial genome. However, until the anticodon sequences of the $tRNAs^{Val}$ (identified by

aminoacylation in a potato mitochondrial tRNA population) have been determined and are shown to be unique, this conclusion remains tentative.

Another tRNA gene absent from the liverwort mtDNA is the $tRNA^{Ser}(GGA)$. The native $tRNA^{Ser}(GGA)$ gene is also lacking in angiosperm mitochondrial genomes, having been replaced with a chloroplast-like $tRNA^{Ser}(GGA)$ gene.

F. Evolution of the mitochondrial tRNA population in plants

Translation in the mitochondria of plants requires minimally 32 tRNAs if the universal genetic code is used and anticodon/codon recognition follows the standard wobble rules. All existing evidence suggests that the universal genetic code is used in mitochondria of both angiosperms and the non-vascular plant, *M. polymorpha* (liverwort). However it is not clear whether plant mitochondria use U:N wobble or a "two out ot three" base pair recognition mechanism between the third nucleoside position of the codon and the first nucleoside position of the anticodon. It is therefore difficult to ascertain whether one has fully characterized a plant mitochondrial tRNA population, or if tRNAs remain to be identified. Certainly the threonine tRNAs of wheat mitochondria have yet to be found, but how many additional tRNAs are required for a complete description of the decoding capacity of this system?

It is clear from the preceding discussion that the angiosperm mitochondrial tRNA population is derived from nuclear, chloroplast-like and mitochondrial genes, whereas the liverwort mitochondrial tRNA population consists of native tRNAs and

possibly a few cytosolic tRNAs. The absence of any chloroplast-like tRNA sequences in liverwort mitochondria suggests that the acquisition of tRNA genes from the chloroplast genome and loss of corresponding native tRNA genes might have occurred only in the mitochondrial genome of land plants after the divergence of bryophytes.

Complete sequence analysis of the liverwort mitochondrial genome has revealed that although this plant mitochondrial genome contains a greater number of mtDNA-encoded tRNAs than does angiosperm mtDNA, it is apparently lacking at least two tRNAs and possibly more if U:N wobble is not proposed. Genes for the two "missing" tRNAs are also absent from angiosperm mtDNA, and therefore these tRNA genes may have been lost from the mitochondrial genome of land plants before the divergence of bryophytes. In any event if the "missing" tRNAs in liverwort mitochondria are identified as cytosolic tRNAs, this will establish the existence of an importation process in an early diverging plant.

Although the genetic origin of the majority of the angiosperm mitochondrial tRNAs is the same in both monocotyledonous and dicotyledonous plants, there is some variation. Considering the commonalities among species of angiosperm mitochondrial tRNAs that are native, chloroplast-like or nDNA-encoded, it is probable that these shared features existed before moncot-dicot divergence. However, the fact that there are differences in the genetic origin of some of the tRNAs suggests that both gain of chloroplast tRNA genes and import of cytosolic tRNAs with concomitant loss of the corresponding native tRNAs are ongoing

processes.

Presumably in the development of a translation system that uses tRNAs from various genetic sources, there must be some conservation of tRNA structure and related sequence components. This functional constraint has been cited as a reason for the general normalcy of plant mitochondrial tRNAs with regard to the standard tRNA configuration (Boer and Gray, 1988). Furthermore, the ablility of tRNAs from various genetic origins to function in plant mitochondrial translation may have provided the milieu for the evolution of the plant mtDNA-encoded tRNA population. It is completely speculative, but interesting to hypothesize, that the presence of both the cytosolic and chloroplast-like $tRNAs^{His}$ in wheat mitochondria represents an intermediary stage in the loss of a mtDNA-encoded tRNA.

As reviewed in the Introduction, RNA import into mitochondria is not limited to plants. Direct or indirect evidence of RNA import has been found in kinetoplastids *(T. brucei* and *L. tarentolae)*, ciliates *(P. aurelia* and *T. pyriformis)*, a chlorophyte algae *(C. reinhardtii)*, an ameboid protist (*Acanthomeba castellani)*, a yeast (*Saccharomyces cerevisiae)*, and several mammals. Although the majority of RNAs presently identified as "imported" are tRNAs, several other types of imported RNAs have been identified including 5S and 5.8S rRNA, and MRP (mitochondrial ribosomal processing) RNA (see section B, Introduction for details). The existence of an RNA import mechanism in such a broad base of organisms, importing a variety of RNA molecules, suggests that the ability to import RNA is not a limited, specialized function of a discrete population but exists in general in

eukaryotes. However it is still relatively early in the study of RNA import and in the identification of imported RNAs. If the pervasiveness of RNA import is indeed further established, it would suggest that the ability to import RNA molecules is an ancestral trait and that it has been retained to different degrees in extant mitochondria.

G. Import

This thesis did not study import mechanism or any of the processes of import directly. Having reviewed the current literature pertaining to import of tRNAs and small RNAs in the Introduction, I will not repeat this information here. However I would like to direct attention to a recent review of import of RNA into mitochondria (Schneider, 1994). In this review, cytosolic and mitochondrial tRNAs in organisms in which importation occurs, are divided into four categories, according to their localization and genetic origin: 1) nDNA-encoded and located only in the cytosol; 2) mtDNA-encoded and located only in the mitochondria; 3) nDNA-encoded and found in both the cytosol and mitochondria; and 4) nDNA-encoded and found only in the mitochondria. All the nDNA-encoded tRNAs of plant mitochondria are thought to be a subset of the cytosolic tRNA population, because cytosolic tRNAs corresponding to some of the mitochondrial tRNAs were identified and found to have identical primary sequence. The slot blot analysis conducted in the research presented here confirms that all the nDNA-encoded mt tRNAs of wheat are also found in the cytosol. Moreover, the majority of these tRNAs represent approximately the same proportion of either the cytosolic or

mitochondrial RNA. However, there were exceptions to this. For example, $tRNA^{Leu4}$(XAA) represents a significantly greater (approximately 5-fold) proportion of the mitochondrial RNA than it does the cytosolic RNA. Conversely, $tRNA^{Leu}$(GAG) represents a much smaller proportion of the mitochondrial RNA than it does the cytosolic RNA.

In both *L. tarentolae* and *T. brucei* the entire mitochondrial tRNA population is thought to be imported from the cytosol (Simpson *et al.*, 1989; Shi *et al.*, 1994; Hancock and Hajduk, 1990). Initial studies of the mitochondrial tRNA populations of these organisms indicated that some of the nDNA-encoded mt tRNAs were unique to the mitochondria. These tRNAs are often suggested to be specifically targeted to the mitochondria. Although specific targeting of a cytosolic tRNA to the mitochondria is a possible explanation, I would like to suggest that differential accumulation of tRNAs within either the cytosol or the mitochondria could also explain the apparent presence or absence of a specific tRNA within a subcellular compartment. It is not necessary to postulate specific targeting of a nDNA-encoded tRNA to the mitochondria if it is not present in significant (detectable) amounts in the cytosol. In more recent work 10 nDNA-encoded tRNAs of *L. tarentolae* were categorized as either: 1) abundant in the cytosol and mitochondria; 2) abundant in the cytosol and scarce in the mitochondria; 3) abundant in the mitochondria and scarce in the cytosol or; 4) scarce in both the cytosol and mitochondria (Shi *et al.*, 1994). This description probably more adequately reflects what is currently known about the localization

of these tRNAs and does not confound amount of tRNA with possible function.

Other evidence that suggests the RNA import mechanism is capable of recognizing a wide variety of cellular RNAs was obtained in studies of RNA import in *Leishmania* (Ghosh *et al.*, 1994). In this system srRNAs (small ribosomal RNAs) and tRNAs were shown to be imported into mitochondria. These RNAs also effectively competed with each other for import suggesting that at least a part of the import mechanism is shared by the various RNAs.

The differential accumulation of tRNAs within mitochondria requires stabilization of certain tRNAs and degradation of others. Stabilization possibly occurs as a result of interaction of the tRNA with cognate aminoacyl-tRNA synthetases or modification enzymes. Small *et al.* (1992) noted a correlation between the amount of mutated tRNA found in transgenic potato plants and the ability of the cognate synthetases to aminoacylate these tRNAs. They suggested that the aminoacyl-tRNA synthetase might be important for *in vivo* stability of the tRNA. Although specific nucleoside modification of tRNAs is apparently not required for import (see section C, Introduction), interaction with modifying enzymes may be involved in adapting imported tRNAs to specific requirements of the mitochondrial translation machinery and in stabilization of the tRNA within the mitochondria.

Having presented evidence that the import mechanism can recognize a variety of RNA molecules does not preclude the fact that RNA import may be specific or that import does not require some form of a generalized identification

mechanism. In yeast a nDNA-encoded $tRNA^{Lys}$(CUU) is thought to be specifically targeted to the mitochondria as neither the nDNA-encoded $tRNA^{Lys}$(UUU) nor the mtDNA-encoded $tRNA^{Lys}$ was detected in yeast mitochondria following electroporation of intact yeast cells to introduce these labelled tRNAs into the cytoplasm (Tarassov and Entelis, 1992). This is perhaps the most convincing evidence that an individual tRNA can be specifically targeted to the mitochondria, although it should be emphasized that tRNAs introduced by electroporation were degraded in the cytosol and efficiency of import into mitochondria was very poor. Radiolabelled imported $tRNA^{Lys}$(CUU) was not detectable until 1 hour after electroporation and at 3 hours approximately 80% of this imported tRNA was degraded. The poor efficiency of importation was attributed to competition from resident tRNAs, some artifactual change due to the electroporation process and/or low efficiency of the targeting process *in vivo*.

Other evidence that the importation process can be selective includes demonstration of import of only two of three nDNA-encoded tRNAs into mitochondria of *Leishmania* (Lye *et al.*, 1993) and selective import of tRNAs containing a variety of mutations (Chen *et al.*, 1994; see section C, Introduction).

Experiments to determine the regions of the tRNA molecule involved in importation are in their infancy, with only a few non-systematic mutational analyses conducted (Schneider *et al.*, 1994a; Small *et al.*, 1992; Chen *et al.*, 1994). Of the mutations introduced into a variety of tRNAs (see section C, Introduction), only a four nucleotide insertion into the variable loop has so far been

shown to block import (Chen *et al.*, 1994). The fact that the import system is able to recognize and import mature tRNAs in yeast and plants suggests that import signals are localized within their coding regions, whereas importation of large precursor tRNAs in trypanosomes (120-180 nucleotides longer than mature tRNAs) (Hancock *et al.*, 1992) suggests that in this case, the import signal may lie in flanking regions. However, these conclusions are very preliminary.

The involvement of proteins in the import process has been implicated in yeast where a soluble cytoplasmic protein-containing extract was required for *in vitro* import of a tRNA (Tarassov and Entelis, 1992). It has often been suggested that aminoacyl-tRNA synthetases may serve as carrier proteins because they already possess a mitochondrial targeting signal and are capable of recognizing specific tRNAs. However, the fact that the yeast cytoplasmic lysyl-tRNA synthetase is not found in yeast mitochondria (which imports only the $tRNA^{Lys}$) (Martin *et al.*, 1979) and that the large precursor tRNAs in trypanosomes are most likely not recognized by their cognate synthetases, suggests that these proteins may not be involved in import. In addition, many of the mutated tRNAs which were demonstrated to be imported cannot be aminoacylated, at the very least suggesting that aminoacyl- or peptidyl-tRNA is not an obligatory substrate for import.

In contrast to the yeast import mechanism a soluble cytoplasmic extract is apparently not required for import of RNA into *Leishmania* mitochondria (Mahapatra *et al.*, 1994). Because preincubation of the RNA with the *Leishmania* mitochondrial suspension resulted in rapid import following the addition of ATP,

it was postulated that a membrane bound protein may be be involved in import. Both the yeast and *Leishmania in vitro* RNA import systems required ATP.

It is apparent from this discussion that little is known about the RNA import mechanism and that in many respects the available information is conflicting. Given the fact that RNA import is found in widely divergent species and that a variety of RNAs are imported, it is entirely possible that different pathways for mitochondrial RNA import exist in different organisms.

H. Future directions

Although my research has substantially extended our knowledge of the wheat mitochondrial tRNA population, there is still at least one tRNA corresponding to the threonine codon family and possibly a $tRNA^{Arg}$(UCU) that remain to be identified if G:U and U:N wobble base pairing occur. I have partial sequence analysis of all major spots and the majority of minor spots appearing in a 2-D gel electrophoretic pattern of the wheat mitochondrial tRNAs. There are tRNAs that so far I have not been able to identify because the anticodon sequences were not discernable and searches of tRNA data banks did not uncover any significant similarity with known tRNA sequences. Because no angiosperm $tRNA(s)^{Thr}$ sequences have yet been determined, it is possible that one or more of the unidentified sequences I have corresponds to the threonine tRNA(s). Due to the apparent variation in the amount of individual tRNA species within wheat mitochondria, it is also possible that some of the minor spots that I have not sequenced represent additional tRNA species. In potato mitochondria the presence

of the threonine tRNA(s) was determined through aminoacylation (Maréchal-Drouard *et al.*, 1990). Isoaccepting tRNAs can also be specifically radioactively labelled (Tarassov and Entelis, 1992) by a method involving aminoacylation with a specific amino acid followed by periodate oxidation, alkaline deacylation and 3' end-labelling with T4 RNA ligase and [5'-^{32}P]pCp. Aminoacylation protects the 3' end of the tRNA during periodate treatment, which prevents 3' end-labelling of the tRNAs not aminoacylated by oxidizing the 2', 3'-*cis* glycol grouping at the 3' end. The labelled tRNA(s) would require gel purification before sequencing.

Another approach to extend sequence information of the wheat nDNA-encoded mt tRNAs would be the polymerase chain reaction (PCR). From the tRNA sequence data provided in this thesis, oligonucleotides corresponding to the 5' and 3' end of each tRNA could be constructed and used as primers for PCR. The cloned PCR product could be sequenced or used to produce high specific activity probes for Southern hybridization. The DNA sequence data obtained would be particularly useful in confirming or expanding information for the anticodon nucleotides not identified through RNA sequence analysis. The successful hybridization of tRNA-specific probes with nDNA would confirm the nuclear origin of the wheat cytosolic-like mitochondrial tRNAs.

Given the U:N and I:N wobble base pairing or "two out of three" codon/anticodon recognition mechanism of chloroplast tRNAs (Pfitzinger *et al.*, 1990), it is entirely possible that following identification of the tRNA(s)^{Thr}, the characterized wheat mitochondrial tRNA population would be complete. Therefore

instead of continuing to search further for novel mitochondrial tRNAs, it may be more productive to determine the nucleoside modifications of the anticodon sequences of those tRNAs already identified, particularly the single tRNA species for four codon families. The $tRNA^{Pro}$(UGG) is of particular interest as a $tRNA^{Pro}$(GGG) has not been found in any angiosperm or liverwort mitochondria. If the uridine residue in the wobble position of the anticodon is found to be unmodified, it would indicate that this tRNA has the potential to wobble base pair with all four codons of the proline family. If it is modified, it still has the potential to recognize all four codons by the "two out of three" mechanism. Determination of the codon recognition mechanism of these tRNAs will require an assay similar to the one used for chloroplast tRNAs, in which polypeptide synthesis directed by an mRNA such as globin is conducted using a tRNA-dependent wheat germ extract. This would require purification of the $tRNA^{Pro}$(UGG) and any additional tRNAs required to translate the mRNA sequence.

The differences between the native, chloroplast-like and nDNA-encoded mitochondrial tRNA species of moncots and dicots suggest that evolution of the angiosperm mitochondrial tRNA population is ongoing. They also suggest to me that the importation of tRNAs is flexible with regard to the species used in mitochondrial translation. Perhaps all cytosol tRNAs are imported into angiosperm mitochondria and only the ones actively used accumulate to detectable levels while those not used are degraded. Such non-specific import of tRNAs would certainly provide the intermediary stage required for translation to continue during loss of

the native tRNA gene. This hypothesis, however, does not address the issue of why mitochondria use nDNA-encoded tRNAs rather than mtDNA-encoded ones. Presumably if both the nDNA-encoded and the mtDNA-encoded tRNAs function equally well in protein synthesis then loss of the mtDNA-encoded tRNA could simply reflect lack of selection pressure to maintain it.

It may be possible to test the hypothesis that specific tRNAs are not selected for import into mitochondria in angiosperms using a system similar to the one developed by Small *et al.* (1992). Through the creation of a transgenic potato plant carrying the bean nDNA-encoded $tRNA^{Leu}$(CAA) gene (which differs from the potato $tRNA^{Leu}$(CAA) at two nucleoside positions in the anticodon loop) Small *et al.* (1992) were able to demonstrate import of the bean $tRNA^{Leu}$(CAA) into mitochondria and thus show that mitochondrial tRNAs are indeed derived from nuclear genes. Using a system similar to this, one may be able to show that the nDNA-encoded $tRNA^{Val}$ of wheat can be imported into the mitochondria of the dicot, potato, which has a mtDNA-encoded $tRNA^{Val}$. However, if the tRNA is rapidly degraded following import it may not be detected in such an experiment. From this prespective it becomes very difficult to ascertain whether the tRNA is not imported, or is imported and then degraded, thus escaping detection.

Further elucidation of the mechanism of import such as possible sequence-specific or structural signals involved in identification of RNA molecules to be imported, should ultimately provide insight into whether individual tRNA species are differentiated by the import mechanism.

V. References

Akama, K., Tanifuji, S. 1990. Sequence analysis of three $tRNA^{Phe}$ nuclear genes and a mutated gene, and one gene for $tRNA^{Ala}$ from *Arabidopsis thaliana*. Plant Mol. Biol. 15:337-346.

Altman, S. 1989. Ribonuclease P: An enzyme with a catalytic RNA subunit. Adv. Enzymol. Relat. Areas Mol. Biol. 62:1-36.

Ambrosini, M., Ceci, L.R., Fiorella, S., Gallerani, R. 1992. Comparison of regions coding for $tRNA^{His}$ genes of mitochondrial and chloroplast DNA in sunflower: a proposal concerning the classification of "CP-like" tRNA genes. Plant Mol. Biol. 20:1-4.

Andachi, Y., Yamao, F., Muto, A., Osawa, S. 1989. Codon recognition patterns as deduced from sequences of the complete set of transfer RNA species in *Mycoplasma capricolum*: resemblance to mitochondria. J. Mol. Biol. 209:37-54.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, J.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., Young, I.G. 1981. Sequence and organization of the human mitochondrial genome. Nature. 290:457-465.

Barciszewska, M., Jones, D.S. 1987. The nucleotide sequence of a valine accepting tRNA from *Lupinus luteus* (lupin) seeds. Nucleic Acids Res. 15:1333.

Barciszewska, M., Keith, G., Dirheimer, G., Barciszewska, J. 1988. The nucleotide sequence of $tRNA^{Ser}$ and $tRNA^{Ile}$ of yellow lupin seeds. Nucleic Acids Res. 16:8175.

Barciszewska, M., Keith, G., Dirheimer, G., Mashkova, T., Kubli, E., Barciszewski, J. 1990. The primary structure of six leucine isoacceptor tRNAs of yellow lupin seeds. The structural requirements for amber tRNA suppressor activity. Biochim. Biophys. Acta. 1048:78-84.

Barciszewska, M., Keith, G., Kubli, E., Barciszewski, J. 1986. The primary structure of wheat germ $tRNA^{Arg}$ - the substrate for arginyl-$tRNA^{Arg}$:protein transferase. Biochimie. 68:319-323.

Beier, H., Barciszewska, M., Krupp, G., Mitnacht, R., Gross, H.J. 1984. UAG readthrough during TMV RNA translation: isolation and sequence of two tRNAs^{Tyr} with suppressor activity from tobacco plants. EMBO J. 3:351-356.

Binder, S., Brennicke, A. 1993a. Transcription initiation sites in mitochondria of *Oenothera berteriana*. J. Biol. Chem. 268:7849-7855.

Binder, S., Brennicke, A. 1993b. A tRNA gene transcription initiation site is similar to mRNA and rRNA promoters in plant mitochondria. Nucleic Acids Res. 21:5012-5019.

Binder, S., Schuster, W., Grienenberger, J.M., Weil, J.H., Brennicke, A. 1990. Genes for tRNA^{Gly}, tRNA^{His}, tRNA^{Lys}, tRNA^{Phe}, tRNA^{Ser} and tRNA^{Tyr} are encoded in *Oenthera* mitochondrial DNA. Curr. Genet. 17:353-358.

Björk, G.R., Kohli, J. 1990. Synthesis and function of modified nucleosides in tRNA. In *Chromatography and Modification of Nucleosides*. eds. D.N. Gehrke, K.C. Kuo. Elsevier Science. Amsterdam. pp. 813-867.

Boer, P.H., Gray, M.W. 1988. Transfer RNA genes and the genetic code in *Chlamydomonas reinhardtii* mitochondria. Curr. Genet. 14:583-590.

Brown, G.G., Auchincloss, A.H., Covello, P.S., Gray, M.W., Menassa, R., Singh, M. 1991. Characterization of transcription initiation sites on the soybean mitochondrial genome allows identification of a transcription-associated sequence motif. Mol. Gen. Genet. 228:345-355.

Burger, G., Plante, I., Lonergan, K.M., Gray, M.W. 1995. The mitochondrial DNA of the amoeboid protozoan, *Acanthamoeba castellanii*. Complete sequence, gene content and genome organization. J. Mol. Biol. 265, in press.

Burkard, G., Steinmetz, A., Keller, M., Mubumbila, M., Crouse, E., Weil, J.H. 1982. Resolution of chloroplast tRNAs by two-dimensional gel electrophoresis. In *Methods in Chloroplast Molecular Biology*. eds. Edelman et al. Elsevier Biomedical Press. pp. 347-357.

Ceci, L.R., Ambrosini, M., Fiorella, S., Gallerani, R. 1994. Detection of a conserved arrangement of three tRNA genes in the sunflower mitochondrial genome. Identification, mapping and expression of trnC-trnN-trnY genes. Biochem. Mol. Biol. Internat. 32:1161-1172.

Ceci, L.R., Ambrosini, M., Siculella, L., Gallerani, R. 1989. Location of a single tRNA-His gene on the master chromosome of sunflower mitochondrial

DNA. Plant Science. 61:219-225.

Ceci, L.R., Dell'Orco, M., Gallerani, R. 1992. Identification of a nuclear-encoded sunflower mitochondrial tRNA-Leu(AAG). Plant Mol. Biol. 19:863-866.

Ceci, L.R., Saiardi, A., Siculella, L., Quagliariello, C. 1993. A $tRNA^{Val}$(GAC) gene of chloroplast origin in sunflower mitochondria is not transcribed. Plant Mol. Biol. 23:727-736.

Chang, D.D., Clayton, D.A. 1987. A mammalian mitochondrial RNA processing activity contains nucleus-encoded RNA. Science. 235:1178-1183.

Chen, D.H.T., Shi, X., Suyama, Y. 1994. *In vivo* expression and mitochondrial import of normal and mutated $tRNA^{thr}$ in *Leishmania*. Mol. Biochem. Para. 64:121-133.

Chu, S., Archer, R.H., Zengel, J.M., Lindahl, L. 1994. The RNA of RNase MRP is required for normal processing of ribosomal RNA. Proc. Natl Acad. Sci. USA. 91:659-663.

Crick, F.H.C. 1966. Codon-anticodon pairing: The wobble hypothesis. J. Mol. Biol. 19:548-555.

Dietrich, A., Weil, J.H., Maréchal-Drouard, L. 1992. Nuclear-encoded transfer RNAs in plant mitochondria. Annu. Rev. Cell Biol. 8:115-131.

Dirheimer, G., Martin, R.P. 1990. Mitochondrial tRNAs; structure, modified nucleosides and codon reading patterns. In *Chromatography and Modification of Nucleosides. Part B: Biological Roles and Function of Modification*. eds. C.W. Gehrke, K.C.T. Kuo. Elsevier Science. Amsterdam. pp. B197-B264.

Dron, M., Hartmann, C., Rode, A., Sevignac, M. 1985. Gene conversion as a mechanism for divergence of a chloroplast tRNA gene inserted in the mitochondrial genome of *Brassica oleracea*. Nucleic Acids Res. 13:8603-8610.

Ellis, J. 1982. Promiscous DNA - chloroplast genes inside plant mitochondria. Nature. 299:678-679.

Fradin, A., Gruhl, H., Feldman, H. 1975. Mapping of yeast tRNAs by two-dimensional electrophoresis on polyacrylamide gels. FEBS Lett. 50:185-189.

Fuchs, T., Beier, D., Beier, H. 1992. The $tRNA^{Tyr}$ multigene family of *Nicotiana rustica*: genome organization, sequence analyses and expression *in vitro*. Plant Mol. Biol. 20:869-878.

Gaillard, C., Strauss, F. 1990. Ethanol precipitation of DNA with linear polyacrylamide as carrier. Nucleic Acids Res. 18:378.

Geliebter, J. 1987. Dideoxynucleotide sequencing of RNA and uncloned cDNA. Focus. 9:5-8.

Ghosh, A., Ghosh, T., Ghosh, S., Das, S., Adhya, S. 1994. Interaction of small ribosomal and transfer RNAs with a protein from *Leishmania donovani*. Nucleic Acids Res. 22:1663-1669.

Ghosh, K., Ghosh, H.P. 1984. Structure and function of tryptophan tRNA from wheat germ. Nucleic Acids Res. 12:4997-5003.

Gokhman, I., Zamir, A. 1990. Nucleotide sequence of $tRNA^{val}$(GUU) from *Arabidopsis thaliana*. Nucleic Acids Res. 18:6729.

Gray, M.W. 1989. Origin and evolution of mitochondrial DNA. Annu. Rev. Cell Biol. 5:25-50.

Gray, M.W. 1991. Origin and evolution of plastid genomes and genes. In *Cell Culture and Somatic Cell Genetics of Plants. The Molecular Biology of Plastids*. eds. I.K. Vasil, L. Bogorad. Academic Press. San Diego. pp. 303-330.

Gray, M.W. 1992. The endosymbiont hypothesis revisited. Inter. Rev. Cytol. 141:233-357.

Gray, M.W., Doolittle, W.F. 1982. Has the endosymbiont hypothesis been proven? Microbiol. Rev. 46:1-42.

Gray, M.W., Hanic-Joyce, P.J., Covello, P.S. 1992. Transcription, processing and editing in plant mitochondria. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:145-147.

Green, G.A., Maréchal-Drouard, L., Weil, J.H., Guillemaut, P. 1987. A *Phaseolus vulgaris* mitochondrial $tRNA^{Leu}$ is identical to its cytoplasmic counterpart: sequencing and in vivo transcription of the gene corresponding to the cytoplasmic $tRNA^{Leu}$. Plant Mol. Biol. 10:13-19.

Grosjean, H.J., de Henau, S., Crothers, D.M. 1978. On the physical bases for ambiguity in genetic coding interactions. Proc. Natl. Acad. Sci. USA. 75:610-614.

Guillemaut, P., Weil, J.H. 1990. Purification and identification of higher plant organellar transfer RNAs. In *Transfer RNAs and Other Soluble RNAs*. ed. J.D. Cherayil. CRC Press. Boston. pp. 55-67.

Hancock, K., Hajduk, S.L. 1990. The mitochondrial tRNAs of *Trypanosoma brucei* are nuclear encoded. J. Biol. Chem. 265:19208-19215.

Hancock, K., LeBlanc, A.J., Donze, D., Hajduk, S.L. 1992. Identification of nuclear encoded precursor tRNAs within the mitochondrion of *Trypanosoma brucei*. J. Biol. Chem. 267:23963-23971.

Hanic-Joyce, P.J., Gray, M.W. 1990. Processing of transfer RNA precursors in a wheat mitochondrial extract. J. Biol. Chem. 265:13782-13791.

Hanson, M.R., Folkerts, O. 1992. Structure and function of the higher plant mitochondrial genome. Inter. Rev. Cytol. 141:129-172.

Heinonen, T.Y.K., Schnare, M.N., Young, P.G., Gray, M.W. 1987. Rearranged coding segments, separated by a transfer RNA gene, specify the two parts of a discontinuous large subunit ribosomal RNA in *Tetrahymena pyriformis* mitochondria. J. Biol. Chem. 262:2879-2887.

Hintz, W.E., Mohan, M., Anderson, J.B., Horgen, P.A. 1985. The mitochondrial DNAs of *Agaricus*: heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. Curr. Genet. 9:127-132.

Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M. 1989. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. 217:185-194.

Iams, K.P., Heckman, J.E., Sinclair, J.H. 1985. Sequence of histidyl tRNA, present as a chloroplast insert in mtDNA of *Zea mays*. Plant Mol. Biol. 4:225-232.

Izuchi, S., Terachi, T., Sakamoto, M., Mikami, T., Sugita, M. 1990. Structure and expression of tomato mitochondrial genes coding for $tRNA^{Cys}$(CGA), $tRNA^{Asn}$(GUU) and $tRNA^{Tyr}$(GUA): a native $tRNA^{Cys}$ gene is present in dicot plants but absent in monocot plants. Curr. Genet. 18:239-243.

Jayabaskaran, C., Puttaraju, M. 1993. Fractionation and identification of cytoplasmic tRNAs and structural characterization of a phenylalanine and a leucine tRNA from cucumber hypocotyls. Biochem. Mol. Biol. Internat. 31:983-994.

Joyce, P.B. 1989. Structure, organization and expression of mitochondrial transfer RNA genes in wheat (*Triticum aestivum*). Ph.D dissertation. Dalhousie University, Halifax, Nova Scotia. pp. 253.

Joyce, P.B.M., Gray, M.W. 1989a. Chloroplast-like transfer RNA genes expressed in wheat mitochondria. Nucleic Acids Res. 17:5461-5476.

Joyce, P.B.M., Gray, M.W. 1989b. Aspartate and asparagine tRNA genes in wheat mitochondrial DNA: a cautionary note on the isolation of tRNA genes from plants. Nucleic Acids Res. 17:7865-7878.

Joyce, P.B.M., Spencer, D.F., Bonen, L., Gray, M.W. 1988. Genes for $tRNA^{Asp}$, $tRNA^{Pro}$, $tRNA^{Tyr}$ and $tRNAs^{Ser}$ in wheat mitochondrial DNA. Plant Mol. Biol. 10:251-262.

Jukes, T.H., Osawa, S. 1990. The genetic code in mitochondria and chloroplasts. Experientia. 46:1117-1126.

Juniper, B.E., Clowes, F.A.L. 1965. Cytoplasmic organelles and cell growth in root caps. Nature. 208:864-865.

Karwan, R., Bennett, J.L., Clayton, D.A. 1991. Nuclear RNase MRP processes RNA at multiple discrete sites: interaction with an upstream B box is required for subsequent downstream cleavages. Genes Dev. 5:1264-1276.

Kiss, T., Filipowicz, W. 1992. Evidence against a mitochondrial location of the 7-2/MRP RNA in mammalian cells. Cell 70:11-16.

Kobayashi, S., Amikura, R., Okada, M. 1993. Presence of mitochondrial large ribosomal RNA outside mitochondria in germ plasm of *Drosophila melanogaster*. Science. 260:1521-1524.

Kumazawa, Y., Yokogawa, T., Tsurui, H., Miura, K., Watanabe, K. 1992. Effect of the higher-order structure of tRNAs on the stability of hybrids with oligodeoxyribonucleotides: separation of tRNA by an efficient solution hybridization. Nucleic Acids Res. 20:2223-2232.

Kusama-Eguchi, K., Watanabe, S., Irisawa, M., Watanabe, K., Igarashi, K. 1991.

Correlation between spermine stimulation of rat liver Ile-tRNA formation and structural change of the acceptor stem by spermine. Biochem. Biophys. Res. Comm. 177:745-750.

Lagerkvist, U. 1978. "Two out of three": an alternative method for codon reading. Proc. Natl. Acad. Sci. USA. 75:1759-1762.

Lagerkvist, U. 1986. Unconventional methods in codon reading. BioEssays. 4:223-226.

Lamppa, G.K., Bendich, A.J. 1984. Changes in mitochondrial DNA levels during development of pea (*Pisum sativum* L.). Planta. 162:463-468.

Lane, B.G. 1963. The separation of adenosine, guanosine, cytidine and uridine by one-dimensional filter-paper chromatography. Biochim. Biophys. Acta. 72:110-112.

Lankat-Buttgereit, B., Gross, H.J., Krupp, G. 1987. Detection of modified nucleosides by rapid RNA sequencing methods. Nucleic Acids Res. 15:7649.

Leaver, C. J., Harmey, M. A. 1973. Plant mitochondrial nucleic acids. Biochem. Soc. Sym. 38:175-193.

Lee, S.L.J., Warmke, H.E. 1979. Organelle size and number in fertile and T - cytoplasmic male-sterile corn. Amer. J. Bot. 66:141-148.

Leon, P., Walbot, V., Bedinger, P. 1989. Molecular analysis of the linear 2.3 kb plasmid of maize mitochondria: apparent capture of tRNA genes. Nucleic Acids Res. 17:4089-4099.

Li, K., Smagula, C.S., Parsons, W.J., Richardson, J.A., Gonzalez, M., Hagler, H.K., Williams, R.S. 1994. Subcellular partitioning of MRP RNA assessed by ultrastructural and biochemical analysis. J. Cell Biol. 124:871-882.

Lonsdale, D.M. 1989. The plant mitochondrial genome. Biochem. Plants 15:229-295.

Lye, L.F., Chen, D.H.T., Suyama, Y. 1993. Selective import of nuclear-encoded tRNAs into mitochondria of the protozoan *Leishmania tarentolae*. Mol. Biochem. Parasitol. 58:233-246.

Mahapatra, S., Ghosh, R., Adhya, S. 1994. Import of small RNAs into *Leishmania*

mitochondria *in vitro*. Nucleic Acids Res. 22:3381-3386.

Marchfelder, A., Schuster, W., Brennicke, A. 1990. *In vitro* processing of mitochondrial and plastid derived tRNA precursors in a plant mitochondrial extract. Nucleic Acids Res. 18:1401-1406.

Maréchal, L., Guillemaut, P., Grienenberger, J.M., Jeannin, G., Weil, J.H. 1986. Sequences of initiator and elongator methionine tRNAs in bean mitochondria. Plant Mol. Biol. 7:245-253.

Maréchal, L., Guillemaut, P., Weil, J.H. 1985. Sequences of two bean mitochondria $tRNAs^{Tyr}$ which differ in the level of post-transcriptional modification and have a prokaryotic-like large extra-loop. Plant Mol. Biol. 5:347-351.

Maréchal-Drouard, L., Guillemaut, P. 1988. Nucleotide sequence of a bean mitochondrial $tRNA^{Leu}4$ and of its cytoplasmic counterpart. Re-examination of the modified nucleotide present at position 12 in bean mitochondrial and cytoplasmic $tRNA^{Leu}1$ sequences. Nucleic Acids Res. 16:11812.

Maréchal-Drouard, L., Guillemaut, P., Cosset, A., Arbogast, M., Weber, F., Weil, J.H., Dietrich, A. 1990. Transfer RNAs of potato *Solanum tuberosum* mitochondria have different genetic origins. Nucleic Acids Res. 18:3689-3696.

Maréchal-Drouard, L., Kuntz, M., Weil, J.H. 1991. tRNAs and tRNA genes of plastids. In *Cell Culture and Somatic Cell Genetics of Plants. The Molecular Biology of Plastids*. eds. I.K. Vasil, L. Bogorad. Academic Press. San Diego/New York. pp. 169-189.

Maréchal-Drouard, L., Weil, J.H., Dietrich, A. 1993. Transfer RNAs and transfer RNA genes in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:13-32.

Maréchal-Drouard, L., Weil, J.H., Guillemaut, P. 1988. Import of several tRNAs from the cytoplasm into the mitochondria in bean *Phaseolus vulgaris*. Nucleic Acids Res. 16:4777-4788.

Marion-Poll, A., Hibbert, C.S., Radebaugh, C.A., Hallick, R.B. 1988. Processing of mono-, di- and tricistronic transfer RNA precursors in a spinach or pea chloroplast soluble extract. Plant Mol. Biol. 11:45-56.

Martin, R.P., Schneller, J.M., Stahl, A.J.C., Dirheimer, G. 1979. Import of nuclear deoxyribonucleic acid coded lysine-accepting transfer ribonucleic acid

(anticodon C-U-U) into yeast mitochondria. Biochemistry. 18:4600-4605.

McClain, W.H. 1993. Transfer RNA identity. FASEB J. 7:72-78.

Muramatsu, T., Nishihawa, K., Nemoto, F., Kuchino, Y., Nishinura, S. 1988. Codon and amino acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. Nature. 336:179-181.

Nicoghosian, K., Bigras, M., Sankoff, D., Cedergren, R. 1987. Archetypical features in tRNA families. J. Mol. Evol. 26:341-346.

Oda, K., Yamato, K., Ohta, E., Nakamura, Y., Takemura, M., Nozato, N., Akashi, K., Kanegae, T., Ogura, Y., Kohchi, T., Ohyama, K. 1992a. Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. J. Mol. Biol. 223:1-7.

Oda, K., Yamato, K., Ohta, E., Nakamura, Y., Takemura, M., Nozato, N., Akashi, K., Ohyama, K. 1992b. Transfer RNA genes in the mitochondrial genome from a liverwort, *Marchantia polymorpha*: the absence of chloroplast-like tRNAs. Nucleic Acids Res. 20:3773-3777.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T. 1986. Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature. 322:572-574.

Okimoto, R., Wolstenholme, D.R. 1990. A set of tRNAs that lack either the TΨC arm or dihydrouridine arm: towards a minimal tRNA adaptor. EMBO J. 9:3405-3411.

Palmer, J.M., Folk, W.R. 1987. Isolation and sequence analysis of a nuclear $tRNA^{met\text{-}i}$ gene from soybean. Plant Mol. Biol. 8:47-51.

Parish, J. H., Kirby, K. S. 1966. Reagents which reduce interactions between ribosomal RNA and rapidly labelled RNA from rat liver, Biochim. Biophys. Acta 129:554-562.

Peattie, D.A. 1979. Direct chemical method for sequencing RNA. Proc. Natl. Acad. Sci. USA. 76:1760-1764.

Pfitzinger, H., Weil, J.H., Pillay, D.T.N., Guillemaut, P. 1990. Codon recognition mechanisms in plant chloroplasts. Plant Mol. Biol. 14:805-814.

Pritchard, A.E., Seilhamer, J.J., Mahalingam, R., Sable, C.L., Venuti, S.E.,

Cummings, D.J. 1990. Nucleotide sequence of the mitochondrial genome of *Paramecium*. Nucleic Acids Res. 18:173-179.

Quetier, F., Lejeune, B., Delorme, S., Falconet, D. 1985. Molecular organization and expression of the mitochondrial genome of higher plants. In *Encyclopedia of Plant Physiology, New Series*. eds. R. Douce, D.A. Bay. Springer. Berlin, Heidelberg, New York. Vol. 18, pp. 27-36.

Reddy, P.S., Padayatty, J.D. 1988. Effects of 5' flanking sequences and changes in the 5' internal control region on the transcription of rice $tRNA^{Gly}$(GCC) gene. Plant Mol. Biol. 11:575-583.

Rubin, G.M. 1973. The nucleotide sequence of *Saccharomyces cerevisiae* 5.8S ribosomal ribonucleic acid. J. Biol. Chem. 248:3860-3875.

Sangaré, A., Weil, J.H., Grienenberger, J.M., Fauron, C., Lonsdale, D. 1990. Localization and organization of tRNA genes on the mitochondrial genomes of fertile and male sterile lines of maize. Mol. Gen. Genet. 223:224-232.

Schinkel, A.H., Tabak, H.F. 1989. Mitochondrial RNA polymerase: dual role in transcription and replication. Trends Genet. 5:149-154.

Schnare, M.N. 1984. Ribosomal RNA structure and evolution revealed by nucleotide sequence analysis. Ph.D. dissertation. Dalhousie University, Halifax, Nova Scotia.

Schnare, M.N., Heinonen, T.Y.K., Young, P.G., Gray, M.W. 1985. Phenylalanine and tyrosine transfer RNAs encoded by *Tetrahymena pyriformis* mitochondrial DNA: primary sequence, post-transcriptional modifications, and gene localization. Curr. Genet. 9:389-393.

Schneider, A. 1994. Import of RNA into mitochondria. Trends Cell Biol. 4:282-286.

Schneider, A., Martin, J., Agabian, N. 1994a. A nuclear encoded tRNA of *Trypanosoma brucei* is imported into mitochondria. Mol. Cell Biol. 14:2317-2322.

Schneider, A., McNally, K.P., Agabian, N. 1994b. Nuclear-encoded mitochondrial tRNAs of *Trypanosoma brucei* have a modified cytidine in the anticodon loop. Nucleic Acids Res. 22:3699-3705.

Schuster, W., Brennicke, A. 1985. TGA-Termination codon in the apocytochrome

b gene from *Oenothera* mitochondria.. Curr. Genet. 9:157-163.

Schuster, W., Brennicke, A. 1987. Plastid, nuclear and reverse transcriptase sequences in the mitochondrial genome of *Oenothera:* is genetic information transferred between organelles via RNA? EMBO J. 6:2857-2863.

Shi, X., Chen, D.H.T., Suyama, Y. 1994. A nuclear tRNA gene cluster in the protozoan *Leishmania tarentolae* and differential distribution of nuclear-encoded tRNAs between the cytosol and mitochondria. Mol. Biochem. Parasitol. 65:23-37.

Shields, R. 1993. Pastoral synteny. Nature. 365:297-298.

Simpson, A.M., Suyama, Y., Dewes, H., Campbell, D.A., Simpson, L. 1989. Kinetoplastid mitochondria contain functional tRNAs which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function. Nucleic Acids Res. 17:5427-5445.

Singh, H., Lane, B.G. 1964. The separation, estimation and characterization of alkali-stable oligonucleate preparations. Can. J. Biochem. 42:87-93.

Singhal, R.P., Fallis, P.A.M. 1979. Structure, function and evolution of transfer RNAs. Progr. Nucleic Acid Res. Mol. Biol. 23:227-263.

Small, I., Maréchal-Drouard, L., Masson, J., Pelletier, G., Cosset, A., Weil, J.H., Dietrich, A. 1992. *In vivo* import of a normal or mutagenized heterologous transfer RNA into the mitochondria of transgenic plants: towards novel ways of influencing mitochondrial gene expression? EMBO J. 11:1291-1296.

Spencer, D.F., Gray, M.W., Schnare, M.N. 1992. The isolation of wheat mitochondrial DNA and RNA. In *Seed Analysis*. eds. H.F. Linskens, J.F. Jackson. Springer-Verlag. Berlin. pp. 347-360.

Stange, N., Beier, H. 1987. A cell-free extract for accurate pre-tRNA processing, splicing and modification. EMBO J. 6:2811-2818.

Steinberg, S., Misch, A., Sprinzl, M. 1993. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. 21:3011-3015.

Stern, D.B., Lonsdale, D.M. 1982. Mitochondrial and chloroplast genomes of maize have a 12-kilobase DNA sequence in common. Nature. 299:698-702.

Stern, D.B., Palmer, J.D. 1984. Extensive and widespread homologies between mitochondrial DNA and chloroplast DNA in plants. Proc. Natl. Acad. Sci. USA. 81:1946-1950.

Sugiura, M., Wakasugi, T. 1989. Compilation and comparison of transfer RNA genes from tobacco chloroplasts. Crit. Rev. Plant Science. 8:89-101.

Suyama, Y. 1986. Two dimensional polyacrylamide gel electrophoresis analysis of *Tetrahymena* mitochondrial tRNA. Curr. Genet. 10:411-420.

Suyama, Y., Hamada, J. 1978. The mitochondrial and cytoplasmic valyl tRNA synthetases in *Tetrahymena* are indistinguishable. Arch. Biochem. Biophys. 191:437-443.

Tapper, D.P., Van Etten, R.A., Clayton, D.A. 1983. Isolation of mammalian mitochondrial DNA and RNA and cloning of the mitochondrial genome. Meth. Enzymol. 97:426-434.

Tarassov, I.A., Entelis, N.S. 1992. Mitochondrially-imported cytoplasmic $tRNA^{Lys}$(CUU) of *Saccharomyces cerevisiae: in vivo* and *in vitro* targetting. Nucleic Acids Res. 20:1277-1281.

Teichmann, T., Urban, C., Beier, H. 1994. The $tRNA^{Ser}$-isoacceptors and their genes in *Nicotiana rustica:* genome organization, expression *in vitro* and sequence analyses. Plant Mol. Biol. 24:889-901.

Topper, J.N., Bennett, J.L., Clayton, D.A. 1992. A role for RNAase MRP in mitochondrial RNA processing. Cell 70:16-20.

Tsudzuki, J., Itoh, S., Nakashima, K., Tsudzuki, T., Wakasugi, T., Sugiura, M. 1994. The complete nucleotide sequence of the black pine chloroplast genome. Abstract 166, 4th International Congress of Plant Molecular Biology, Amsterdam. June 19-24, 1994.

Utz, E.D., Siravo-Sagraves, G.M., Trewyn, R.W., Morgan, C.J. 1994. Detection of human tRNAs with antisense oligonucleotides. Anal. Biochem. 216:110-117.

Vahrenholz, C., Riemen, G., Pratje, E., Dujon, B., Michaelis, G. 1993. Mitochondrial DNA of *Chlamydomonas reinhardtii*: the structure of the ends of the linear 15.8-kb genome suggests mechanisms for DNA replication. Curr. Genet. 24:241-247.

Vestweber, D., Schatz, G. 1989. DNA-protein conjugates can enter mitochondria

via the protein import pathway. Nature. 338:170-172.

Waldron, C., Wills, N., Gesteland, R.F. 1985. Plant tRNA genes: putative soybean genes for $tRNA^{asp}$ and $tRNA^{met}$. J. Mol. Appl. Genet. 3:7-17.

Walseth, T.F., Johnson, R.A. 1979. The enzymatic preparation of [α-^{32}P]nucleotide triphosphates, cyclic [^{32}P]AMP and cyclic [^{32}P]GMP. Biochim. Biophys. Acta. 562:11-31.

Ward, B.L., Anderson, R.S., Bendich, A.J. 1981. The mitochondrial genome is large and variable in a family of plants (*Cucurbitaceae*). Cell 25:793-803.

Weber, F., Dietrich, A., Weil, J.H., Maréchal-Drouard, L. 1990. A potato mitochondrial isoleucine tRNA is coded for by a mitochondrial gene possessing a methionine anticodon. Nucleic Acids Res. 18:5027-5030.

Weber-Lotfi, F., Maréchal-Drouard, L., Folkerts, O., Hanson, M., Grienenberger, J.M. 1993. Localization of tRNA genes on the *Petunia hybrida* 3704 mitochondrial genome. Plant Mol. Biol. 21:403-407.

Wilson, A.C., Cann, R.L., Carr, S.M., George, M., Gyllensten, U.B., Helm-Bychowski, K.M., Higuchi, R.G., Palumbi, S.R., Prager, E.M., Sage, R.D., Stoneking, M. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc. 26:375-400.

Wolfe, K.H., Li, W.H., Sharp, P.M. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proc. Natl. Acad. Sci. USA. 84:9054-9058.

Wolstenholme, D.R. 1992. Animal mitochondrial DNA: Structure and evolution. Inter. Rev. Cytol. 141:173-216.

Wong, T.W., Clayton, D.A. 1986. DNA primase of human mitochondria is associated with structural RNA that is essential for enzymatic activity. Cell 45:817-825.

Yoshionari, S., Koike, T., Yokogawa, T., Nishikawa, K., Ueda, T., Miura, K., Watanabe, K. 1994. Existence of nuclear-encoded 5S-rRNA in bovine mitochondria. FEBS Lett. 338:137-142.

Zeff, R.A., Geliebter, J. 1987. Oligonucleotide probes for genomic DNA blots. BRL Focus 9:1-2.

Zimmer, M., Lückemann, G., Lang, B., Wolf, K. 1984. The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe* 3. Gene mapping in strain EF1 (CBS 356) and analysis of hybrids between the strains EF1 and ade7-50h$^-$. Mol. Gen. Genet. 196:473-481.