MITOCHONDRIAL GENOME REARRANGEMENTS IN SORGHUM

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I wish to dedicate this thesis to my grandmother Valborg M. Bailey, in memory of my generous grandfather Lloyd Evan Bailey, who sowed the seed for my academic enrichment.

Who Dares ?

Who dares to twist and base-match
atoms to life?
God's hand tied molecules neatly into bows
nucleotide by nucleotide
α-helix by α-helix
Genetic code designed and deciphered,
Montage of life.

Who dares to touch, base mis-match,
clone and culture?
Genetic engineers pull and unravel
DNA into data
mystery from mutations
Hypotheses are formed and tested,
New questions revealed.

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I have written this thesis on my own but I owe a great deal to those people whose ideas have given me inspiration.

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Abstract

Interspecific variation in mitochondrial genome organization and expression was examined in *Sorghum bicolor*. Cytoplasmic genotypes were classified into eleven groups on the basis of restriction endonuclease digestion of mitochondrial DNA (mtDNA) and five groups on the basis of *in organello* translation of mitochondrial polypeptides. The interspecific variation in genome organization was critically examined by hybridization of *Eco*RI digested mtDNA with gene specific probes.

The gene (*COX*I) encoding cytochrome *c* oxidase subunit I (COI) is located on a 4.3 or 10.4 Kb *Eco*RI generated mtDNA restriction fragment in different cytoplasms. Lines in which *COX*I is located on a 4.3 Kb fragment synthesize a major *COX*I transcript of 1.8 Kb and a polypeptide with an apparent molecular weight of 38,000 (38K). In contrast, lines in which the gene is located on a 10.4 Kb fragment synthesize a single *COX*I transcript of 2.2 Kb and a variant polypeptide with an apparent molecular weight of 42,000 (42K). Sequence analysis of the genes which encode the two forms of COI revealed they are identical from position -100 bp 5' to the start of translation, to position +1579 bp *within* the coding region of the gene. The *COX*I gene sequences 5' and 3' to these divergence points are non-homologous. Thus, the *COX*I gene can be located in one of two distinct environments in the mitochondrial genome.

The normal, 38K COI is encoded by a 530 codon ORF (58K daltons) which shares 98% sequence homology with and is similar in length to the corresponding maize gene. The variant 42K COI is encoded by a 631 codon ORF (70K daltons). Genome rearrangement within *COX*I resulted in the 101 codon continuation of the ORF beyond the 3' divergence point. The sequence of this C-terminal extension is not homologous to any known gene. No differences were detected in the cytochrome spectrum and cytochrome *c* oxidase activity of seedling mitochondria containing the normal and variant COI.

The variant COI is synthesized by male fertile and cytoplasmic male sterile (CMS) lines and therefore is apparently not correlated with male sterility. The CMS phenotype is observed when a cytoplasm is transferred to a foreign nuclear background and is often associated with alterations in mitochondrial protein synthesis. The CMS phenotype could result from the absence of nuclear 'fertility' genes which co-evolved with mitochondrial genome rearrangements and compensate for the expression of a mitochondrial polypeptide, such as an altered COI.

ABBREVIATIONS

A_N	Absorbance at wavelenth (nm)=N
pAB - HCL	ρ - aminobenzamide hydrochoride
AMPS	Ammonium persulfate
ATPA ATP6, ATP8, ATP9	Genes encoding mitochondrial ATPase Subunits α, 6, 8, 9
ATPase	Adenosine 5' triphosphatase (ATPsynthase)
b.	nucleotide
bis acrylamide	N, N' - methylene bis acrylamide
BME	ß - mercaptoethanol
bp	Base pair(s)
BPB	Bromo Phenol Blue
BSA	Bovine Serum Albumin (FractionV)
CCMV	Cowpea chlorotic mosaic virus
CMS	Cytoplasmic male sterile
COB	Apocytochrome b
COB	Mitochondrial gene encoding Apocytochrome b
CO I - VIII	Cytochrome c oxidase Subunits I - VIII
COXI, COXII, COXIII	Mitochondrial gene encoding cytochrome c oxidase Subunits I, II, III
cpDNA	Chloroplast DNA
d(A,G,C,and T)TP	2' deoxy (Adenosine, Guanosine, Cytidine and Thymidine) 5' triphosphate
dd(A,G,C, and T)TP	2', 3' deoxy (Adenosine, Guanosine, Cytidine and Thymidine) 5' triphosphate
DCCD	N, N' Dicyclohexylcarbodiimide
DNA;(r,t)DNA	Deoxyribonucleic acid; DNA encoding rRNA, tRNA
DNase	Deoxyribonuclease

DTT	Dithiothreitol
EM	Electron microscope
FPLC	Fast Protein Liquid Chromotography
X $g_{ave(max)}$	Average (maximum) relative gravitational force
hr	Hour(s)
IEF	Isoelectric focusing
IPTG	Isopropyl thio ß D - galactoside
Kb	Kilobase (pair(s))
MOPS	Morpholinopropanesulphonic acid
mtDNA, mtRNA	Mitochondrial DNA, RNA
$NaPO_4$	Mixture of varing quantities of NaH_2PO_4 and Na_2HPO_4 at a given pH.
ND1 - 8	Gene encoding mitochondrial NADH-ubiquinone oxidoreductase subunits 1 - 8
NP-40	Nonidet P -40
OD	Optical density
ORF	Open reading frame
PAS	4 amino 2 - hydroxybenzoic acid (sodium salt)
PEG	Polyethylene glycol
PIPES	Piperazine - N,N' bis [2 - ethanesulphonic acid]: 1, 4 - piperazine diethane sulphonic acid
PVP	Polyvinylpyrrolidone
PMSF	Pheny - methyl - sulphonyl - flouride
Rf,rf	Nuclear alleles of maize which restore male fertility
RF	replicative form
RNA; (m,t and r)RNA	Ribonucleic acid; (messenger, transfer and ribosomal)RNA

RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	second(s)
SSC	Standard saline citrate (defined in Section 2.7)
ssDNA	Single stranded DNA
TBE	Tris Borate EDTA buffer (defined in section 2.7)
TEMED	N,N,N',N' - tetramethylethylene-diamine
TNS	Tri-iso-propylnaphthalene sulphonic acid (sodium salt)
Tricine	N-tris (hydroxy methyl) methyl glycine
Triton X-100	Octylphenoxpolethoxethanol
Tween-20 (-80)	Polyoxethylene sorbitan mono-laurate (mono-oleate)
URF	Unidentified reading frame
v/v	Volume per volume (as percent)
w/v	Weight per volume (as percent)
Xgal	5 - bromo, 4 chloro, 3 indolyl D -galactoside

Standard abbreviations were used for scientific units, nucleic acids, polynucleotides and chemicals as recommended by The Handbook of Biochemistry, Selected for Molecular Biology, (1968) Ed. H.A. Sober, The Chemical Rubber Co., Cleveland, Ohio.

EQUIVALENTS

1 μm DNA = 3.3 kilo base pairs (Kb)

1 base pair DNA = 100 X 10^{-23} grams = 658 daltons

1 base RNA = 57.6 X 10^{-23} grams = 345 daltons

1 dalton = 1.67 X 10^{-24} grams

Chapter I

MITOCHONDRIAL BIOGENESIS: THE ORGANELLAR CONTRIBUTION

1.1 Introduction

Higher plant cells possess three separate genomes: nuclear, chloroplast and mitochondrial. The chloroplast and mitochondrial genomes are small and contain only a portion of the genetic information necessary for organellar biogenesis and maintenance of a genetic system. In mitochondria, about 5 - 10% of the total number of proteins are encoded by mitochondrial DNA (mtDNA) and translated on mitochondrial ribosomes. The gene products of mtDNA include rRNAs, tRNAs and a few hydrophobic subunits of the inner mitochondrial membrane enzyme complexes. The remaining mitochondrial proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and imported into mitochondria. Thus, the biogenesis and function of mitochondria involves the expression of two separate genomes (Tzagoloff, 1982).

Although the major function of mitochondria as the site of oxidative phosphorylation is invariant, the mitochondrial genome is variable in size, shape and genetic content. The entire mitochondrial genome of a number of animals has been sequenced, as well as a major portion of the mtDNA of a number of fungi. In contrast, the large mitochondrial genome of higher plants has been characterized to a much lesser extent. The purpose of this chapter is to:

1) review mitochondrial genome structure and expression in higher plants in reference to what is known for other organisms and,
2) introduce mitochondrial and nuclear mutations associated with altered mitochondrial biogenesis or function in fungi and higher plants.

1.2 A. Size and Conformation and Genome Organization of Mitochondrial DNA

The mitochondrial genomes of animals, protozoa, fungi and higher plants vary in size, conformation and organization (Table 1.1) (for review, Sederoff, 1984). The mitochondrial genome of animals is a circular molecule of about 16 Kb which contains a compact organization of gene sequences (Anderson et al., 1981; Anderson et al., 1982; Clary and Wolstenholme, 1984; Bibb et al., 1981). The functional mitochondrial genome of protozoa is either a circular or linear molecule and is also relatively small in size. In contrast, the mitochondrial genome of fungi is large and variable in size. The mtDNA of Saccharomyces cerevisiae has been mapped as a 75 - 85 Kb circular molecule (de Zamoroczy and Bernardi, 1985), whereas the fission yeast, Schizosaccharomyces pombe, has a circular mitochondrial genome of only 17 - 19 Kb (Anziano et al., 1983; Wolf et al., 1982). The mitochondrial genome of filamentous fungi is also large: Neurospora crassa, 60 - 73 Kb (Burke and RajBhandary, 1982); Podospora anserina, 94 Kb (Wright et al., 1982); Aspergillus nidulans, 33 Kb (Brown et al., 1985). The observed variation in genome size in yeast mitochondria is due in part to non-coding A + T rich intergenic regions and optional intragenic sequences ("introns"). In Neurospora, interspecific size variation is due to optional introns and G + C rich palindromic, non-coding sequences (Burke et al., 1984; Collins and Lambowitz, 1983). The physical and genetic maps of the mitochondrial genomes of fungi are different, probably due to numerous insertion, deletion and reorganization events (Dujon, 1983).

Table 1.1
Structural Characteristics of Various Mitochondrial DNAs

ORGANISM	GENOME SIZE (Kb)	GENOME CONFORMATION	REFERENCES
ANIMALS			
Human	16.57	circular	Anderson et al., 1981
Bovine	16.33	circular	Anderson et al.,1982
Drosophila	15.7 - 19.5	circular	Wolstenholme et al., 1979; Clary and Wolstenholme, 1985
PROTOZOA			
Tetrahymena	31.5	linear	Goldbach et al., 1979 Flavell and Jones,1970
Trypanosoma maxicircle	18.5	circular	Fouts et al., 1975
FUNGI			
Saccharomyces	74 - 82	circular	de Zamaroczy and Bernardi, 1985
Torolopsis	18.5	circular	Clark-Walker etal.,1981a
Schizosaccharomyces	18.5	circular	Anziano et al.,1983 Wolf et al., 1982
Hansenula	25	circular	Wesolowski and Fukahara, 1981
Kluyvermyces lactis	37	circular	O'Conner et al., 1975
Neurospora	60 - 73	circular	Bernard et al., 1975
Podospora	94	circular	Wright et al.,1982
Aspergillus	33	circular	Brown et al., 1985
ALGAE			
Chlamydomonas	16	linear	Grant and Chiang, 1980
HIGHER PLANTS			
Zea	570	circular	Lonsdale et al., 1984
Brassica	218	circular	Palmer and Shields, 1984
Triticum	440	circular	Quetier and Vedel, 1977
Cucurbitaceae	330-2400	?	Ward et al., 1981

Additional References: Sederoff (1984), Gray (1982).

1.2 B Higher Plant Mitochondrial Genomes

The mitochondrial genome of higher plants is the largest and most variable in size described. Plant genome size has been calculated by reassociation kinetics, restriction enzyme digestion analysis, and genome mapping studies. The smallest genome reported is in Brassica (217 Kb) and the largest in watermelon (2400 Kb) (for review, Bendich, 1985). Extreme variation in size (330 - 2400 Kb) has been observed within the curcurbit family alone and is not correlated with differences in nuclear genome size or mitochondrial volume (Ward et al., 1981). Although, the large estimate of genome size is not due to large, highly repetitive DNA sequences or methylated nucleotides (Spruill et al., 1980; Ward et al., 1981; Bonen et al., 1980), molecules as large as the predicted genome have not been observed by electron microscopy (for review: Leaver and Gray, 1982; Bendich, 1985).

MtDNA extracted from plants is mostly linear in conformation, but in many plants a small portion of mtDNA molecules are circular. In general, closed circular molecules are observed more frequently in mtDNA isolated from plant cell cultures than from meristematic cells (Dale, 1981; Dale et al., 1981; Brennicke and Blanz 1982). The size distribution of circular mtDNA molecules of higher plants fall into two categories:

1) Circular molecules of distinct size classes, which are sometimes observed in oligomeric series:
   a) large subgenomic circles: Zea mays (maize), 80 Kb, 67 Kb, 47 Kb (Levings et al., 1979); Oenothera (Evening primrose) 6.3, 7.0, 8.2, 9.9, 13.5 Kb (Brennicke and Blanz, 1982); Linum (flax) 36 - 56 Kb (Bailey-Serres, unpublished),
   b) minicircular molecules (Table 1.2).
2) Circular molecules with a broad size distribution: Nicotiana (tobacco) (Sparks and Dale, 1980); Citrus (Fontarnau and Hernandez-Yago, 1982); and others (Quetier and Vedel, 1977; Bendich, 1985).

The significance of the circular mtDNA populations in higher plants is not clearly understood. Large circular molecules may, or may not represent the entire mitochondrial sequence complexity (Bendich, 1985; Sparks and Dale, 1980; Dale et al., 1983). Circular molecules may be replication intermediates or autonomously replicating mtDNAs which arise from recombination events and are subsequently amplified. Subgenomic mtDNA circular molecules arise from recombination in petite mutants of yeast (Locker et al., 1974), stopper mutants of N.crassa (Manella et al., 1978; Bertrand et al., 1980; deVries et al., 1981), senescent DNA (senDNA) of Podospora (Wright et al., 1982) and ragged mutants of A.amstelodami (Lazarus et al., 1980).

Additional genetic elements are observed in numerous higher plants and some fungal mitochondria. These are minicircular and minilinear molecules of distinct size, termed 'plasmid-like' mtDNAs (Table 1.2). The origin and function of these purported autonomously replicating molecules is not known. Certain 'plasmid-like' mtDNAs appear to be transcribed and/or share homology to the main band mtDNA (Table 1.2), and may be associated with expression of cytoplasmic male sterility (Goblet et al., 1985; Leroy et al., 1985; See section 1.7).

Restriction digestion analysis and genome mapping studies have revealed that the mitochondrial genomes of higher plants are extremely large in size, and perhaps more surprisingly, that mitochondrial genome organization is different in closely related plants. For example, the restriction digestion pattern of mtDNA from normal fertile (N) and cytoplasmic male sterile (CMS) lines (CMS T, S, or C) of maize are different (See also section 1.7; Levings and Pring, 1976). Spruill et al. (1980) predicted on the basis of hybridization of mtDNA fragments to endonuclease digested mtDNA of N and CMS T maize that the observed variations are due to either rearrangements or point mutations, and not to large repeated sequences.

Table 1.2
Minicircular and Minilinear DNAs in Plant Mitochondria

PLANT CYTOPLASM	Kb	FORM	TRANSCRIBED (mtRNA)	HOMOLOGY TO MAIN MTDNA	REFERENCES
Maize					
CMS S	6.4 (S1)	linear	+	+	Pring etal., 1977;
CMS S	5.4 (S2)	linear	+	+	Kemble and Bedbrook,
fertile N CMS C, S	2.35	linear	?	+	1980;Kemble and Thompson, 1982
CMS T	2.15	linear	?	+	
CMS C	1.57	circular	?	-	
CMS C	1.42	circular	+	-/+	
fertile N some S CMS C, T	1.94	circular	+	-/+	Ludwig etal.,1984 Carlson and Kemble, 1985
South American Maize	7.5 (R1)	linear	+	+	Weissinger et al.,1982
	5.4 (R2)	linear	+	+	
	2.0	circular	?	?	
Teosinte	7.5 (D1)	linear	+	+	Timothy
	5.4 (D2)	linear	+	+	etal.,1982
Sorghum					
IS1112C	5.7 (N1)	linear	+	-	Pring etal.,
M35-1	5.3 (N2)	linear	+	-	1982; Dixon Leaver,1982
	2.3	circular	+	-	Chase and Pring, 1985
all	1.36	circular	?	?	Chase and Pring, 1986
IS1112C	1.7	circular	+	?	
Brassica					
B.campestris	11.3	linear	?	+	Palmer and Shields,1982
Sugarbeet					
fertile	1.44	circular	+	-	Munk-Hansen etal., 1984
CMS	1.5	circular	?	?	Powling,1981
some fert.	1.3	circular	?	?	Powling and Ellis,1983

Table 1.2 (continued)

PLANT CYTOPLASM	Kb	FORM	TRANSCRIBED (mtRNA)	HOMOLOGY TO MAIN BAND MTDNA	REFERENCES
Broad Bean					
all	1.70 (F)	circular	+	?	Goblet *etal.*,
	1.42	circular	?	?	1985; Negruk *et*
CMS	1.70 (S)	circular	?	?	*al.*, 1982;
					Boutry and
350	1.54	circular	?	?	Briquet, 1983
Sunflower					
MF HA89	1.45	circular	?	?	Leroy *etal.*, 1985
Fungi					
N.intermedia					
Labelle	4.2	circular	-	-	Stohl *etal.*,
Fiji	5.2	"	-	-	1982
N.crassa					
Mauriceville	3.6	circular	+	-	Nargang *etal.*,1985 Collins *etal.*,1981
Podospora					
α sen DNA	2.6	circular			Wright *et*
ß sen DNA	9.8	"			*al.*, 1982
ϑ sen DNA	6.3	"			
Claviceps					
purpurea	6.6	linear			Tudzynski
	7.4	"			*etal.*, 1983
	1.1	"			
Agaricus	7.4	linear	-		Mohan *et*
	3.7	"	-		*al.*,1984
Cochliobolis heterostrophus (*Helminthosporium maydis*)	1.9	circular			Garber *et al.*, 1984

N - normal, male fertile maize; T,C,S, - CMS maize

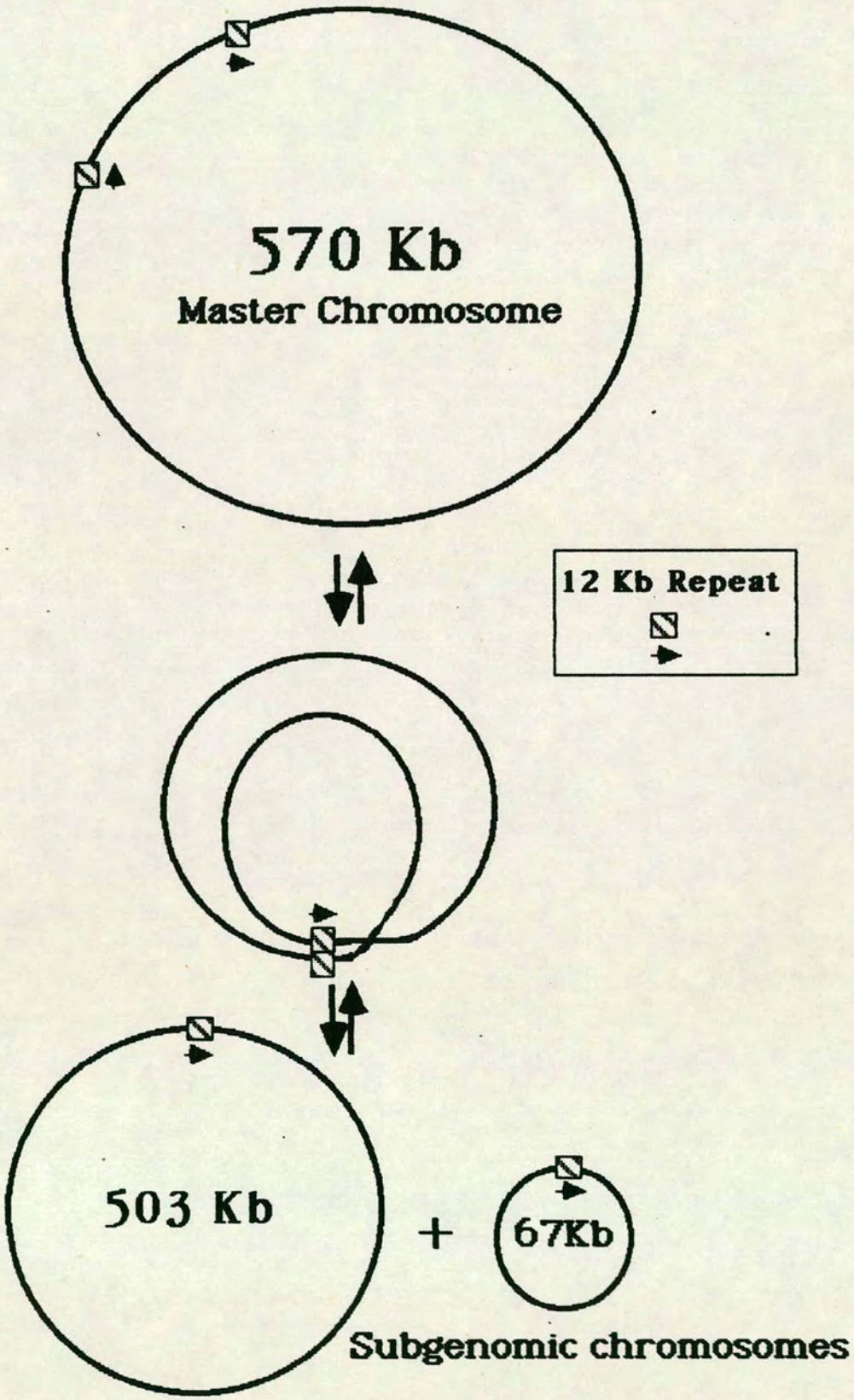
The mitochondrial genome of maize has been mapped by cloning large mtDNA fragments into cosmid recombinant DNA vectors and restriction mapping of the clones (Lonsdale et al., 1984). The mitochondrial genome map of N maize is a single circular molecule ('master chromosome' or 'master circle') of 570 Kb which contains five directly repeated sequences (termed the "12, 10, 3, 2, 1 Kb repeats") and one indirectly repeated sequence (the "14 Kb repeat"). Recombination between these repeated sequences is thought to generate subgenomic populations of circular molecules. For example, recombination across the 12 Kb repeat would form the 67 Kb circle which is the predominant circular molecule observed by electron microscopy and a 503 Kb circle (Figure 1.1) (Lonsdale et al., 1984; Levings et al., 1979). The partially completed mitochondrial genome map of the CMS T line differs from N maize due to specific deletions and regions of genome reorganization (C. Fauron, personal communication).

A single circular mitochondrial chromosome has also been mapped for Brassica campestris (218 Kb) and Brassica oleracea (217 Kb) (Palmer and Shields, 1984; Chetrit et al., 1984). The 'master chromosome' of Brassica campestris contains two 2 Kb direct repeats which are separated by 135 and 83 Kb. It has been proposed that homologous recombination across these repeats may give rise to subgenomic circular molecules of 135 and 83 Kb which are present in equal amounts (Palmer and Shields, 1984). Permutations of this recombination event (ie. recombination between two 83 Kb molecules) may give rise to a population of mtDNA molecules which is visible as endonuclease digested mtDNA fragments present in sub-stoichiometric amounts.

As in maize and Brassica, the Triticum (wheat) mitochondrial genome has been mapped as a circular chromosome (440 - 450 Kb) which contains at least 10 repeated sequences (B. Lejeune, personal communication).

Thus, recombination across repeated DNA sequences and deletion events may cause the observed differences in

Figure 1.1: Theoretical Recombination Across the 12 Kb Repeats of Maize MtDNA



mtDNA restriction pattern amongst related plants. Mitochondrial genome size may be proportional to the number of DNA sequences which are active in recombination. The large sequence complexity may be generated by the scrambling of small DNA sequences which are not detected as repeated sequences by reassociation kinetic or restriction digestion analyses (Bendich, 1985). However, it must be stressed that recombination in higher plant mtDNA has not been demonstrated *in vivo* or *in vitro*: genomic recombination is a theoretical model used to explain the restriction maps of mtDNA cosmid clones. The molecular basis of genomic rearrangement in higher plant mitochondria will be discussed in Chapter VI.

1.3 A Mitochondrial Genes, Translation Products and Genome Organization

Despite the large variation in size and organization, the mitochondrial genomes of animals, fungi and higher plants encode a similar but nonidentical set of rRNA, tRNA and protein genes (Table 1.3). The proteins encoded by mtDNA are mainly hydrophobic subunits of the inner mitochondrial membrane enzyme complexes (Figure 1.3).

Animal mitochondrial genes are tightly packed on the circa 16 Kb circular chromosome and do not contain introns (for review: Attardi, 1982). In fungi, mitochondrial genes are separated by non-coding regions and may contain a variable number of intervening sequences (introns) of two distinct classes (for review: Dujon, 1983; de Zamoroczy and Bernardi, 1985; Grivell, 1983b; Michel and Dujon, 1983). The most well characterized plant mitochondrial genome is that of maize. A number of genes have been mapped on the N maize 570 Kb mitochondrial chromosome (Figure 1.2), of these only one is known to contain an intron (Dawson *et al.*, 1986b).

In animals and fungi mitochondrial genes are mostly encoded by a single DNA strand, whereas in maize genes are encoded on either strand. The maize *COB*, *COX*II, *COX*I

Table 1.3
Mitochondrial Genome Size and Gene Products

	Animals	FUNGI *N.crassa*/Yeast	HIGHER PLANTS
GENOME SIZE (Kb)	16 - 17	17 - 103	200-2400
GENE PRODUCT (Gene Name)			
RNAs			
large rRNA	16S	23S/21S	26S
small rRNA	12S	17S/15S	18S
5S rRNA	-	-	+
tRNAs	22	27/25	30 (?)
tRNA synthesis locus	-	?/+	?
Proteins			
ribosomal			
Var1 (*Var1*)	-	-/+	?
S5	-	+/-	?
Complex I			
Subunit I (*ND1*)	+	+/-	+
Subunit II (*ND2*)	+	+/-	*
Subunit III (*ND3*)	+	?/-	
Subunit IV (*ND4*)	+	?/-	
(*ND4L*)	+	?/-	
Subunit V (*ND5*)	+	+/-	*
Complex III			
apocytochrome*b* (*COB*)	+	+	+
Complex IV			
Subunit I (*COXI*)	+	+	+
Subunit II (*COXII*)	+	+	+
Subunit III (*COXIII*)	+	+	+
Complex V			
αF_1ATPase (*ATPA*)	-	-	+
F_oATPase			
Subunit 6 (*ATP6*)	+	+	+
Subunit 8 (*ATP8*)	+	+	?
Subunit 9 (*ATP9*)	+	-/+	+
RNA maturases	-	+	?
URFs	-	+	+

For simplicity, mitochondrial protein genes will be referred to by the nomenclature used here and not by that used for specific organisms. *Hybridization identification.

Figure 1.2

Map of the Maize Mitochondrial Genome

The physical map of the normal (WF9 N) fertile maize mitochondrial genome was determined by cross hybridization of cosmid clones containing mtDNA restriction fragments. Linkage groups of cosmid clones were established and eventually a circular mitochondrial chromosome map of 570 Kb was completed (Lonsdale et al., 1984).

6 repeated mtDNA sequences ('1, 2, 3, 10, 12 and 14 Kb repeats') were identified and mapped. The genomic locations of mitochondrial genes, sequences homologous to plasmid-like mtDNA (S1, S2, R1 and R2), and chloroplast DNA (16S rRNA, LS (Large Subunit 1,5-Ribulose bis-phosphate Carboxylase)) were identified by hybridization of specific probes to the cosmid mtDNA library (Dawson et al., 1986b). For the mtDNA genes studied by sequence analysis, the direction of transcription has been determined by restriction mapping of the cosmid clones containing the gene. The location of the mtDNA homologous to ND1 has been determined by hybridization analysis but this gene has not been sequenced to date. The gene encoding F_oATPase subunit 6 has not been mapped but probably contains the '2Kb repeat', since a portion of the ATP6 ORF is homologous to the sequence just 5' to the COXII ORF (Dewey et al., 1986).

In addition to the 570 Kb 'master circle' a number of sub-genomic circular molecules were mapped. The most common sub-genomic molecules are those theoretically derived from recombination across the,

1) 12 Kb repeats: the 67 and 503 Kb sub-genomic circles,
2) 3 Kb repeats: the 250 and 253 Kb sub-genomic circles.

The identification of sub-genomic circular molecules has led to the prediction that the maize mitochondrial genome has a complex multipartite structure which arises from homologous recombination across repeated DNA sequences (Lonsdale et al., 1984).

Figure 1.2:
The Maize Mitochondrial Genome Map
Adapted from Dawson et al., 1986b

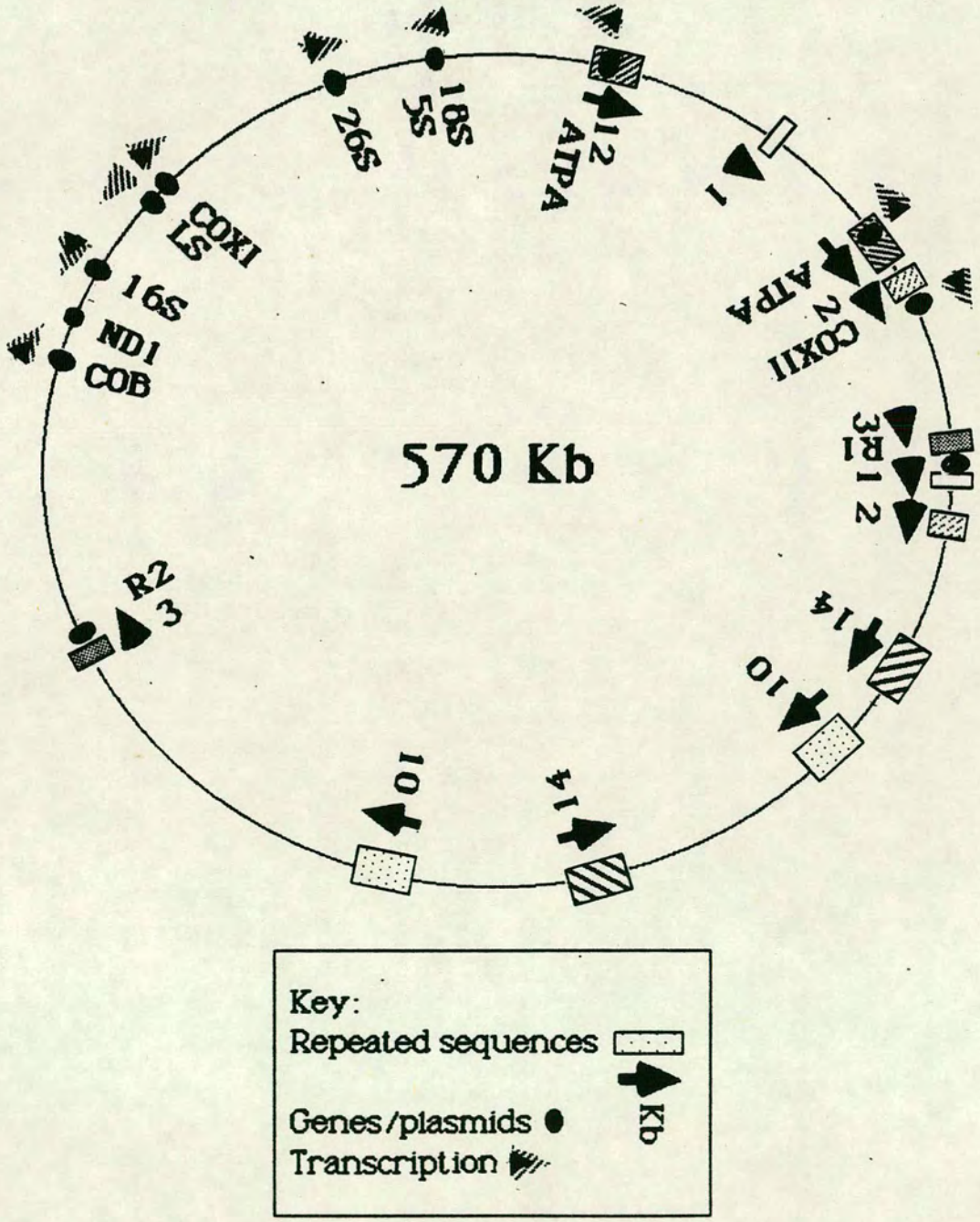


Figure 1.3

Cartoon of the Enzyme Complexes of the Inner Mitochondrial Membrane

Mitochondrial encoded subunits of the inner membrane enzyme complexes of higher plants have been determined by identification of *in organello* synthesized mitochondrial polypeptides and DNA sequence analysis of mitochondrial genes. This is a schematic representation of the protein subunits of the inner mitochondrial membrane, the known gene products of higher plant mitochondria are represented in black.

Complex I: NADH:ubiquinone oxidoreductase
> 20 subunits in fungi (Ise *et al.*, 1985)

Mitochondrial gene products (to date):
Subunits ND1, ND2, ND5

Complex II: Succinate:ubiquinone oxidoreductase
No mitochondrial gene products

Complex III: Ubiquinol:cytochrome *c* oxidoreductase, or cytochrome bc_1 complex
8 - 10 subunits in yeast (Kreike, 1982)

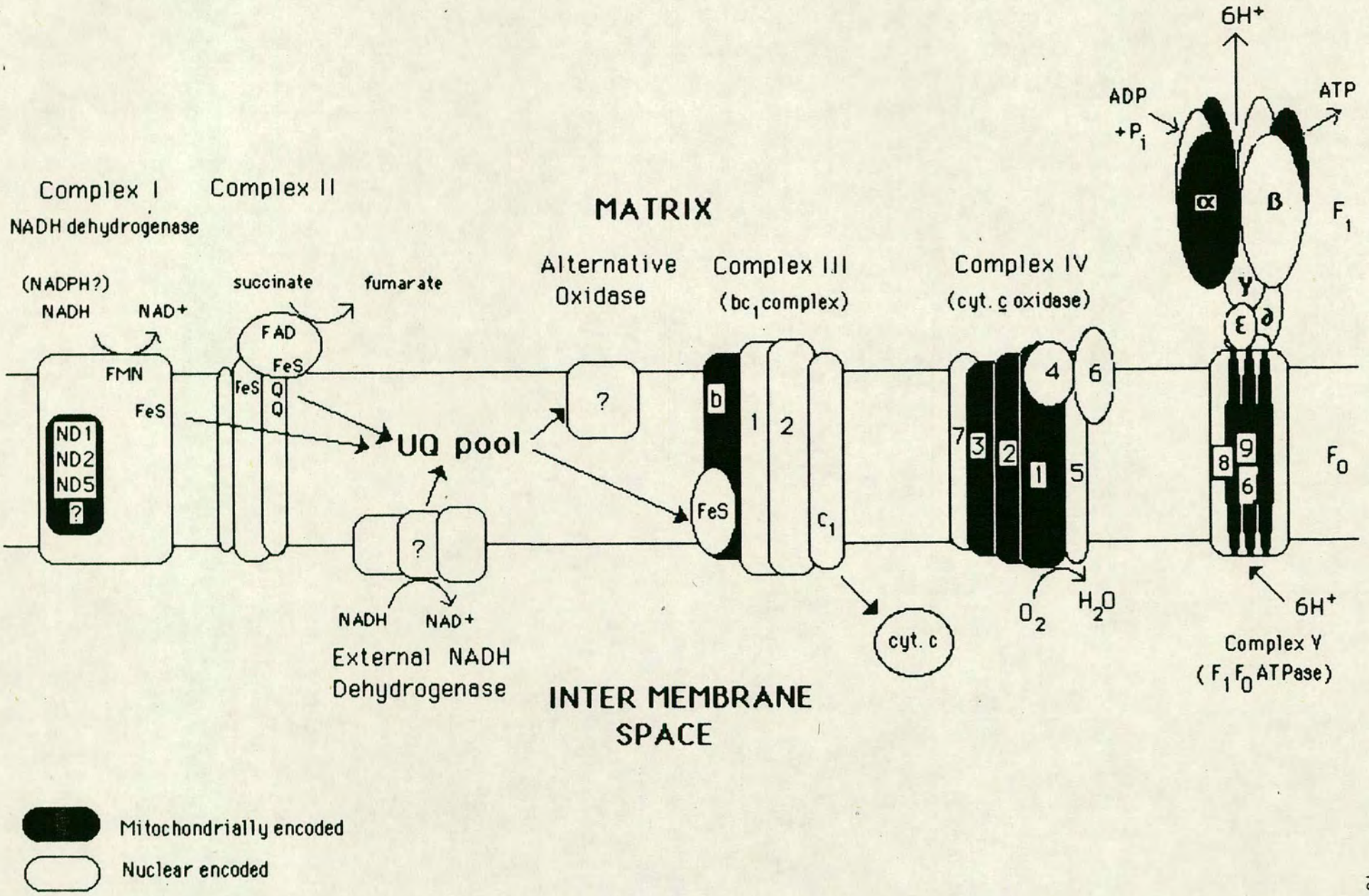
Mitochondrial gene product: Apocytochrome *b*

Complex IV: Cytochrome *c*: O_2 oxidoreductase, or cytochrome *c* oxidase
9 Subunits in yeast (Power *et al.*, 1984)

Mitochondrial gene products: COI, COII and COIII

Complex V: Oligomycin-sensitive ATPase, or
F_1 - F_o ATPase
5 F_1ATPase Subunits: α, β, ,δ and ϵ.
(Hack and Leaver, 1983)
Mitochondrial gene product: α-subunit
(Isaac *et al.*, 1985b)
5 F_oATPase Subunits: ATP5 - 9
Mitochondrial gene products: ATP6, ATP8(?), ATP9

Alternate Oxidase and External Dehydrogenase:
Cyanide insensitive oxidase activity has been observed in higher plant mitochondria (Solomos, 1977). Evidence for an external dehydrogenase has also been put forth (Palmer, 1976).


6H+
ADP
+Pi
ATP
Complex I
NADH dehydrogenase
Complex II
MATRIX
Alternative
Oxidase
Complex III
(bc1 complex)
Complex IV
(cyt. c oxidase)
F1
(NADPH?)
NADH
NAD+
succinate
fumarate
FAD
FeS
FMN
FeS
ND1
ND2
ND5
?
feS
Q
Q
UQ pool
?
?
b
1
2
FeS
c1
7
3
2
4
6
1
5
α
β
γ
ε
∂
9
8
6
F0
NADH
NAD+
External NADH
Dehydrogenase
cyt. c
O2
H2O
6H+
Complex V
(F1F0 ATPase)
INTER MEMBRANE
SPACE
Mitochondrially encoded
Nuclear encoded

and 26S rRNA are transcribed from one strand and the 5S -18S rRNAs cistron and ATPA are transcribed from the opposite strand (Dawson et al., 1986b) (Figure 1.2). An additional feature of higher plant mtDNA is that repeated mtDNA sequences which contain partial or complete copies of rRNA, tRNA and protein genes may be present. In wheat, the 18S and 5S rRNA and 26S rRNA genes are located within repeated mtDNA sequences (Falconet et al., 1984; Falconet et al., 1985). Hybridization studies indicate that at least a portion of the 26S rRNA gene is repeated in spinach, pokeweed and Oenothera (Stern and Palmer, 1984; Stern and Newton, 1984; Manna and Brennicke, 1986). In comparison, the rRNA genes of maize are not repeated (Stern and Lonsdale, 1982; Iams and Sinclair, 1982). A portion of the COXII ORF is repeated in wheat and not in maize (Bonen et al., 1984), but surprisingly 122 bp of the ATP6 gene ORF is also located just 5' to the COXII ORF in maize (Dewey et al., 1986). In fertile N maize the ATPA gene is present in two genomic locations, but as a single copy in sunflower and artichoke (Isaac et al., 1985b; P.G. Isaac, personal communication). Thus, it seems possible that these and other short repeated mtDNA sequences may contribute to the large plant mitochondrial genome size, and may have escaped detection during reassociation kinetic analyses (Bonen et al., 1984).

Mitochondrial Ribosomes, rRNA Genes and Ribosomal Proteins: Mitochondrial ribosomes: Mitoribosomes vary in size from 80S in Tetrahymena to 55 - 60S in animals and may even be smaller in Typanosomes (Gray, 1982; Tzagoloff, 1982). The mitochondrial ribosomes of higher plants sediment at 77 - 78S and can be dissociated into large and small subunits which include a 26S, 18S and 5S rRNA, a 4S RNA and many ribosomal proteins (Leaver and Harmey, 1976).

rRNA genes: Mitochondrial rRNA subunits range in size from 12S and 9S in Trypanosoma and Crithidia to 26S and 18S in higher plants. The molecular weight of plant

mitochondrial rRNAs varies between species (Leaver and Harmey, 1976). The large and small rRNA genes of animals and N.crassa are linked and probably co-transcribed. In contrast, the rRNA genes of yeast, A.nidulans and higher plants are separated. The wheat 18S rRNA gene shares 94% DNA sequence homology with the E.coli large rRNA, which supports a possible prokaryotic (eubacterial) origin for mitochondria (Gray, 1982; Gray and Doolittle, 1982).

In contrast to other eukaryotes examined, higher plant mitochondria encode a 5S rRNA which is not associated with the ribosomes of other mitochondria (Leaver and Harmey, 1976; Spencer et al., 1981). The 5S and 18S rRNA genes are linked in all higher plants examined to date (references: Table 1.4).

Ribosomal Proteins: Mitoribosomal proteins are numerous (>85 mitoribosomal proteins in mammals) and are nuclear encoded with a few exceptions (Scheiber and O'Brien, 1983). 1) A yeast mitochondrial gene encodes (Var1) a ribosomal protein (Var1) (Terpstra et al., 1979; Hudspeth et al., 1982). A product of maize mitochondrial protein synthesis is associated with mitoribosomes and may be homologous to Var1 (Leaver et al., 1982). 2) A mitochondrial encoded ribosomal protein, not homologous to Var1, called S5 has been identified in N.crassa (Breitenberger and Raj Bhandary, 1985).

Mitochondrial tRNAs, tRNA Synthesis Locus and Codon Usage: tRNAs: The number, genomic location and structure of the tRNAs encoded by different organisms is variable. The tRNA genes are clustered in yeast, N.crassa and A.nidulans and dispersed in animals and higher plants (Sederoff, 1984; Dujon, 1983; Breitenberger et al., 1984; Bonen and Gray, 1980; D. Lonsdale, personal communication). Saccharomyces mtDNA encodes 25 tRNAs which are mostly grouped in one quarter of the mitochondrial genome (Tabak et al., 1983a). In contrast, the tRNA genes of S.pombe are located mainly between protein coding genes (Lang et al., 1983). Maize mtDNA contains approximately 30 tRNA

genes, some of which may be duplicated (eg. tRNAs associated with DNA repeats (D. Lonsdale, personal communication)). Maize mtDNA also contains at least one chloroplast tRNA gene ($tRNA^{his}$) but this gene may not encode a functional tRNA (Iams et al., 1985).

tRNA synthesis locus: A gene required for tRNA maturation present in the yeast mitochondrial genome is probably nuclear encoded in animals (Millar et al., 1983; Attardi, personal communication). The tRNA synthesis locus encodes a 9S RNA which has a similar secondary structure to the RNA component of E.coli RNase P.

Codon usage: Mitochondrial codon usage differs from the 'universal' genetic code of nuclear and chloroplast DNA (for review, Grivell, 1983). The codon UGA encodes tryptophan in all mitochondrial systems examined except higher plants. This 'universal' stop codon is used as such in the COB gene of Oenothera and the ATPA gene of maize (Schuster and Brennicke, 1985; Isaac et al., 1985b). In higher plants the codon CGG encodes tryptophan and not arginine as in other mitochondrial systems. Variations in the 'universal' codon assignment observed in mitochondria are summarized in Table 1.4.

Table 1.4
Mitochondrial Variations from the 'Universal' Genetic Code

CODON(S)	UNIVERSAL	MITOCHONDRIAL	ORGANISM(S)
CGG	arginine	tryptophan	higher plants S.cerevisiae (?) N.crassa (?)
AGA	arginine	serine	Drosophila
AGA/G	arginine	stop	mammals
CUN	leucine	threonine	S.cerevisiae
AUA	isoleucine	methionine	mammals, Drosophila, S.cerevisiae
UGA	stop	tryptophan	mammals, Drosophila, protozoa S.cerevisiae, N.crassa

(References: Breitenberger and RajBhandary, 1985 and references therein).

Complex I: Complex I is NADH-ubiquinone oxidoreductase (NADH dehydrogenase), a multisubunit complex of the inner mitochondrial membrane (Figure 1.3). The mitochondrial genome of mammals and *Drosophila* encodes six subunits of the NADH dehydrogenase which are apparently not synthesized by yeast mitochondria (Chomyn *et al.*, 1985; Clary and Wolstenholme, 1984; Grivell, 1983). These subunits are encoded by six of the seven previously unidentified ORFs of mammalian mtDNA (URF1 = ND1; URF2 = ND2; URF3 = ND3; URF4 = ND4; URF4L = ND4L; URF5 = ND5). *N.crassa* mitochondria synthesize six polypeptides which co-purify with NADH-dehydrogenase (Ise *et al.*, 1985) and mitochondrial genes encoding at least three of these subunits (ND1, ND2 and ND5) have been identified (Burger and Werner, 1983; Macino, G. and Nelson, M., personal communication). *A.nidulans* mtDNA encodes genes homologous to human ND1, ND3, ND4 and ND5 (Brown *et al.*, 1985), *Chlamydomonas* mtDNA encodes ND2 and ND5 (Boer *et al.*, 1985), and sequences homologous to ND1, ND2 and ND5 have been detected in higher plant mtDNA (Scazzocchio *et al.*, 1983; Walbot *et al.*, 1983; L. Bonen, personal communication).

Complex III: The apocytochrome *b* protein is the only subunit of Complex III encoded by the mitochondrial genome (Table 1.3; Figure 1.3). The yeast apocytochrome *b* gene (COB) is interrupted by 2 to 5 optional introns in the "short" and "long" forms, respectively (Labouesse and Slominski, 1983). Introns Ib2, Ib3 and Ib4 of the long form of COB contain an URF which is contiguous with the ORF of the preceding exon and encodes a protein necessary for intron splicing (RNA maturase) (MacKeller *et al.*, 1983; Jacq *et al.*, 1984). In *N.crassa* the COB gene is interrupted at sites different from those in yeast and *Aspergillus*, but the second intron of the *N.crassa* COB gene also encodes a protein which may also be a RNA maturase (Burke *et al.*, 1984).

Complex IV: The three largest and most hydrophobic subunits of cytochrome c oxidase (Complex IV) are encoded by the mtDNA of all organisms so far examined (Table 1.3; Figure 1.3), with the exception of COII and COIII in Chlamydomonas reinhardtii (Boer et al., 1985). The genes encoding COI, COII and COIII (which will be referred to as COXI, COXII, and COXIII, respectively) contain no intervening sequences in animals or N.crassa (Anderson et al., 1981; Burger et al., 1982).

COXI is interrupted in S.cerevisiae (oxi3), S.pombe (COX3), Podospora (COI), A.nidulans (oxiA) and N.intermedia (CoI), but not in N.crassa (CoI) (Bonitz et al., 1980; Lang, 1984; Kuck et al., 1985; Waring et al., 1984; Collins and Lambowitz, 1983; Burger et al., 1982). In the laboratory yeast strain D272-10B, COXI contains seven or eight exons and eight or nine introns. The first four introns (aI1, aI2, aI3 and aI4) contain an URF which is in the same reading frame as the preceding exon. These intron URFs may encode RNA maturases required for the excision of intervening sequences (Groudinsky et al., 1983). Despite the overall variation in COXI structure, the amino acid sequence of COI is relatively well conserved except at its carboxy terminus. Due to variations at the 3' terminus of COXI the predicted molecular weight of COI ranges from 56K in S.cerevisiae (510 amino acids) to 61K in N.crassa (555 amino acids) (Wikstrom et al., 1985; Isaac et al., 1985).

COXII is not interrupted in the animals, fungi or dicotyledonous plants examined to date, but contains a centrally located intron in monocotyledonous plants. The COXII gene of maize, wheat and rice is interrupted by a Class II intron of 793, 1216 and 1265 bp, respectively (Table 1.5). The variable size of the COXII intron is due to an inserted or deleted sequence within the intron (Bonen et al., 1984; Kao et al., 1984). The predicted molecular weight of COII ranges from 25.5 K to 30.8 K (227 to 260 amino acids) in mammals and maize, respectively (Fox and Leaver, 1982; Anderson et al., 1982; Wikstrom et

al, 1985 and references therein).

COXIII of animals and fungi is contiguous and encodes a polypeptide with a predicted molecular weight of 30K (261 - 271 amino acids) (Anderson et al., 1982; Thalenfeld and Tzagoloff, 1980). A mitochondrial gene in Oenothera which shares homology with yeast COXIII has been identified (A. Brennicke, personal communication).

Complex V: The F_1 and F_o portions of ATPase (Complex V) contains subunits that are either mitochondrial or nuclear encoded (Figure 1.3, Table 1.3).

F_1ATPase: The F_1 portion of ATPase contains five protein subunits which are synthesized on cytosolic ribosomes in all organisms examined to date with the exception of the α-subunit of F_1ATPase in higher plants and the unicellular heterotroph Prototheca zopfii (Neupert and Schatz, 1981; Boutry et al., 1983; Hack and Leaver, 1983; Deters and Ewing, 1985). The mitochondrial gene encoding the α-subunit has been sequenced in maize (Isaac et al., 1985b; Braun and Levings, 1985).

F_oATPase: The cellular location of the genes encoding three subunits of F_oATPase (ATP6, ATP8, ATP9) is variable. The gene encoding subunit 6 of F_o (ATP6) is a mitochondrial gene of fungi, protozoa, animals and maize (Grivell, 1983; Dewey et al., 1986). The gene encoding subunit 8 of F_o (ATP8) is a mitochondrial gene in all organisms examined to date, but has only been tentatively identified in a higher plant (Oenothera: Hiesel and Brennicke, 1985). The gene encoding subunit 9 of F_o (ATP9) is present in the mitochondrial genome of fungi and maize (Grivell, 1983; Dewey et al., 1985). In N.crassa and A.nidulans this gene is apparently not expressed but, as in animals is encoded by a nuclear gene, synthesized on cytosolic ribosomes and imported into mitochondria (Anderson et al., 1981; van den Boogaert et al., 1982; Scazzocchio et al., 1983).

Additional Protein Coding Genes which are not homologous to other mitochondrial genes have been identified in fungi and plant mtDNA. These include:

1) Intron encoded proteins of fungal COB, COXI and large rRNA genes. a) Certain introns of COB and COXI contain an ORF which may encode a mRNA maturase (Burke et al., 1984; Jacq et al., 1984; Groudinsky et al., 1983). b) The intron of the N.crassa 24S rRNA gene may encode the S5 ribosomal protein (Breitenberger and RajBhandary,1985). c) The 21S rRNA intron of S.cerevisiae encodes a protein involved in transposition of the intron (Zinn and Butow, 1985; Macreadie et al., 1985; Jacquier and Dujon, 1985).

2) URFa of S.pombe (Lang, 1984).

3) Two URFs, encoded by N.crassa mtDNA (URF N and U).

4) Six URFs in A.nidulans (Brown et al., 1985).

5) An ORF encoding a circa 13K polypeptide in CMS T maize which is not present in fertile N maize (Dewey et al., in press).

1.3 B Genes and Gene Products of Higher Plant Mitochondria

Plant mitochondrial genes have been mainly identified by hybridization with [^{32}P]-labelled gene probes from fungal and animal mitochondria (Dawson et al., 1986a). A number of plant mitochondrial rRNA, tRNA and protein genes have been sequenced (Table 1.5). The DNA sequences of protein coding genes so far examined share 40 - 60% sequence homology to the corresponding mitochondrial or nuclear gene of fungi and animals (however the tentative ATP8 gene of Oenothera shares only 28% homology), and in comparison to animals and fungi, appear to be more highly conserved between species. The isolation of plant mitochondrial genes has been useful in the study of mitochondrial genome organization, expression, evolution and plant development (Leaver et al., 1983a; Gray, 1982; Dawson et al,. 1986b). Gene probes have been used to examine variations in mitochondrial genome structure and transcription in CMS lines of maize and sorghum (Isaac et al., 1985; See Chapters IV, and VI).

Table 1.5
rRNA, tRNA and Protein Coding Genes Isolated from Plant Mitochondria

GENE	PLANT	LENGTH (amino acids)		REFERENCE
18S + 5S rRNA	maize			Chao et al., 1983; Chao et al., 1984
	wheat			Bonen et al., 1977; Spencer et al.,1981; Gray and Spencer, 1983; Bonen and Gray, 1980
	soybean			Morgens et al., 1984
	Oenothera			Brennicke et al., 1985
26SrRNA	maize			Dale et al., 1984
	wheat			Gray, personal communication
	Oenothera			Manna and Brennicke, 1985
$tRNA_f^{met}$	wheat			Spencer et al., 1984
	Oenothera			Gottschalk and Brennicke,1985
$tRNA^{trp}$	maize			Parks et al., 1985
cp $tRNA^{his}$	maize			Iams et al., 1985
ND1	maize			Scazzocchio et al.,1983
	wheat			L. Bonen, pers. comm.
ND2	maize			Walbot et al., 1983
ND5	wheat			L. Bonen, pers. comm.
COB	maize	388		Dawson et al., 1983
	Oenothera	395		Schuster and Brennicke,1985
	wheat	398		Boer et al., 1985b
COXI	maize	528	X	Isaac et al., 1985
	(Chlamydomonas			Boer et al., in press)
COXII	maize	260	X	Fox and Leaver, 1981
	wheat	260		Bonen et al., 1984
	Oenothera	259		Hiesel and Brennicke, 1983
	rice	259		Kao et al., 1984
	pea	259		Moon et al., 1985
ATPA	maize	508	X	Isaac et al., 1985b; Braun and Levings, 1985
ATP6	maize	291		Dewey et al., in press
ATP8 (?)	Oenothera	58		Hiesel and Brennicke, 1985
ATP9	maize	74	X	Dewey et al., 1985

X A mitochondrial translation product encoded by this gene has been tentatively identified.

Proteins synthesized by isolated mitochondria have been identified by immunological and biochemical techniques (Hack and Leaver, 1983; Hack and Leaver, 1984; Forde and Leaver, 1980). Mitochondria, isolated from seedlings and other tissues, synthesize polypeptides from accumulated mRNA when placed in a suitable medium (Forde *et al.*, 1978). Eighteen to twenty polypeptides can be distinguished by SDS-polyacrylamide gel electrophoresis of mitochondrial translation products, of which five have been tentatively identified. Cytochrome *c* oxidase subunit I (COI), subunit II (COII) and the α-subunit of F_1ATPase have been immunoprecipitated from [^{35}S]-labelled maize mitochondrial translation products with antisera against the homologous yeast protein (Forde and Leaver, 1980; Hack and Leaver, 1983). The α-subunit of F_1ATPase has also been identified as a mitochondrial translation product in *Vicia faba* (Boutry *et al.*, 1983). This contrasts with the situation in other organisms where this subunit is encoded by the nuclear genome, synthesized as a precursor on cytosolic ribosomes and imported into mitochondria (Yaffe and Schatz, 1984). In addition, a maize mitochondrial translation product with an estimated molecular weight of 8,000, which is soluble in organic solvents and specifically binds ^{14}C-DCCD, has been tentatively identified as F_o ATPase Subunit 9 (DCCD binding protein) which is encoded by *ATP9* (Hack and Leaver, 1984; Dewey *et al.*, 1985). A soluble ca. 40,000 dalton mitochondrial translation product which co-purifies with mitochondrial ribosomes may be equivalent to the yeast ribosomal Var1 protein has been identified (Leaver *et al.*, 1982), but a gene encoding this protein has not been reported.

The identification of other proteins synthesized by plant mitochondria (eg. proteins encoded by identified genes (Table 1.5)) awaits the development of linked transcription-translation system and/or the purification of plant mitochondrial enzyme complexes and the preparation of specific antibodies.

1.4 Cellular Location of Genes Encoding Mitochondrial Proteins

It is evident from the previous discussion that the cellular location and site of expression of genes which encode mitochondrial proteins varies amongst organisms (Table 1.3). Whether these variations in mitochondrial genome content have resulted from progressive movement of mitochondrial genes to nuclear chromosomes or vica versa has not been determined. The endosymbiont hypothesis predicts that the proto-mitochondrial genome has been reduced by the transfer of genes to the nucleus (for review: Gray and Doolittle, 1982). Indeed, a large number of sequences homologous to mtDNA and cpDNA are present in nuclear DNA (for review: Fox, 1983; Whisson and Scott, 1985). It has recently been suggested that transfer of mtDNA to the nuclear chromosomes occurs during senescence in Podospora (Wright and Cummings, 1983; Kück et al., 1985). If mitochondrial genes are indeed transferred to the nucleus then sequence alterations may be required before expression can occur since the mitochondrial genetic code is not identical to the 'universal' genetic code.

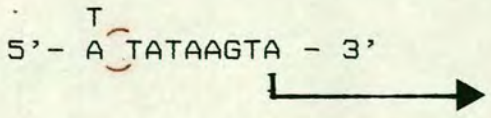
An additional striking observation is that numerous DNA sequences homologous to chloroplast genes are located on the maize master chromosome (Figure 1.2) and within 'plasmid-like' mtDNA molecules of numerous higher plants, and may contribute to the large sequence complexity (For review: Ellis, 1982; see also: Stern and Lonsdale, 1982; Lonsdale et al., 1983; Kemble et al., 1983; Stern and Palmer, 1983; Whisson and Scott, 1985; Iams et al., 1985). To date, no evidence of transcription of cpDNA sequences in mtDNA has been presented and the mechanisms responsible for interorganellar genetic transfer are not known.

1.5 Transcription of Mitochondrial Genes

Mitochondrial genome organization and structure plays an important role in the mode of gene expression. The distinctive features of mtDNA transcription in mammals and fungi have been reviewed by Attardi et al. (1983), Hixon and Clayton (1985) and Tabak et al. (1983a). Relatively little is known about higher plant mtDNA transcription. Since the genome size and organization of higher plants resembles that of fungi, a review of transcription and transcript processing in fungal mitochondria and what is known about transcription in plant mitochondria is presented here.

1.5 A Transcription of Fungal MtDNA

Transcript Initiation: The mitochondrial genes of fungi examined to date are encoded by the same DNA strand (de Zamaroczy and Bernardi, 1985; Breitenberger and RajBhandary, 1985). Transcription of mitochondrial genes occurs at a number of genomic locations and the transcripts are large and often polycistronic. In S.cerevisiae, 19 sites of transcript initiation have been identified to date by labelling the 5'-terminal diphosphate of primary transcripts with γ-[^{32}P]GTP and guanylyltransferase (Edwards et al., 1983; Christianson and Rabinowitz, 1983; Tabak et al., 1983b). DNA sequence analysis and S_1 transcript mapping studies around sites of transcript initiation in S.cerevisiae, Torulopsis glabrata and Kluyveromyces lactis has revealed that transcription initiates at the 3' terminal A of a nonanucleotide consensus sequence:



(Tabak et al., 1983a; Clark Walker et al., 1985; Osinga et al., 1982). Homology to this consensus sequence has also been identified near to the 5' terminus of the putative primary transcript of COXI from N.crassa and maize

(de Vries et al., 1983; Isaac et al., 1985). Site directed mutagenesis within and around this nonanucleotide box and in vitro transcription analyses have determined that the box and surrounding sequence constitutes a promoter in yeast mtDNA (Osinga et al., 1984a).

Transcript Processing: The maturation of the large polycistronic primary transcripts of fungi requires processing of 5' and 3' non-coding sequences and the removal (splicing) of intervening sequences (Grivell, 1983). Since primary transcripts are in low abundance and numerous gene specific transcripts are present, it has been suggested that transcript processing is a rapid, multi-step procedure. In S.pombe and N.crassa, as in animals, tRNA genes appear to punctuate genes and function as transcript processing sites (Lang, 1984; Breitenberger and RajBhandary, 1985). In yeast however, transcript processing is associated primarily with a conserved dodecanucleotide consensus sequence (5'- AAUAAUAUUCUU - 3') (Osinga et al., 1984b) and tRNA genes play only a minor role in RNA processing.

Intron Splicing: The precise excision (splicing) of mitochondrial introns from RNA is a complex process and may require:

1) intron and/or nuclear encoded subunits of an RNA maturase,
2) formation of complex 'core' structure which brings adjacent exons into close proximity,
3) mitochondrially encoded proteins from a different loci (eg. In S.cerevisiae the gene product of COB bI4 is necessary for splicing intron aI4 of COXI (Netter et al., 1982; Groudinsky et al., 1981).

Mitochondrial introns can be placed into two groups (I and II) based on computer predictions of potential RNA secondary structure formation (Michel et al., 1982; Michel and Dujon, 1983) and URF amino acid sequence (Hensgens et al., 1983a). Group I introns may contain an URF which is contiguous with the upstream exon, and share substantial 3' sequence homology. Genetic and molecular evidence

indicates that splicing of Group I introns *in vivo* requires the URF gene product and/or specific nuclear gene products. Since certain Group I introns can self-splice *in vitro* it is possible that the putative intron encoded maturases assist in the formation of a stable core structure *in vivo* (van der Horst and Tabak, 1985). Group II introns are found in yeast and in the *COX*II gene of maize, wheat and rice. In yeast these introns form covalently closed circular RNA molecules upon excision (Michel *et al.*, 1982, Hensgens *et al.*, 1983b; Arnberg *et al.*, 1980).

The origin and function of mitochondrial introns is unknown. Because introns share structural and sequence homology is has been suggested that they may have a common origin (Waring *et al.*, 1984; Lang, 1984; Hensgens *et al.*, 1983a). In support of this, a mtDNA plasmid-like molecule in *Neurospora* (kalDNA) which integrates into the mitochondrial chromosome during senescence and has similarities to transposable elements and introns has been identified (Bertrand *et al.*, 1984). As for their function, introns may be'selfish DNA' which has integrated into mitochondrial genes or additional regulators of gene expression (Hensgens *et al.*, 1983a; Nargang *et al.*, 1984).

1.5 B Transcription of Higher Plant MtDNA

Plant mitochondrial gene transcription has not yet been studied in detail. Northern hybridization analysis has revealed that the transcript pattern complexity varies between genes and amongst species. For example, transcription of maize *COX*II and *COB* is complex and involves multiple transcripts which may be generated by transcript processing events (Fox and Leaver, 1981; Dawson *et al.*, 1983), whereas transcription of pea and *Oenothera* *COX*II involves one or two transcripts (Moon *et al.*, 1985; Hiesel and Brennicke, 1983, 1985). In maize, transcription of *COX*I involves two gene specific transcripts. The 5' termini of these have been determined by S_1 nuclease transcript mapping analysis. The 5' terminus of

the larger transcript maps to a sequence that shares homology with the yeast nonanucleotide promoter sequence (Isaac et al., 1985), as do the 5' termini of the two pea COXII transcripts (Moon et al., 1985). To date, little is known about mtRNA processing in higher plants since only the 3'.transcript terminus of pea COXII has been mapped by S_1-nuclease transcript analysis (Moon et al., 1985). Since the wheat $tRNA_f^{met}$ and 18S rRNA genes are separated by a single basepair, Spencer et al (1984) have suggested that the tRNA gene may function as a RNA processing signal as in mammalian mitochondria. In wheat, a gene cluster which may represent a single transcription unit including the 18S rRNA, $tRNAf^{met}$ and 5S rRNA genes has been identified (Spencer et al., 1984). It remains to be determined if plant mitochondrial transcripts are synthesized as polycistronic messages, as in fungi.

On the basis of DNA-RNA hybridization studies it has been shown that the amount of mtRNA species present in seedling mitochondria is proportional to the genome size (Stern and Newton, 1985; Bendich, 1985). Since the relative number of mitochondrial translation products appears to be constant (18 - 20 polypeptides are resolved by SDS-polyacrylamide gel electrophoresis), this observation has led to the tenuous prediction that most of the higher plant mtDNA is transcribed and that gene expression is controlled at a post-transcriptional level.

1.6 Nuclear-Cytoplasmic Interactions Associated with Mitochondrial Biogenesis and Function

The study of mitochondrial genome organization and expression has generated a wealth of evidence that mitochondrial biogenesis and function requires the expression of both nuclear and mitochondrial genes. In addition to genes which encode structural mitochondrial proteins and metabolic factors, evidence of numerous nuclear gene products which regulate mitochondrial gene expression has been obtained. These include factors required for DNA

replication, transcription, post-transcriptional processing, intron splicing, tRNA modification and translation (Dujon, 1981; Tzagoloff, 1982). In addition, nuclear gene mutations which suppress mitochondrial (mit^-) mutations have been identified (Groudinsky et al., 1981). To complete this review of the organellar contribution to mitochondrial biogenesis it is necessary to mention the nuclear genes which control mitochondrial gene expression and function. Although such nuclear genes have only been identified in fungi to date, evidence of specific nuclear cytoplasmic interactions have also been observed in higher plants. In particular, nuclear cytoplasmic interactions associated with expression of the cytoplasmic male sterile (CMS) phenotype in maize and sorghum will be discussed.

1.6 A Nuclear Genes Which Regulate Mitochondrial Gene Expression in Yeast

The ability of respiratory deficient yeast to grow by fermentation has allowed the identification of numerous nuclear and mitochondrial genes which are necessary for mitochondrial biogenesis and function (For review: Dujon, 1981, 1983). Nuclear mutations, known as pet^- mutations, which affect mitochondrial respiration have been grouped by genetic complementation analysis and according to their specific effect on mitochondrial protein synthesis. A large number of pet^- mutants of yeast have been examined by genetic crosses and have established at least two-hundred different complementation groups (Michaelis et al., 1982; Dieckmann et al., 1982, Pillar et al., 1983; Myers et al., 1985). Analysis of mitochondrial protein synthesis by pet^- strains has revealed that mitochondrial protein synthesis in these mutants can be absent, normal or deficient in one or few mitochondrial translation products. The examination of pet^- mutants has identified nuclear genes required for normal mitochondrial function. These genes include subunits of mitochondrial enzyme complexes (eg. COXV (Clumsky et al., 1983)),

metabolic factors (eg. haem and cytochrome c (Gaurente and Mason, 1983)), proteins required for maintenance of the mitochondrial genome (Myers et al., 1985) and gene expression. Three examples of nuclear gene products which are necessary for mitochondrial gene expression are reviewed below.

1) **Nuclear gene products required for** translation and stability of mRNA: The translation of COXIII in yeast is dependent upon the expression of the nuclear gene termed PET494. Pet$^-$494 yeast accumulate COXIII mRNA and fail to synthesize COIII. This nuclear mutation apparently effects the normal translation of the COXIII pre-mRNA (Müller et al., 1984). A surprising finding is in mitochondrial mutants which suppress the pet$^-$494 mutation, mitochondrial genome rearrangements which position the 5' untranslated leader sequence of the ATP8 (aap1) or ATP9 (oli1) gene upstream of COXIII permit the normal expression of COXIII in pet$^-$494 yeast (Costanza and Fox, 1985). Thus, PET494 gene appears to encode a mitochondrial protein which interacts with the 5' untranslated leader of the COXIII transcript. Similarly, a nuclear gene which promotes the processing of COB pre-mRNA has been identified through the analysis of the pet$^-$ mutant MK2. COB pre-mRNA accumulates in pet$^-$ MK2 yeast. In a mitochondrial mutant which suppresses this nuclear mutation a recombination event has replaced the 5' leader sequence of COB with the leader of the ATP9 gene. Thus, the deficient nuclear gene product in pet$^-$ MK2 probably interacts with the 5' untranslated leader sequence of COB pre-mRNA (Rödel et al., 1985). A second nuclear gene (CBP6) is required for translation of COB mRNA has been identified (Dieckmann and Tzagoloff, 1985).

2) Nuclear gene products required for mRNA processing: Nuclear genes which appear to be necessary for processing of the 5' untranslated leader sequence or splicing of mitochondrial introns have been identified. The nuclear gene product of CBP1 is required for proper 5' end processing of the COB transcript (Dieckmann et al., 1984) and the product of MSS51 is required for processing of COXI pre-mRNA (Simon and Faye, 1984). Two nuclear genes, CBP2 and MIP1 appear to be required for the correct splicing of introns bI5 and bI3 of the 'long' form of COB, respectively (Hill et al., 1985; Pillar et al., 1983; Kreike et al., 1985).

3) A nuclear gene required for postranslational processing: A nuclear mutation in the temperature sensitive mutant ts2858 results in the accumulation of a precursor of COII with an additional 15 amino acids at its amino terminus. It has been suggested that the defective nuclear gene is associated with the proteolytic cleavage of the COII precursor (Pratje et al., 1983).

From these examples, it is clear that nuclear genes are required for mitochondrial gene expression in fungi. An indication of the specificity of the nuclear regulation of mitochondrial gene expression has been obtained by examining the effect of a foreign nucleus on mitochondrial function. Slonimski and co-workers, have found that when the Saccharomyces douglasii nucleus is placed into the S.cerevisiae cytoplasm by a heterokaryon cross (Jinks, 1964) the resulting yeast are normal, whereas the reciprocal cross produces respiratory deficient yeast. Yeast with the genotype S.cerevisiae nucleus in S.douglasii cytoplasm accumulate pre-mRNA transcripts of COXI, probably because of a failure in RNA processing. Perhaps nuclear factor(s) required for S.douglasii mitochondrial gene expression are not expressed by the S.cerevisiae nucleus (P. Slonimski, personal communication).

1.6 B Mutations Affecting Organelle Biogenesis and Function in Higher Plants

Mutations which affect mitochondrial or chloroplast function and show non-mendelian (maternal) or Mendelian inheritance have been described (for review: Grun, 1976). These include mutations affecting plastid development (eg. the nuclear mutation, iojap (Walbot and Coe, 1979)), or associated with altered mitochondrial protein synthesis (eg. the cytoplasmic mutation, non-chromosomal stripe (Newton and Coe, 1984)). The most widespread cytoplasmic mutation of higher plants is cytoplasmic male sterility. This is the maternally inherited failure to produce and release functional pollen and has been described in at least 170 plant species (Laser and Lersten, 1972). Cytoplasmic male sterile (CMS) lines have been reported in natural populations (Duvick, 1965; Edwardson, 1970) and generated through interspecific crosses in which a nucleus of one race or species is placed into a foreign cytoplasm by backcrossing. Thus, the CMS phenotype appears to be caused by nuclear-cytoplasmic incompatibility which results in abnormal pollen development.

The CMS phenotype has been used extensively in the commercial production of F_1 hybrid seed of maize, sorghum, sugarbeet, Brassica and sunflower. In hybrid production, CMS lines are used as the female parent and crossed with a male which carries nuclear genes which restore pollen fertility (termed Rf alleles in maize and Ms alleles in sorghum) (Figure 1.4). Nuclear genes which restore pollen fertility are normally present in the natural nucleus of a given cytoplasm and sometimes in unrelated nuclei. Since nuclear genes restore fertility, the CMS phenomenon may be analogous to the types of mutations which affect nuclear-mitochondrial interactions in fungi.

An abundance of circumstantial evidence suggests that the mitochondrial genome, and not the chloroplast genome, is the site of the mutation(s) which lead to the expression of the CMS phenotype in maize and a number of

other plants. CMS cytoplasms can be distinguished by 1) genetic analysis of nuclear alleles which restore male fertility and 2) by molecular analysis of mitochondrial genome organization and expression. In particular, mtDNA restriction endonuclease digestion patterns, the presence (or absence) of 'plasmid-like' mtDNAs and *in organello* synthesis of variant proteins have been used to classify CMS cytoplasms (for review: Leaver and Gray, 1982; Hanson and Conde, 1985).

1.6 C Cytoplasmic Male Sterility in Maize

In maize, three CMS cytoplasms (S (USDA), T (Texas) and C (Charrua)) have been identified on the basis of nuclear genes which restore pollen fertility. These cytoplasms differ from one another and from normal, fertile N cytoplasm maize (for review, Laughnan and Gabay-Laughnan).

CMS-S: Maize containing S cytoplasm is sterile unless the nuclear allele Rf3 is present (Duvick, 1965; Beckett, 1971). The restoration of CMS-S maize is gametophytic since pollen grains containing the rf3 allele abort, whereas those with the Rf3 allele are normal (Laughnan and Gabay, 1974). A particular characteristic of CMS-S lines is frequent spontaneous reversion to fertility which can be due to either nuclear or cytoplasmic mutations (termed nuclear or cytoplasmic revertants) (Singh and Laughnan, 1972).

CMS-T: Maize containing T cytoplasm develops normal pollen when two nuclear restorer alleles are present (Rf1, Rf2) (Duvick *et al.*, 1961). Restoration of CMS-T differs from that of CMS-S since the genotype of the sporophyte, and not the pollen, appears to determine whether pollen development is normal. An additional characteristic of CMS-T cytoplasm maize is susceptibility to infection by the fungal pathogen *Helminthosporium maydis*, Race T (also known as *Cochliobolis heterostrophis* or *Drechslera maydis*) which produces 'T-toxin' and disrupts mitochondrial

function (Gengenbach et al., 1973; Matthews et al., 1979; Gregory et al., 1980). Evidence suggests that the CMS-T phenotype and T-toxin sensitivity are linked, maternally inherited phenotypes. When CMS-T maize calli were grown on media with or without toxin and subsequently regenerated into plants, some male fertile and toxin resistant regenerants were obtained. In these 'revertants' male fertility and toxin resistance were maternally transmitted, indicating that a cytoplasmic mutation had ocurred (Gengenbach et al., 1977; Gengenbach et al., 1981; Brettell et al., 1980).

CMS-C: Maize containing C cytoplasm is fertile when one or two nuclear genes are present (Laughnan and Gabay-Laughnan, 1983). As in CMS-T, restoration of CMS-C is sporophytic, however, fertile revertants of C cytoplasm maize have not been reported.

Molecular analysis of the mtDNA restriction endonuclease digestion pattern and mitochondrial translation products from fertile N cytoplasms, CMS cytoplasms (S, T or C), nuclear restored CMS lines (termed SRf, TRf or CRf) and cytoplasmic revertants of CMS lines (termed Srev or Trev) has been reported. These data reveal that:

1) The restriction digestion pattern of N cytoplasm mtDNA differs from that of CMS S, T and C cytoplasms (Pring and Levings, 1978).

2) The mtDNA restriction digestion patterns of CMS and nuclear restored CMS lines appear to be identical (Kemble et al., 1980; Levings et al., 1980).

3) The mtDNA restriction digestion patterns of cytoplasmic revertants differ from the original CMS line and N cytoplasm (Kemble et al.,1982; Kemble and Mans,1983).

4) Characteristic variant polypeptides are synthesized by mitochondria isolated from CMS cytoplasms and nuclear restored lines (Forde et al., 1978; Forde and Leaver, 1980; Leaver and Forde, 1980) (Table 1.6).

Table 1.6
Variant Mitochondrial Proteins Associated with CMS Cytoplasms of Maize

CYTOPLASM	PHENOTYPE	VARIANT POLYPEPTIDE(S) (daltons)
N	MF	none
T	CMS	13K present, 21K absent
TRf	MF	13K suppressed, 21K absent
Trev	MF	13K suppressed, 21K ?
S	CMS	8 polypeptides from 42 - 85K
SRf	MF	8 polypeptides from 42 - 85K
Srev	MF	none
C	CMS	17.5K present, 15K absent
CRf	MF	17.5K present, 15K absent

MF- male fertile; rev- cytoplasmic revertant; Rf- nuclear restored

To determine whether the CMS phenotype indeed results from mitochondrial mutations associated with the observed differences in mitochondrial genome organization and expression, these variations have been further examined.

CMS-S cytoplasm maize has a distinct mtDNA restriction pattern, synthesizes 8 variant mitochondrial translation products (Table 1.6) and two double stranded, linear 'plasmid-like' mtDNA species called S1 (6397 bp) and S2 (5453 bp) (Pring and Levings, 1978; Forde and Leaver, 1980; Pring et al., 1977; Kemble and Bedbrook, 1980). Sequence analysis of S1 and S2 has revealed that they have identical 208 bp terminal inverted repeats (TIRs) and share 1462 bp at one end (Levings and Sederoff, 1983; Paillard et al., 1985). Both S1 and S2 have transcribed sequences (ORFs), one of which may encode the protein which is bound to the 5' terminus of these 'plasmid-like' mtDNA molecules and may be involved in

their autonomous replication (Kemble and Thompson, 1982, Levings, personal communication). Sequences homologous to the TIRs of these plasmids are found at a number of positions within the mitochondrial chromosomes of N and CMS-S cytoplasms, but not in CMS-T and CMS-C cytoplasms (Thompson et al., 1980; Lonsdale et al., 1981; Koncz et al., 1980).

Genome mapping of CMS-S cytoplasm mtDNA has revealed that most of the mitochondrial chromosomes of S cytoplasm are linear and possess a copy of S1 or S2 at one end (Schardl et al., 1984). It has been proposed that recombination across the TIRs of S1 or S2 and homologous sequences located within the mtDNA results in the linearization of the normally circular mitochondrial chromosomes. In contrast, it has been found that the spontaneous reversion to fertility (cytoplasmic revertants) is often (but not always) concomitant with the loss of free S1 and S2 and the linear chromosomes characteristic of CMS-S cytoplasm (Levings et al., 1980; Laughnan et al., 1981; Schardl et al., 1985). Although S1 and S2 sequences located within mitochondrial chromosomes are not lost, a portion of the two terminal inverted repeats of integrated S2 and the transcript corresponding to the S2 URF-1 is absent (Schardl et al., 1985).

In relation to what is known about nuclear control of mitochondrial gene expression in fungi, it is of interest to note that nuclear genotype appears to control a number of mitochondrial characteristics in CMS-S cytoplasm maize: 1) **The amount of S1 and S2 'plasmid-like' mtDNAs and the synthesis of variant polypeptides:** S1 and S2 are present in about fivefold greater amounts than the higher molecular weight DNA in most CMS-S lines (Thompson et al., 1980). However, in the line containing S cytoplasm and M825 nucleus, the ratio of S1 to S2 is 5:1, whereas with the nuclear background 38-11 the ratio is 1:3. Laughnan et al., (1981) have demonstrated that when 38-11 nucleus is replaced with M825 by backcrossing, the ratio of S1 to S2 is changed from 1:3 to 5:1. In addition, the change in

nuclear background coincides with specific quantitative and qualitative changes in synthesis of variant mitochondrial polypeptides (A. Liddell et al., in preparation).

2) The genomic location of COXI and ATPA: The genomic location of the COXI and ATPA genes appears to be under nuclear control in S cytoplasm (Isaac et al., 1985; P.G. Isaac, personal communication). In N maize the COXI is present as a single copy, whereas in CMS-S the COXI is present in different genomic locations depending upon the nuclear genotype. Leaver et al. (1985) predict that the multiple genomic locations of COXI in CMS-S cytoplasm may arise from recombination between the TIR of S1 or S2 and a 186 bp sequence located 5' to the COXI gene.

3) The frequency of nuclear and cytoplasmic reversion to fertility: Spontaneous reversion to fertility by CMS-S can be nuclear or cytoplasmic heritable mutation. The frequency of spontaneous nuclear revertants varies from about 1% in WF9 to 11% in M825 nuclear background. The frequency of spontaneous cytoplasmic revertants varies from 17% in WB4 to 94% in M825 (Laughnan and Gabay-Laughnan,1983).

CMS-T: Mitochondria isolated from T cytoplasm do not synthesize a 21,000 (21K) polypeptide observed in other cytoplasms of maize, but synthesize an additional 13,000 dalton (13K) polypeptide. The synthesis of the 13K polypeptide is suppressed in lines which have fertility restorer alleles Rf1 and Rf2 (TRf) (Forde and Leaver, 1980) (Table 1.6). Since no difference in the mtDNA restriction pattern of CMS-T and TRf lines has been found it appears that the synthesis of the 13K polypeptide is suppressed by the TRf nucleus at some level of transcription or translation. In the case of male fertile maize derived by regeneration of CMS-T callus culture (Trev), alterations in mtDNA restriction pattern are coincident with reduced synthesis of the 13K polypeptide.

When synthesis of the 13K polypeptide by plants regenerated from callus culture is >33% the level synthesized by CMS-T lines, toxin sensitivity and the CMS phenotype is observed (Dixon et al., 1982). This observation has led to the suggestion that toxin sensitivity and male sterility may cause (associated) with synthesis of the variant 13 K polypeptide (Forde and Leaver, 1980; Dixon et al., 1982).

Genome mapping studies of the mitochondrial genome in T cytoplasm maize have revealed that the T mitochondrial genome is characterized by specific deletions and rearrangements in comparison to the N mitochondrial genome (C. Fauron, personal communication). Mitochondrial genome rearrangements associated with cytoplasmic reversion to fertility include the loss of a 6.6 Kb XhoI mtDNA restriction fragment which is characteristic of CMS-T mtDNA (Umbeck and Gengenbach, 1983). Recently, this 6.6 Kb XhoI of CMS-T maize has been cloned, and portion which generates a transcript unique to CMS-T has been sequenced. Within this transcribed region an ORF which could produce a 13 K polypeptide has been identified (C.J. Levings personal communication).

In conclusion, the mitochondrial genomes of CMS cytoplasms differ from N cytoplasm maize because of specific genome rearrangements and deletion events which can be correlated with the synthesis of variant mitochondrial polypeptides. When nuclear genes which restore male fertility are present the synthesis of variant polypeptides is reduced (CMS-T) or unaltered (CMS-S and CMS-C). Whereas, in cytoplasmic revertants of both CMS-S and CMS-T the mitochondrial genome has undergone specific alterations and the expression of variant polypeptides is reduced (CMS-T) or absent (CMS-S). Thus, both mitochondrial genome organization and nuclear background appear to play an role in mitochondrial gene expression and the CMS phenotype in maize.

1.6 D Cytoplasmic Male Sterility in Sorghum

Stephens and Holland (1954) produced the first CMS sorghum [Sorghum bicolor (L.) Moench] line by fertilizing an emasculated Milo plant (Milo nucleus in Milo cytoplasm) with pollen from Kafir (Kafir nucleus in Kafir cytoplasm), and crossing the subsequent progeny repeatedly with Kafir until a line with Kafir nucleus in Milo cytoplasm was generated (Figure 1.4). Somewhat surprisingly, the reciprocal cross (Kafir ♀ X Milo ♂) did not yield male sterile progeny. It was subsequently determined that three nuclear genes which suppress male sterility are present in Milo and absent in Kafir. Plants which contain one or more of these dominant alleles ($Ms_{=1}$ (Maunder and Picket, 1959), $Ms_{=2}$ (Erichsen and Ross, 1963), and $Ms_{=3}$) are partially to completely fertile (Quinby and Schertz, 1970). Thus, nuclear-cytoplasmic combinations which display the CMS phenotype lack dominant nuclear "fertility" genes.

The CMS phenotype is used extensively in the commercial production of hybrid sorghum seed since the flowers are bi-sexual, self-fertile and difficult to hand emasculate. There is little genetic diversity in sorghum crops because nearly all hybrids are produced using Milo cytoplasm and its restorers (Stephens and Holland, 1954; Harvey, 1977). This uniformity is undesirable because susceptibility to fungal pathogens and herbicides can be cytoplasmically inherited (Ullstrup, 1972; Grun,1976). To increase the genetic diversity of hybrid sorghum, additional CMS lines have been bred from diverse sorghum cytoplasms by the strategy shown in Figure 1.4. A number of cytoplasms which display CMS in combination with Kafir nucleus have been reported Table 1.7. These cytoplasms have been differentiated taxanomically, as well as genetically on the basis of fertility restoration characteristics. The CMS lines are maintained with the related inbred cultivars Kafir and Martin which lack dominant fertility alleles (Figure 1.4 and Table 1.7).

TABLE 1.7 TAXONOMIC CLASSIFICATION OF SORGHUM CYTOPLASMS

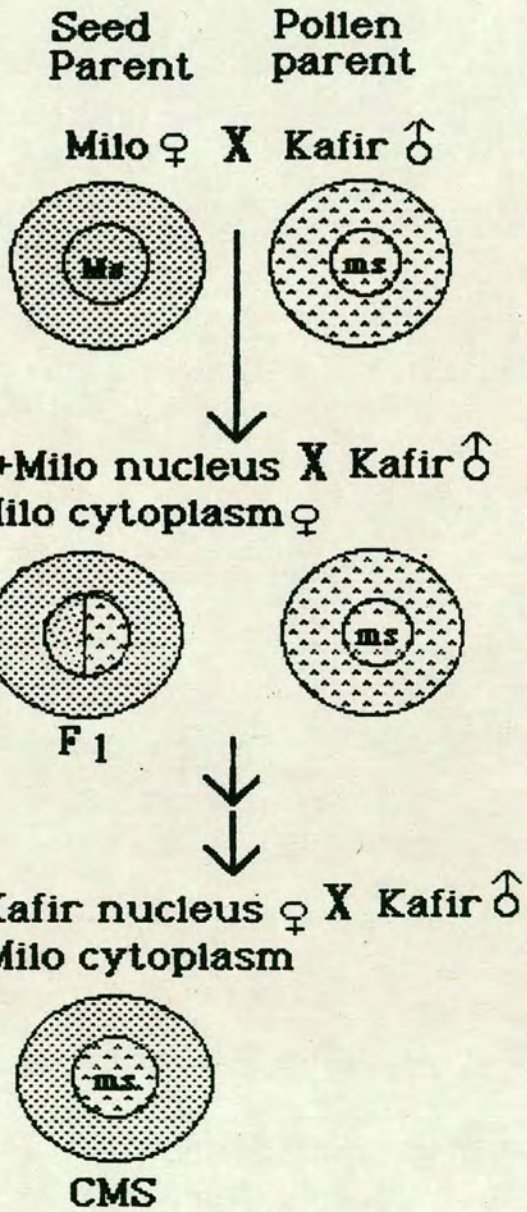
COMMON NAME	INTERNATIONAL SORGHUM NO.	ORIGIN	RACE	GROUP
M35-1[d]	-	India	?	?
A3[a]	IS1112C	India	Durra	Durra-Subglabrenscens
Milo[c] ATx3197	-	Africa	Durra	Milo-Subglabrenscens
A2[a]	IS12662C	Ethiopia	Caudatum	Zerazera
A4[a]	IS7920C	Nigeria	Guinea	Conspicuum
9E[b]	IS17218	Nigeria	Guinea	Conspicuum
9ED*	IS2483C	Sudan	Bicolor	?
Kafir[c] BTx3197	-	So. Africa	Kafir	Cafforum
Martin[c]	-	So. Africa	Kafir	Cafforum

REFERENCES: a - Schertz and Ritchey, 1978; b - Webster and Singh, 1964; c - Stephens and Holland, 1954; d - Rao, 1962; d - K. Schertz, personal communication.

* The cytoplasm IS2483C has no common name. A line referred to as 9ED, but with the mtDNA restriction pattern of IS2483C has been identified (Chapter IV).

Figure 1.4

Development of CMS Sorghum Lines



I. A hybrid is produced from two fertile lines. The female parent is hand emasculated.

II. The F_1 hybrid is emasculated and backcrossed again with the pollen parent. This process is repeated until male sterile progeny are obtained.

III. The male sterile line is maintained by the original male parent and can be restored to fertility by nuclear genes of the original Milo parent or a restorer line.

IV. Thus, CMS lines are hybrids in which a nucleus is maintained in a foreign cytoplasm and nuclear genes which restore fertility (Ms) are absent.

1.7 Mitochondrial Genome Rearrangements and Cytoplasmic Male Sterility in Sorghum: Scope of this Thesis

Preliminary molecular analyses have shown that as in maize, CMS lines of sorghum synthesize variant mitochondrial polypeptides (Dixon and Leaver, 1982) and have characteristic mtDNA restriction digestion patterns (Pring et al., 1982; Conde et al., 1982). My investigations were undertaken to elucidate the molecular basis of the CMS phenotype in sorghum. Two general questions were asked:

1) Is variability in mitochondrial genome organization related to expression of specific genes?
2) Do differences in mitochondrial genome structure and expression underlie the CMS phenotype?

The results obtained are presented in three chapters: In Chapter III the interspecific variability in sorghum mitochondrial genome organization and expression is examined. Specific mitochondrial gene probes were isolated to be used to characterize this heterogeneity in later studies. In Chapter IV the molecular basis of the synthesis of a variant form of cytochrome c oxidase was investigated. It was revealed that a mitochondrial genome rearrangement leads to altered gene expression and synthesis of a larger polypeptide. In Chapter V the effect of nuclear genotype on mitochondrial genome organization and expression was examined in relation to expression of the CMS phenotype. It was determined that mitochondrial genome rearrangements underlie genome variability and altered gene expression in sorghum. In addition, when a cytoplasm is transferred from its normal nuclear background (in which the phenotype is male fertile), to a foreign nuclear background (in which the phenotype is CMS) changes in mitochondrial gene expression may occur. In Chapter VI the molecular mechanisms and biological consequence of mitochondrial genome rearrangements is discussed.

Chapter II

MATERIALS AND METHODS

Part I. MATERIALS

2.1 Genetic Stocks

Sorghum [*Sorghum bicolor* (L.) Moench] was obtained from Dr. Gene Dalton of Pioneer Hi-bred International, Inc., Plainview, TX, Dr. Keith Schertz, USDA ARS, College Station, Texas A & M University, TX, and Dr. Orin Webster, Arizona State University, Tucson, AZ.

Sorghum lines are classified by nuclear and cytoplasmic genotype. For example, the line containing Kafir nucleus in Kafir cytoplasm is written as Kafir/Kafir (nucleus/cytoplasm). The sorghum lines examined are listed according to nuclear and cytoplasmic genetic background, male fertility status, and International Sorghum number in Appendix I.

2.2 Chemicals

Reagents were purchased from British Drug Houses (BDH, Poole, England) or from Sigma Chemical Co. (Poole, England) unless indicated otherwise.

Acrylamide and bis acrylamide:BDH Electran grade

Agarose:a) Miles, for standard gel electrophoresis

b) SeaKem Agarose, for preparative gel electrophoresis and fractionation of mtDNA.

Agar: a) Difco Bacto Agar, for minimal media plates.

b) Oxoid Technical Agar, for L Broth agar plates.

Ampholines: LKB Ltd.

Ampicillin ("Penbritin"):Beecham Research Laboratories

Cesium Chloride: Fisons Scientific Apparatus

Coomassie Brilliant Blue R: Raymond A. Lamb, Ltd.

Deoxy-N-5'triphosphates, yeast tRNA Boehringer Manheim GmBH

Dideoxy-N-5' triphosphates: P and L Biochemicals Inc.

Herring Sperm DNA: Serva Feinbiochemica, Heidelberg

PVP: L. Light and Co., Ltd.
Sephadex: Pharmacia Fine Chemicals
TNS: Eastman Chemicals
Triton X-100: Hopkin and Williams

2.3 Enzymes

Restriction enzymes were obtained from the following sources:
Amersham International,plc; Bethesda Research Laboratories Ltd. (BRL); Boehringer Manheim GmBH; New England Biolabs (NBL); P & S Biochemicals Ltd. and NBL Enzymes Ltd.
Other enzymes were obtained from the source(s) name(d) below:
CIP: gift of Dr. P. G. Isaac.
DNase I (free of RNase) and AMV reverse transcriptase: Pro Mega Biotec, P & S Biochemicals Ltd.
DNA Pol I (*E. coli*): New England Biolabs.
DNA Pol I, Klenow Fragment (*E. coli*): Boehringer, Bethesda Research Laboratories or Amersham, plc.
Lysozyme, T_4 DNA ligase: Boehringer
S_1 nuclease: Bethesda Research Laboratories.
Enzymes were stored at -20°C.

2.4 Radioisotopes

Purchased from Amersham International plc.:
α-[^{32}P]dCTP triethylammonium salt (in a stabilized aqueous solution), 400 Ci/mmol, 10 μCi/μl.
L-[^{35}S]methionine (in aqueous 20 mM potassium acetate, 1 mM DTT solution), 1150 Ci/mmol, 5 μCi/μl.
A gift from Dr. D. Apps, Edinburgh University Biochemistry Department:
[^{125}I]- *Staphylococcus aureus* Protein A, 2 μCi/μl.

2.5 Film

X-Ray film: Dupont Cronex-4 and Fuji FX Saftey.
Negative Film: Ilford HP-4 and Kodak 2415.

2.6 Supplies

Nitrocellulose: type BA-85, pore size 0.45 um (Schleicher and Schuell)

3MM chromatography paper (3MM paper) (Whatman)

Saran Wrap (Dow Chemical Corp.)

2.7 Stock Buffers and Solutions

TE80: 10 mM Tris HCl (pH 8.0), 1 mM EDTA.

10X TBE: 0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA (pH 8.0).

20X SSC: 3.0 M NaCl, 0.3 M Sodium citrate trisodium salt, pH 7.0.

100X Denhardts: 2% (w/v) BSA, 2 % (w/v) Ficoll type 400, 2% (w/v) PVP-40 (Denhardt, 1966).

1M $NaPO_4$ buffer (pH 7.0): 0.24 M Na_2HPO_4, and 0.76 M NaH_2PO_4.

3 M NaOAc: pH adjusted to 5.0 with glacial acetic acid.

2.8 Bacterial Growth Media

Minimal agar: 1.5% (w/v) Difco bacto agar, 0.2% (w/v) $(NH_4)_2SO_4$, 1.4% (w/v) KH_2PO_4, 0.6% (w/v), K_2HPO_4, 0.1% (w/v) Sodium citrate, 0.02% (w/v) $MgSO_4$, 0.2% (w/v) glucose, 2.5 X 10^{-4} (w/v) thiamine hydrochloride.

BBL top agar: 1% (w/v) Difco bacto agar, 0.65% (w/v) Baltimore Biological Laboratories trypticase, 0.5% (w/v) NaCl.

L Broth : 1% (w/v) Difco Bacto Tryptone, 0.5% (w/v) Difco Bacto yeast extract, 0.5% (w/v) NaCl, pH 7.2 adjusted with 5 M NaOH.

5X Spitzizen salts: 1% (w/v) $(NH_4)SO_4$, 7% (w/v) K_2HPO_4, 3.0% (w/v) KH_2PO_4, 0.5% (w/v) sodium citrate, 0.1% (w/v) $MgSO_4$.

L agar: 1.5 % (w/v) Difco agar, 1% (w/v) Difco Bacto Tryptone, 0.5% (w/v) Difco Bacto yeast extract, 0.5% (w/v) NaCl. pH adjusted to 7.2 with 5 M NaOH.

2.9 Centrifuges and Centrifuge Tubes

Reactions were carried out in 1.5 and 0.75 ml polypropylene tubes (Starstedt or Treff) which fit into a 12 or 16 place rotor in a microfuge (Eppendorf, or MSE Micro-Centaur). Tubes were centrifuged at a fixed angle at 12,000 X g_{max}. Centrifugation of larger volumes was in Corex tubes or polycarbonate bottles in the SS-34 rotor or GSA fixed angle rotor, in the Sorvall RC5B centrifuge. Ultracentrifugation was in the appropriate centrifuge tubes in the AH627, TFT.65.1, or Ti70.1 rotor, in the Sorvall OTD65B.

Part II. METHODS

2.10 Isolation of Mitochondria

A. Seed Sterilization, Growth and Preparation of Material

Sorghum seeds were re-hydrated overnight in cold running tap water, surface sterilized in a 1:15 dilution of sodium hypochlorite (14% (w/v)) for 15 minutes and rinsed six times with sterile distilled water. Sterile 12 ply cellulose wadding (Robinsons and Sons Ltd.) was placed on 45 X 27 cm seed trays and moistened with sterile water containing 0.1 mM $CaCl_2$. 20 grams of seed were distributed per tray and covered with 1/2 thickness of lightly moistened cellulose wadding. The seed trays were covered with aluminum foil and the seeds germinated for five days at 28°C in complete darkness.

Coleoptiles, free of obvious fungal or bacterial contamination, were harvested into pre-weighed beakers on ice. The tissue was weighed, rinsed 4 times with ice-cold sterile water, cut into 0.5 - 1 cm sections and chilled at 2°C prior to homogenization.

2.10 B Isolation of Mitochondria

Grinding Medium: 0.4 M Mannitol, 0.1 M EGTA, 25 mM MOPS (pH 7.8), 8 mM cystine, 0.1% (w/v) BSA. (BSA was included only when mitochondria were to be used for *in organello* protein syntheis).

Mitochondria were extracted from 5 - 50 g of coleoptiles by homogenization in Grinding Medium (2 ml/g harvest weight) with a mortar and pestle and purified by two rounds of differential centrifugation as described previously (Leaver *et al.*, 1983). Mitochondria to be used for *in organello* protein synthesis, immunolabelling experiments, and isolation of nucleic acids were purified further by centrifugation through a sucrose gradient in the Sorvall AH 627 swinging bucket rotor, at 68,300 X g_{max} for 1 hr. Mitochondria were used immediately for *in vitro* protein synthesis and for extraction of mtRNA (total nucleic acids). Mitochondria were stored at -80°C prior to extraction of mtDNA, protein fractionation and enzyme assays. This protocol yielded 1.0 - 1.5 µg mitochondrial protein/g coleoptiles.

PROTEIN TECHNIQUES

2.11 *In Organello* Protein Synthesis by Isolated Mitochondria

Buffers and Solutions

Resuspension Medium: 0.4 M Mannitol, 10 mM Tricine (pH 7.2), 1 mM EGTA.

Incubation Buffer: 250 mM KCl, 0.4 M Mannitol, 90 mM $MgCl_2$, 10 mM Tricine (pH 7.2), 10 mM potassium phosphate (pH 7.2), 5 mM EGTA, 1 mM 19 amino acids except methionine, 20 µM DTT, 2 mM GTP, 1mM; L-[^{35}S]-methionine (1150 Ci/mmole, 20 µCi).

CP/CPK energy mix: 35mM Creatine Phosphate, 200 µg/µl Creatine phosphokinase (final: 8 mM creatine phosphate, 25 µg creatine phosphokinase, 6 mM ATP).

Succinate energy mix: 50 mM succinate, 10 mM ADP (final: 10 mM sodium succinate and 2 mM ADP).

Acetate energy mix: 100 mM sodium acetate (final: 20 mM sodium acetate).

Protein synthesis by isolated mitochondria was carried out essentially as described by Lerver et al. (1983). 200 - 500 µg of sorghum mitochondrial protein was resuspended in Resuspension Medium in 1.5 ml centrifuge tubes. 150 µl of Incubation Buffer and 50 µl of an energy mix was added. Sodium acetate, a non-oxidizable energy source, was used to detect protein synthesis by contaminating bacteria. The tubes were incubated at 25°C for 90 min in a shaking waterbath and incorporation of L-[^{35}S]methionine into mitochondria was terminated by the addition of 1 ml ice-cold, unlabelled methionine (10 mM) in Resuspension Medium. The mitochondria were pelleted by centrifugation for 5 min, the supernatant was removed and the mitochondria stored at -80°C.

The incorporation of [^{35}S]-methionine was determined by scintillation counts of TCA precipitable protein (Leaver, et al., 1983). Usually sorghum mitochondria, isolated from 5 day old coleoptiles, incorporated 8 - 12% of the [^{35}S]-methionine present in the reaction.

2.12. Cytochrome c Oxidase Enzyme Assay

Buffer and Cytochrome c stock

Assay Buffer: 20 mM MOPS (pH 7.2), 1% (v/v) Tween-80.

Reduced cytochrome c (Horse heart, Fraction III): 5 mM cytochrome c, 10 mM sodium ascorbate, 20 mM MOPS (pH 7.2), 1% (v/v) Tween-80; passed through a G-50 Sephadex spin column (Section 2.27 D) which had been equilibrated in Assay Buffer.

Mitochondrial protein was resuspended to 0.5 µg/µl in Assay Buffer. 1 ml of Assay Buffer and 2 µl of reduced cytochrome c were pipetted into two quartz cuvettes. The cuvettes were placed into the spectrophotometer chambers, the assay was initiated by addition of 5 - 20 µl of

mitochondrial protein to the cuvette in the sample chamber and the increase in A_{550} was measured immediately. Oxidase activity was shown to be KCN sensitive.

The oxidase activity was calculated given that an increase in 1 OD unit absorbance at A_{550} is equivalent to 0.52×10^{-8} moles cytochrome oxidase (Mason et al., 1970):

$$\frac{OD_{550}}{sec} \times \frac{0.52 \times 10^{-8} \text{ moles}}{OD} \times \frac{1}{\mu g \text{ mitochondrial protein}}$$

2.13 ATPase Enzyme Assay

The liberation of inorganic phosphate by isolated mitochondria was assayed as described by Hack and Leaver (1983).

2.14 Estimation of Protein Concentration

Solutions

Reagent Mix: 0.1% (w/v) $CuSO_4$, 0.25% (w/v) Sodium Tartate, 1.5% (w/v) $NaCO_3$ in 0.05M NaOH. Reagent mix made fresh from stock solutions.

Folin and Ciocalteu's Phenol Reagent (B.D.H.) was diluted to 0.3 N (1:7 dilution) with distilled water, prior to use.

An estimation of mitochondrial protein content was determined by an adaptation of the method described by Lowry et al. (1951). Mitochondrial protein was precipitated with an equal volume of 8% (w/v) TCA at 4°C overnight in 1.5 ml centrifuge tubes. The precipitate was pelleted by centrifugation for 2 min and hydrolyzed in 300 µl of 0.4 N NaOH at RT for 1 hr. 2 ml of Reagent Mix were added and the sample was incubated for 10 min at RT prior to the addition of 1 ml of 0.3 N Folin and Ciocalteu's Phenol reagent. A Corning colorimeter 252 with a red (600 nm) filter was used to determine the coloration of the solution. Protein concentration was determined relative to a curve of 10, 25, 50, 75, 100 and 150 µg BSA.

2.15 SDS-Polyacrylamide Gel Electrophoresis

Buffers and Solutions

2X SDS loading buffer: 12.6% (v/v) glycerol, 4% (w/v) SDS, 0.06 M Tris-HCl (pH 6.8), 1% (w/v) BPB, stored at -20°C.

Acrylamide solution: 30% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, stored at 4°C in darkness.

5X Separating gel buffer: 1.875 M Tris-HCl (pH 8.8), stored at 4°C.

10X Stacking gel buffer: 0.6 M Tris-HCl (pH 6.8), stored at 4°C.

Laemmli electrophoresis buffer: 1.92 M glycine, 0.25 M Tris-HCl, (pH 7.2), 0.1% (w/v) SDS.

Coomassie Blue stain: 50% (v/v) MeOH, 7% (v/v)glacial acetic acid, 5% (w/v) Coomassie Brilliant Blue R50.

Destain: 40% (v/v) MeOH, 7% (v/v) glacial acetic acid.

Discontinuous Polyacrylamide Gel Composition:

5% Stacking Gel	16% Separating Gel
5% (w/v) acrylamide	16% (w/v)acrylamide
0.33% (w/v) bis acrylamide	0.1% (w/v) bis-acrylamide
0.06 M Tris-HCl (pH 6.8)	0.375 M Tris-HCl (pH8.8)
0.1% (w/v) SDS	0.1% (w/v) SDS
0.1% (v/v) TEMED	0.1% (v/v) TEMED
0.05% (w/v) AMPS	0.05% (w/v) AMPS

Mitochondrial proteins (50 - 100 µg) were solubilized in 2X SDS loading buffer at 37°C for 5 min, or 100°C for 2 min. BME was added to 1% (v/v) and the proteins were centrifuged for 2 min, loaded into the wells of a discontinuous SDS-polyacrylamide gel and fractionated by electrophoresis (Laemmli, 1970). Typically, 16% (w/v) SDS-polyacrylamide vertical slab gels were used to examine mitochondrial polypeptides. Gels were electrophoresed at 8mA until the BPB dye front had traversed the entire length of the gel. Unless otherwise indicated, gels were fixed, stained with Coomassie Blue stain, and destained. Gels were dried onto 3MM paper under vacuum at 70°C on a gel dryer (Zabona, Basel) and exposed to X-ray film.

Molecular weight markers (Table 2.1) were run on gels to determine the apparent molecular weight of mitochondrial proteins. Stock solutions of standards were made in 2X SDS loading buffer and 0.5 M DTT and stored at -20°C for 2 months. Molecular weights were determined from the regression of electrophoretic mobility against the Log_{10} molecular weight.

Table 2.1 Molecular Weight Markers

protein	concentration (µg/µl)	molecular weight *
BSA	0.5	68,000
catalase (bovine liver)	0.5	60,000
ovalbumin	1.25	43,000
aldolase	0.5	40,000
carbonic anhydrase	0.25	29,000
Trypsin inhibitor (soybean)	1.0	20,000
Myoglobin	0.5	17,000
Lysozyme	1.0	14,200
Aprotinin	1.25	6,500
Insulin	1.25	3,400

* (Weber and Osborn (1969) and Lambin (1978).

2.16 Fractionation of Mitochondrial Polypeptides

Soluble and membrane-bound mitochondrial polypeptides were fractionated by the following procedure. Mitochondrial proteins were resuspended to 40 µg/µl in 5 mM Tris-SO_4 (pH 7.6), 1 mM PMSF, 0.5 mM pAB-HCl, 0.25 M sucrose and osmotically shocked by a 10-fold addition of the same buffer without 0.25M sucrose. The protein sample was frozen and thawed 3 times and pelleted at 10,000 X g_{av} for 10 min at 4°C to obtain a mitochondrial membrane pellet. The supernatant was removed and stored. The pellet was resuspended to 2 µg/ml and recentrifuged at 10,000 X g_{av} to remove any residual soluble proteins. The supernatant was removed, combined with the first supernatant and centrifuged at 100,000 X g_{av} for 30 min at 4°C, to exclude membranes from the soluble protein

fraction. The mitochondrial subfractions were resuspended in SDS loading buffer (to a establish a concentration of 2% (w/v) SDS) and heated at 100°C for 2 min prior to the addition of BME to 1% (v/v). Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (Section 2.15).

2.17 Partial Proteolytic Degradation of Mitochondrial Proteins

Mitochondrial proteins, labelled by *in organello* protein synthesis, were subjected to partial proteolytic digestion according to the technique of Cleveland *et al*. (1977). Polypeptides were first fractionated by SDS-polyacrylamide gel electrophoresis, identified by autoradiography (Randerath, 1970) and excised from the dry gel. A SDS-polyacrylamide gel, with a 5 cm, 5% stacking and a 13 cm, 15 - 20% (v/v) gradient resolving gel was prepared. The gel slices were placed into the wells of the gel and overlayed with 0.5 ml of equilibration buffer (0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 1 mM EDTA) for 1 hr prior to electrophoresis. The equilibration buffer was removed and the top buffer reservior was filled with Laemmli electrophoresis buffer. The gel slices were then overlayed with the specified protease solution in 6 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 1 mM EDTA and 10% (v/v) glycerol. Electrophoresis was at 4mA for 20 - 24 hours at 30°C. Gels were equilibrated in a 1M solution of water soluble sodium salicylate, rinsed in distilled water and flourographed using X-ray film (Chamberlin, 1979).

2.18 Two-Dimensional Isoelectric Focussing, SDS-Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis of mitochondrial polypeptides was carried out essentially as described by Hack and Leaver (1984) adapted from O'Farrell (1975) and Horst *et al*.(1980). The method described here gives high resolution of most mitochondrial proteins. Polypeptides were first solubilized in a non-ionic detergent and urea and separated according to isoelectric

point. In the second dimension, proteins were fractionated according to their apparent molecular weight.

A. Buffers and Solutions

Lysis buffer: 9.5 M urea, 10 mM K_2CO_3, 1 mM PMSF, 2% (v/v) NP-40, 1.6% (v/v) pH 5 - 7 Ampholines and 0.4% (v/v) pH 3.5 - 10 Ampholines, stored at -20°C.

Acrylamide stock: 28.4% (w/v) acrylamide: 1.6% (w/v) bis-acrylamide, stored at 4°C.

Anolyte electrophoresis buffer (bottom tank): 0.02 M NaOH.

Catholyte electrophoresis buffer (top tank): 0.01 M H_3PO_4.

Gel overlay: 9.5 M urea, 2% (v/v) NP-40, 1.6% (v/v) pH 5 - 7 Ampholines, and 0.4% (v/v) pH 3.5 - 10 Ampholines, stored at -20°C.

Gel equilibration buffer: 10% (v/v) glycerol, 5% (v/v) BME, 2.0% (w/v) SDS, 6.25 mM Tris-HCl (pH 6.8) and 0.01% (w/v) BPB, stored at -20°C.

Sample overlay: 7.1 M urea, 7.5 mM K_2CO_3, 0.75 mM PMSF, 1.2% (v/v) pH 5 - 7 Ampholines, and 0.3% (v/v) pH 3.5 - 10 Ampholines, stored at -20°C.

Gel composition:
9.5 M urea
80% (v/v) pH 5 - 7 Ampholines
20% (v/v) pH 3.5 - 10 Ampholines
3.8% (w/v) acrylamide
2.0% (v/v) NP-40 (Shell)
0.1% (w/v) AMPS 0.1 M TEMED.

B. First Dimension - IEF Gel Preparation

Isoelectric focusing (IEF) gels contained a low percentage of polyacrylamide, a high concentration of urea, and a wide pH range of Ampholines. The low acrylamide gel composition allowed large proteins to migrate to their isoelectric point and the high concentration of urea provided structural stability, and maintained proteins in a denatured form. The optimal resolution of mitochondrial proteins was obtained when gels were loaded at the acid end and electrophoresed towards the anode.

Gels (11cm) were cast in vertical 13 X 0.3 cm siliconized glass tubes, overlaid with 8M urea and allowed to polymerize for 1 hr. Following polymerization, the 8M urea overlay was removed and the gels were placed in a BRL tube gel electrophoresis apparatus, overlaid with 10 µl of Gel overlay and electrophoresis buffer, then pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. Solution above the gels was removed prior to loading of sample.

C. Sample Preparation and Electrophoresis

Mitochondrial proteins (200 - 300 ug) were solubilized by vortexing at RT in 10µl of Lysis buffer. Following solubilization, DTT was added to a final concentration of 0.5 M, together with a few crystals of urea. The sample was centrifuged for 3 min to pellet any insoluble protein and urea. The sample was loaded, overlaid with 10µl of Sample overlay and Catholyte electrophoresis buffer, and electrophoresis was carried out at 400 V for 16 hr and 800 V for 1 hr.

D. 2nd Dimension Electrophoresis

Following IEF electrophoresis gels were removed from the glass tubes and equilibrated for 1 hr at RT in Equilibration buffer to remove Ampholines and urea and add SDS. 2nd dimension electrophoresis was performed in 16% (w/v) SDS-polyacrylamide gels similar to those described by Laemmli et al., (1970) as modified by O'Farrell (1975). The gels were stained with Coomassie Blue stain, destained and flourographed (Chamberlin, 1979).

2.19 Immunologial Identification of Mitochondrial Proteins

Mitochondrial polypeptides were identified by their ability to cross react with rabbit antibodies raised against yeast mitochondrial proteins. Two techniques were used: A) Western blotting and B) Immunoprecipitation.

A. Western Blotting

Mitochondrial proteins were fractionated by SDS-polyacrylamide gel electrophoresis (Section 2.15). Two gel tracks, one of protein molecular weight markers and the other mitochondrial proteins, were excised from the gel and stained as usual. The unstained portion of the gel was soaked in WTB (20% (v/v) MeOH, 0.25 M glycine, 1.92 M Tris-HCl (pH 7.0), 0.1% (w/v) SDS) for 30 min at RT and transferred to nitrocellulose according to the method described by Towbin et al., (1979). Electrophoretic transfer was at 0.4 Amps for 2 hr. The nitrocellulose filter replica or "western blot" was washed in T-20 buffer (0.01% (v/v) Tween-20, 10 mM Tris-HCl (pH 7.2), 0.9% (w/v) NaCl) (Batteiger et al., 1982) for 1 hr, then incubated in T-20 buffer containing an antiserum, overnight at RT. Non-specifically bound antibody was removed by two 10 min washes in T-20 buffer. The antibody-antigen complex was subsequently labelled by incubation of the western blot in T-20 buffer containing 0.2 μCi/ml of [I^{125}]-S. aureus Protein A, for 1 hr at RT. Unbound Protein A was removed by three 10 min washes with T-20 buffer and the western blot was exposed to X-Ray film at -80°C.

B. Immunoprecipitation

Buffers and Solutions

Resuspension buffer: 10 mM Tricine (pH 7.2), 2 mM ATP, 1 mM PMSF, 5 mM pAB, stored at -20°C.

TNET: 1% (v/v) Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris (pH 7.2).

Immunoprecipitation loading buffer: 50 mM Na_2CO_3, 25% (w/v) SDS, 12% (w/v) sucrose, 0.04% (w/v) BPB, stored at -20°C.

Mitochondrial proteins, labelled by in organello protein synthesis, were immunoprecipitated with rabbit antiserum prepared against yeast cytochrome c oxidase Sub-unit I. Samples containing 2.5 - 5 x 10^6 c.p.m. (250 - 500 μg) of mitochondrial protein were diluted to 2.5 mg/ml with Resuspension buffer, mixed well and centrifuged at

100,000 X g_{max} for 30 min at 2°C, in order to remove soluble proteins. The mitochondrial membrane pellet was resuspended in 1 ml of TNET and PMSF was added to 1 mM. 20 µl of antiserum (DD-2T) was added and the sample was rotated overnight at 4°C. The sample was centrifuged briefly to remove any insoluble protein and the supernatant transferred to a fresh tube. 100 µl of Protein-A Sepharose CL 4B (Sigma) solution (0.1 mg/µl in TNET) was added and the mixture was incubated for 1 hr at 4°C. The Protein-A sepharose - antibody - antigen complex was pelleted by centrifugation at low speed (1,300 X g_{max}) in the MSE Mistral 4L centrifuge. The supernatant was discarded and the pellet was washed with 1 ml of TNET, 5 times. The final pellet was solubilized in 80 µl of Immunoprecipitation loading buffer and heated at 37°C for 5 min. BME was added to 1% (v/v) and the sample was fractionated by SDS--polyacrylamide gel electrophoresis. Labelled polypeptides were detected by flourography.

RNA TECHNIQUES

2.20 Isolation of Mitochondrial RNA from Purified Mitochondria and Treatment with DNAse

Total mitochondrial nucleic acid was extracted by the method of Parish and Kirby, (1966). Mitochondria were prepared from 5 - 50 g of tissue as described in Section 2.10. The extraction procedure was carried out at 2°C in sterile glass and plasticware. The mitochondrial pellet was resuspended in 1 ml of extraction buffer (6% (w/v) PAS, 1% (w/v) TNS, 0.1 M Tris-HCl (pH 8.5), made fresh) in a 15 ml Corex tube. 1 ml of phenol, saturated with TE80, was added and the solution was mixed vigorously. The phenol and aqueous layers were separated by centrifugation in the SS-34 rotor at 2,000 X g_{max} for 5 min. The aqueous layer was removed and transferred to a fresh tube and re-extracted twice with phenol, or until the aqueous-phenol interface was clean. The final aqueous volume was determined and the nucleic acids were precipitated overnight at -20°C following the addition of 0.1 volume of

3 M potassium acetate (pH 5.4) and 2.5 volumes of EtOH. The nucleic acids were pelleted by centrifugation in the SS-34 rotor at 8,000 X g_{max} for 10 min, washed twice with 70% EtOH, dried under vacuum and resuspended in 100 µl of TE80. The concentration and purity of the mitochondrial nucleic acids was determined by a UV absorbance scan from 220 to 320nm. 1 OD at 260 nm was assumed to be eqivalent to 40 µg of RNA (Maniatis, et al., 1982). Typically, 2 µg of mitochondrial nucleic acid was recovered from 1 g of sorghum coleoptiles. Sample purity was assessed from the ratio of A_{260}:A_{280}, which should be approximately 2:1.

DNA was removed from the nucleic acid sample by incubation with RNase free DNase I (1unit/µg RNA). 10 µg of nucleic acid was incubated with 10 units of DNase I in Medium digestion buffer (Section 2.26) at 37°C for 30 min, The mtRNA was extracted with phenol and precipitated at -80°C for 20 min, with 0.1 volume of 3 M NaOAc (pH 5.0) and 2.5 volumes of EtOH. The mtRNA was resuspended in TE80 and the OD of the solution at 260 nm was measured. MtRNA was stored at -20°C.

2.21 Gel Electrophoresis of RNA and Northern Blotting

The procedure used for the fractionation of RNA was essentially that of Lehrach et al., (1977) as modified by Maniatis et al., (1982). Gels were transferred to nitrocellulose as described by Thomas (1980) and probed with labelled DNA probes (Section 2.27).

Buffers

10X MOPS Buffer: 0.2 M MOPS (pH 7.0), 50 mM NaOAc, 10 mM EDTA, stored at 4°C.

2.5X Ficoll Dye Buffer (FDB): 0.1 M EDTA (pH 7.0), 0.3% (w/v) Ficoll, 0.01% (w/v) BPB, stored at 4°C.

5X Formamide sample buffer (FSB): 50 mM MOPS, 12.5 mM NaOAc, 47% (v/v) formamide, 10.6% (v/v) formaldehyde, 2.5 mM EDTA (pH 7.0), stored at -20°C.

Gel composition:1.3% (w/v) agarose, 17.3% (v/v) formaldehyde, 1X MOPS buffer.

A. Gels, Sample Preparation and Electrophoresis

Gels were cast in a horizontal slab gel apparatus (usually, 16 X 11 X 0.5 cm) and electrophoresed submerged in an electrophoresis tank containing 1X MOPS Buffer. 5 - 15 µg of mtRNA in 10 µl of distilled water was diluted with an equal volume of FSB, heated at 60°C for 5 minutes and quenced on ice. RNA samples were loaded into preformed wells and electrophoresed at 150 V for 4 hr, or until the BPB marker had traversed 2/3 of the gel length. The electrophoresis buffer was not circulated because the pH of 1X MOPS buffer was stable under these electrophoresis conditions.

B. Gel Staining and Photography

Molecular weight markers were E. coli rRNA (2904 and 1514 bp) (Brosuis et al., 1979; Brosuis et al., 1980) and CCMV RNA (3200, 2900, 2300, 825 b) (Davis and Verduin, 1979; Dasgupta and Kaesberg, 1982). The gel tracks containing molecular weight markers were excised and stained (Maniatis et al., 1982). The gel was illuminated with 245 nm UV light (Ultraviolet Products, San Gabriel, CA) and photographed through a Kodak Wratten gelatin filter (No. 234) onto negative film (Section 2.5). Molecular weight was estimated from mobility relative to that of the molecular weight markers.

2.22 Northern Blotting and Hybridization Buffer

Northern Hybridization Buffer: 50% (v/v) formamide, 5X SSC, 50 mM $NaPO_4$ buffer (pH 7.0), 250 µg/ml denatured herring sperm DNA, 1X Denhardt's, stored at -20°C.

A. Northern Blotting

RNA was transferred to nitrocellulose without staining since staining reduces the efficiency of RNA transfer by 50% (Thomas, 1980). 2 layers of 3MM filter paper where draped over two sides of a glass plate and into a trough of 20X SSC. The gel trimmed to include only

the tracks of interest and placed on top of the filter paper wick. A sheet of nitrocellulose was cut to the exact dimensions of the gel and wetted on the surface of a dish of 2X SSC. The nitrocellulose sheet was placed on top of the gel and a glass pipette was rolled over the surface of the nitrocellulose to exclude any trapped air bubbles. The transfer assembly was completed by placing 2 pre-cut sheets of 3 MM filter paper over the nitrocellulose sheet, 6 cm of absorbent towels and a weight. Transfer of the RNA to the nitrocellulose was allowed to proceed overnight. The nitrocellulose "northern blot" was removed and baked at 80°C for 2 hr under vacuum.

B. Northern Blot Hybridization

Northern blots were prehybridized in polyethlene bags for at least 4 hr at 42°C in Northern Hybridization Buffer (Thomas, 1980). Prehybridization for less than 4 hr resulted in an increase of the background radioactivity on Northern blots. Radioactive DNA probes were made by varying methods as described in Section 2.27. Hybridization was in fresh Northern Hybridization Buffer containing the thermally denatured radioactive probe at 42°C for 12 - 24 hr. The hybridization solution was then removed and the filter washed with two changes of 2X SSC, 0.1% (w/v) SDS at RT for 15 min and two changes of 0.1X SSC, 0.1 (w/v) SDS, each at RT for 15 min. The filter was blotted dry, wrapped in Saran Wrap and exposed to X-ray film at -80°C (Randerath, 1970).

Probes were removed from the filters by washing in bath of boiling, sterile distilled water which was allowed to cool to room temperature while rotating slowly.

2.23 S_1 Nuclease Transcript Mapping

S_1 nuclease transcript mapping was performed essentially by the method described by Berk and Sharp, (1977) as modified by Weaver and Weissman, (1979). S_1 nuclease transcript mapping requires the hybridization of

RNA to a well defined, labelled DNA fragment. Single stranded DNA and RNA is removed by digestion with S_1 nuclease. The resultant hybrid is sized by gel electrophoresis and autoradiography of the [^{32}P]-labelled DNA.

Buffers and Solutions

Alkaline agarose gel composition: 50 mM NaCl, 1 mM EDTA, 2% (w/v) agarose (SeaKem or Sigma Low Melting Temperature), equilibrated in 30 mM NaOH for 1 hr.

NaOH loading buffer: 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll-400. 0.025% (w/v) bromocresol green.

5X Hybridization buffer: 2 M NaCl, 200 mM Pipes-NaOH (pH 6.4), 5 mM EDTA, stored at -20°C.

S_1 nuclease buffer: 250 mM NaCl, 30 mM NaOAc (pH 4.6), 1 mM $ZnSO_4$, 20 µg/ml denatured herring sperm DNA, stored at -20°C (Vogt, 1973).

A. Preparation of the Radioactive DNA template

[^{32}P]-labelled DNA, complementary to the RNA transcript, was synthesized from a ssM13 DNA template, as described in Section 2.27 B. The DNA was digested with a restriction enzyme (Section 2.26) to separate the cloned insert from the vector and then passed through a G-50 Sephadex spin column to remove unincorporated α[^{32}P]dCTP (Section 2.27 D). 10 µg of *E. coli* tRNA, 0.1 volume of 3M NaOAc and 2 volumes of ethanol were added and the DNA precipitated at -80°C for 20 min. The DNA was resuspended in 15 µl of NaOH loading buffer, loaded into the preformed wells of a 2% (w/v) alkaline low melting temperature agarose gel (Mc Donnell *et al.*, 1977; Maniatis *et al.*, 1982) and electrophoresed at 200 V for 3 hr. The gel was wrapped in Saran Wrap and exposed to X-ray film for 30 min to 18 hr. The [^{32}P]-labelled restriction fragment containing the insert DNA (and a portion of the M13 polylinker) was cut out of the gel and the DNA was eluted (Section 2.28).

B. Calculation of Hybridization Temperature

The temperature of DNA:RNA hybridization was determined emperically for each S_1 transcript mapping study. Casey and Davidson (1977) suggest that the thermal melting point (Tm) of a DNA:RNA hybrid is 5 - 10°C higher than the Tm of a DNA:DNA hybrid. The thermal melting point of a DNA hybrid is determined by the equation:

$$°C = 81.5 + 0.41\ (GC) - 0.72\ F + 16.6\ Log_{10}\ M,$$

where GC is the percent G + C of the DNA fragment, F is the formamide concentration and M is the monovalent cation concentration of the solution. Thus, the temperature used for DNA:RNA hybridizations was:

°C Tm DNA:DNA + 7.5 +/- 2.5 = °C Tm DNA:RNA

Preliminary S_1 nuclease transcript mapping expriments were carried out at three temperatures: °C = Tm DNA:RNA, Tm DNA:RNA +2.5°C, Tm DNA:RNA -2.5°C.

C. S_1 Nuclease Protection Assay

The [^{32}P]-labelled DNA (2500-20,000 c.p.m./assay), mtRNA (2.5 - 5 µg/assay) and carrier E. coli tRNA (10µg/assay), desiccated under vacuum, were resuspended in 2 µl of 5X hybridization buffer by vortexing. As a control, a second tube containing the labelled [^{32}P]-DNA and carrier E coli tRNA was treated similarly. Eight µl of deionized formamide (Robberson et al., 1971) was added and the samples were heated in a dry-block at 85°C for 5 min in 1.5 ml centrifuge tubes. The tubes were immediately submerged in a water bath at the hybridization temperature (Casey and Davidson, 1977), and incubated for 4 to 16 hr. After hybridization, 25 units (250 units/ml) of S_1 nuclease in 100 µl of ice cold S_1 nuclease buffer were added. The tube was quickly transferred to ice for 10 sec and then incubated at 37°C for 30 min. The S_1-protected hybrid was precipitated at -80°C for 20 min, following the addition of 0.25 ml EtOH and 10 µg of carrier E. coli tRNA. The nucleic acid pellet was resuspended in 75 µl of 4M NH_4OAc and 75 µl of TE80, reprecipitated with 0.375 ml EtOH, washed once with 70% EtOH and dried.

D. Electrophoresis of S_1 Protected [^{32}P]-labelled DNA Fragments

The [^{32}P]-DNAs were analyzed on 8% (w/v) polyacrylamide - 8.3 M urea DNA sequencing gels (Section 2.31). Typically, an aliquot of the S_1 protected DNA:mtRNA hybrid (1,000 - 50, 000 c.p.m.), the DNA:E. coli tRNA hybrid (2,000 - 100,000 c.p.m.) and the electroeluted DNA fragment (1,000 - 50,000 c.p.m.) were fractionated on a gel alongside labelled molecular weight markers, (Section 2.27 C), and the M13 sequencing ladder of a well defined clone (Section 2.31) The size of the S_1 protected DNA fragments was determined from their mobility relative to the DNA markers and sequencing reactions run in parallel tracts.

DNA TECHNIQUES

2.24 Purification of Mitochondrial DNA

Mitochondria were isolated from 5 - 100 g of tissue as described (Section 2.10) pelleted in tarred Corex tubes and resuspended in 300 µl of lysis buffer (2 mg/ml Protease K (Tritriachium album, Type XI; final concentration 0.1 µg/ml), 0.1 M Tris-HCl (pH 8.0), 0.1 M EDTA) and 0.25 ml of 10% sarkosyl. The solution weight was adjusted to 6.79 g by the addition of 0.1 M Tris (pH 8.0), 0.1 M EDTA and was incubated at 37°C for 30 min prior to the addition of 6.4 g of CsCl and 300 µg of EtBr. The solution was centrifuged at 140,000 X g_{max} in the Ti 70.1 rotor (Beckman) at 15°C for 24 - 48 hr. The centrifuge tube was placed in an adjustable test tube clamp and illuminated with 366 nm UV light. Two distinct UV fluorescent bands, less than 1 mm apart, were observed in the preparation of sorghum mtDNA. The centrifuge tube was punctured with a medium gauge needle 25 mm above the fluorescent bands and the DNA was carefully drawn into a 2 ml syringe. The solution was transferred to a 15 ml Corex tube and extracted 3 times with n-butanol, saturated CsCl and TE80, to remove the EtBr. The CsCl saturated DNA

solution was diluted by the addition of 2 volumes of TE80 and 3 volumes of EtOH and precipitated at -20°C for 4 hr. The DNA was pelleted by centrifugation in the SS-34 rotor at 10,000 X g_{max} for 10 min. The pellet was dissolved in 300 µl of TE80, transferred to a 1.5 ml test tube and re-precipitated by the addition of 0.7 ml cold EtOH and centrifugation at 10,000 X g_{max} for 10 min. The pellet was washed once with cold 70% EtOH and desiccated under vacuum. The mtDNA was resuspended in 50 µl of TE80 and the concentration was determined from the OD at 260 nm.

2.25 Preparation of Plasmid and M13 'Phage DNA

A. Bacterial Stocks, Plasmids and Growth Conditions

E. coli derived plasmids pBR322 (Bolivar *et al.*, 1977), pBR325 (Bolivar 1978), pBR328 (Soberon *et al.*, 1980) pNS1 (Nikolnikov, *et al.*, 1984), and recombinant DNA plasmids (Section 2.29) were maintained in HB101 cells (*E. coli* stain HB101 (F^-, *hsdS20*, (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Sm^r), *xyl-5 mt-1*, supE44, λ^-). Bacterial colonies were grown on L Broth agar plates or L Broth containing permissible antibiotics (10 µg/ml tetracycline and/or 50 µg/ml ampicillin), at 37°C overnight and stored at 4°C (Maniatis *et al.*, 1982). Liquid cultures were grown at 37°C on an orbital shaker (New Brunswick) at 300 r.p.m., for 8 - 18 hr, and harvested immediately for isolation of plasmid DNA.

B. Large Scale Preparation of Plasmid DNA

Plasmid DNA was prepared from a single bacterial colony by the alkaline precipitation procedure described by Birnbiom and Doly (1979) and Maniatis *et al.* (1982). Plasmid DNA was further purified by centrifugation in 1.55 g/ml CsCl, 0.6 mg/ml EtBr gradients (Radloff *et al.*, 1967). The plasmid DNA band, 1 cm below the chromosomal DNA band, was collected as described previously (Section 2.24). Typically, 100 µg of plasmid DNA was recovered from 500 ml cells grown to stationary phase.

C. Plasmid Mini-Preps

Mini-preps of plasmid DNA were performed exactly as described by Birnboim and Doly (1979). A single bacterial colony was used to inoculate 2 ml of L Broth, containing the appropriate antibiotic(s), in a 5 ml bottle. From 1 ml of bacteria, 1 - 2 µg of plasmid DNA was obtained.

D. JM101 and M13 'Phage Maintenance Growth Conditions

M13 is a filamentous DNA phage specific for 'male' E. coli and has been modified by Messing et al. (1977) to contain the promoter and operator and the first 145 amino acid residues of the ß-galactosidase gene (lac DNA) as well as a strain specific polynucleotide linker. M13mp phage was grown in the E. coli F^+ host strain JM101 [Δ(lac pro) thi, supE, F'traD36, proAB, $lacI^q$, ZΔ M15] (Messing, 1979). JM101 was maintained on minimal agar plates and a single colony was used to inoculate 2 ml of L broth in a 5 ml bottle prior to the preparation of cells for transformation with recombinant DNA. JM101 was grown in BBL top agar on minimal plates or in L Broth medium.

E. Single Stranded M13 'Phage DNA Mini-Preps

Single stranded M13 'phage DNA was prepared from a single 'phage plaque essentially as described (Yamamoto et al., 1970), except that JM101 was grown in L Broth and phage precipitation was carried out by the addition of 20% PEG-6000, 2.5 M NaOAc (pH 5.5) (Messing, 1983).

F. Preparation of Double Stranded M13 'Phage DNA

Double stranded (RF) M13 'phage DNA was prepared from infected JM101 cells as described by Crouse et al., (1983). JM101 cells were grown to log phase, inoculated with M13 phage and grown for an additional 4 hr. RF DNA was purified by centrifugation in CsCl-EtBr gradients (Radloff et al., 1967).

2.26 DNA Digestion, Gel Electrophoresis, Southern Blotting and Hybridization to Radioactive DNA Probes

A. Buffers and Solutions (All stored at RT)

EBOGLY (EDTA - BPB - Glycerol electrophoresis loading buffer): 10 mM EDTA (pH 8.3), 0.01% (w/v) BPB, 10% (v/v) glycerol, stored at 4°C.

Southern Hybridization Buffer: 0.75 M NaCl, 75 mM sodium citrate, 50 mM $NaPO_4$ (pH 5.5), 0.2% (w/v) SDS, 2 mg/ml denatured and sonicated herring sperm DNA, 1X Denhardt's.

Restriction Enzyme Digestion Buffers (All stored at -20°C.):

1X High Salt: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1 mM DTT.

1X Medium Salt: 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1 mM DTT.

1X Low Salt: 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1 mM DTT.

1X EcoRI buffer: 50 mM NaCl, 100 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 7 mM DTT, 0.01% (w/v) BSA.

1X SmaI buffer: 15 mM KCl, 15 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$.

B. Digestion and Electrophoresis of MtDNA

1 - 3 µg of mtDNA was digested in a 20 µl solution which containing the appropriate digestion buffer (ie. High, Med, Low, SmaI, EcoRI, for reference see Maniatis et al. (1982)) and 10 - 30 units of the restriction enzyme, at 37°C for at least 1 hr. 2 µl of EBOGLY was added and the sample was loaded into a preformed well of a 0.8% (w/v) (SeaKem) agarose, 1X TBE gel. Usually, horizonal slab gels were cast in a BRL 25 x 20 cm gel apparatus. Gels were run submerged in 1X TBE buffer at 50 V until the BPB had migrated 20 cm. Gels were stained by immersion in an solution of 0.5 µg/ml EtBr and photographed as described for RNA gels (Section 2.21 B).

C. Digestion and Electrophoresis of Plasmid and M13 RF DNA

Plasmid and M13 RF DNA was digested, electrophoresed and photographed in a manner identical to that of mtDNA except that 1 - 5 units of restriction enzyme was used to digest 1 µg of DNA and the DNA was fractionated in agarose gels of varying composition. The guidelines for the percent (w/v) agarose, 1X TBE gel used are listed below:

Fragments to be Resolved (Kb)	% (w/v) agarose
< 1	2
> 1	1
> 2.5	0.8

D. Molecular Weight Calculation of DNA Fragments

The molecular weight of digested DNA was determined from the mobility relative to that of molecular weight standards run in parallel tracts. For fragments between 1 and 10 Kb, λts857 digested with HindIII was used as a molecular weight standard. The λts857 fragments generated by HindIII digestion are 23, 9.4, 6.6, 4.3, 2.3, 2.0, 0.6, and 0.1 Kb (Rickwood and Hames, 1982). Fragments <1 Kb were sized using pBR322 DNA fragments generated by digestion with various endonucleases (Sutcliffe, 1979).

E. Transfer of DNA Fragments to Nitrocellulose

Following digestion with restriction endonucleases and electrophoresis, DNA was routinely transferred to nitrocellulose by the protocol of Southern, (1975), as modified by Wahl, et al. (1979) or Palmer and Shields, (1982) and probed with radioactive DNA probes.

F. Prehybridization and Hybridization

Southern blots were placed in a polyethlene bag containing Southern hybridization buffer (0.5ml/cm^2) and prehybridization was carried out at 65°C for at least 30 min. Thermally denatured probes were injected directly into the bag containing the filter and hybridization was carried out at 65°C for 18 - 48 hr on a rocking table. Blots were washed at 65°C in two changes of 2X SSC, 0.1%

(w/v) SDS and two changes of 0.1X SSC, 0.1% (w/v) SDS. Each wash was for 15 min in 65°C wash solutions. Blots were dried between paper towels,covered with Saran Wrap and exposed to X-ray film at RT or -80°C.

Southern bolts were washed at 85°C for 1 hr in two changes of 0.1X SSC, 0.1% SDS, prior to hybridization with a second probe.

2.27 Radioactive Labelling of DNA

Buffers

10X Nick-translation buffer: 0.5M Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 10 mM DTT, stored at -20°C.

DNase I solution: 22.5 mM Tris-HCl (pH 7.5), 45 mM $(NH_4)SO_4$, 0.45 (w/v) BSA, 4.5 mM BME, 50% glycerol, 5×10^{-7} µg/ml DNAse I, stored at -20°C.

10X anneal buffer: 100 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$.

Second strand synthesis buffer: 1.5 µM dATP, 1.5 µM dGTP, 1.5 µM dTTP, 5mM Tris-HCl (pH 8.0), 0.1 mM EDTA, stored at -20°C

Sanger Chase: 0.5 µM dATP, 0.5 µM dCTP, 0.5 µM dGTP, 0.5 µM dTTP, stored at -20°C.

A. Nick-translation of DNA

Nick-translation utilizes E. coli DNA Pol I for repair synthesis of DNA which has been nicked by DNase I (Rigby et al., 1977). Reactions contained 0.1 - 1µg of DNA, 3 µl of 10X nick translation buffer, 10 µCi α-[^{32}P]dCTP, 3 µl each of 1 mM dATP, dGTP, and dTTP, and distilled water to give a final volume of 28 µl. 1 µl of DNase I solution was added and the reaction was incubated on ice for 1 min. 1 µl of E. coli DNA Pol I (1 unit/µl) was added and the reaction mixture was incubated at 14°C for 15 min and stopped by the addition of 30 µl of TE80. Unincorporated α-[^{32}P]-dCTP was removed by passage through a G-50 Sephadex spin column in a 1ml syringe (See below). This protocol routinely gave 2 - 5 X 10^6 c.p.m./µg DNA.

B. Second Strand Synthesis of DNA

Single stranded M13 DNA clones were labeled with α-[^{32}P]dCTP by the in vitro DNA synthesis procedure described by Messing (1983). The DNA was hybridized to a synthetic primer, either the 'Probe Primer' or the 'Sequencing Primer', and synthesis of labelled DNA was promoted by Klenow Large Fragment Polymerase of E coli. The 'Probe Primer' hybridizes 5' to the cloned insert and promotes the synthesis of M13 DNA. In contrast, the 'Sequencing Primer' promotes synthesis of labelled copy of the cloned insert.

8 µl (ca. 0.5 µg) of single stranded M13 DNA, which had been prepared as described (Section 2.25 B) was mixed with 1 µl of 10X anneal buffer and 1 µl of a 15 bp primer (New England Biolabs)(2.5ng/µl) in a 1.5 ml centrifuge tube and heated to 65°C for 15 min in a dry block. The dry block was removed from the heat source and allowed to cool to 40°C prior to removal of the reaction tube. The tube was centrifuged for 30 sec to pellet any solution which had condensed on the lid of the tube and the following reagents were added to the 10 µl of annealed DNA mix: 9 µl of second strand synthesis buffer, 9 µl of 10 mM Tris-HCl (ph 8.0), 10 µCi of α-[^{32}P]dCTP, and 1 unit of Klenow Large Fragment Polymerase. The reaction was centrifuged for 30 sec and incubated at RT for 30 min. Then, 5µl of Sanger chase and 5 µl of TE80 were added and the reaction incubated for an additional 30 min prior to the removal of unincorporated mononucleotides by passage through G-50 Sephadex spin colum (See Section D below).

Radioactive DNA probes made in this manner using the 'Probe Primer' were not heated above 60°C, in order not to separate the radiolabelled M13 DNA from the M13 DNA strand which contained the cloned insert. Probes made using the 'Sequencing Primer' were thermally denatured at 100°C for 5 min and quenched on ice prior to hybridization.

C. End-labelling of DNA with 3' Recessed Ends:

DNA digested with a restriction enzyme which generates 3' recessed ends (such as Sau3A, HindIII, TaqI, HinfI, EcoRI) were labelled using the Klenow fragment of E. coli DNA polymerase (Maniatis et al., 1982). 1.5 µg of DNA was digested and 0.5 µg was electrophoresed on an agarose gel to check that the digestion was complete. The restriction enzyme was denatured by heating at 65°C for 5 min and the DNA labelled by the addition of 10 µCi α-[^{32}P]dCTP, 0.5 mM dATP, dGTP, dTTP, and 1 unit of the Klenow fragment of DNA polymerase. The reaction was carried out at RT for 30 min.

D. Removal of Mononucleotides from DNA with G-50 Sephadex

G-50 Sephadex 'spin columns' were used to remove unincorporated mononucleotides from labelling reactions (Maniatis et al., 1982). G-50 Sephadex (fine) was swollen by autoclaving in TE80. A 1 ml syringe was plugged with glass wool and filled with 1 ml of G-50 Sephadex solution. The spin column was prepared by centrifugation in a swing-out rotor at 500 X g_{max} for 2 min. The radioactive sample was pipetted on top of the column and the elutant was recovered in a 1.5 ml tube by centrifugation at 750 X g_{max} for 3 min. The incorporation of radioactive nucleotides into DNA samples was determined by Cherenkov counting (Marshall, 1952).

2.28 Recovery of DNA from Agarose Gels

DNA fragments were routinely recovered from agarose (SeaKem) gels by electroelution or from low melting temperature agarose gels (Maniatis et al., 1982). DNA recovered by these protocols was sufficiently clean for cloning, labelling and use in S_1 transcript mapping experiments. The efficiency of electroelution was determined by electrophoresis of an aliquot of the elutant or by monitoring of radioactive elutant.

2.29 Construction of Recombinant Plasmids Containing MtDNA Fragments

A recombinant DNA library was constructed in the plasmid pNS1 (Nikolnikov et al.,1984), in E. coli HB101 cells (Section 2.25). Specific DNA fragments, which had been eluted from agarose gels (Maniatis et al., 1982) were also cloned into pNS1.

pNS1 is a pBR327 derivative which contains a 906 bp Sau3A fragment of lambda-434 C_I repressor gene. The promoter of the tetracycline resistance gene of pBR327 has been removed, and therefore the expression of tetracycline resistance is under the control of the C_I gene. E.coli cells containing pNS1 are ampiclillin resistant and tetracycline sensitive unless the C_I gene has been inactivated by cloning into the unique EcoRI, HpaI or HindIII site. Positive selection of bacterial transformants was carried out on plates containing 10 µg/ml tetracycline and 50 µg/ml ampicillin.

A. Ligation Reaction

Buffers and Solutions

10X Ligase buffer: 660 mM Tris-HCl (pH 7.2), 100mM $MgCl_2$, 100 mM DTT, 1mM ATP, 10 mM EDTA, stored at -20°C.

TEN buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl.

1 µg of mtDNA and 1 µg of pNS1 was digested to completion with EcoRI. The enzyme was deactivated by heating at 65°C for 10 min. The digests were checked by agarose gel electrophoresis of an aliquot of each reaction. The ligation of restricted mtDNA and plasmid DNA was set up as follows: 300 ng mtDNA, 100 ng pNS1, 2µl 10X ligase buffer, TEN buffer to 19 µl and 1 unit of T_4 DNA ligase. The mixture was incubated at 14°C overnight, and stored at 4°C. A ligation of 100 ng of pNS1 was set up as a control.

B. Transformation of HB101

Competent HB101 cells were prepared by the technique of Dagert and Ehrlich (1979). The $CaCl_2$ treated cells were used immediately or were frozen in 100 mM $CaCl_2$, 10% glycerol on dry ice and stored at -80°C. 100 µl of competent cells were used to transform:

1) 5 µl of ligated plasmid,
2) 100 µl of ligated plasmid, and
3) 100 ng of uncut plasmid.

The transformations were carried out in a sterile glass test tubes on ice for 30 min. The cells were then heat-shocked at 37°C for 5 min, 2 ml of L broth was added and incubation continued at 37°C for 1 hr. 200 µl of cells were spread onto L broth agar plates containing 10 µg/ml tetracycline and 50 µg/ml ampicillin and incubated overnight at 37°C. As a control, 200 µl of cells were spread onto L broth agar plates containing 50 µg/ml ampicillin.

Transformation and antibiotic selection efficiency was determined by comparison of the control and selection plates. If few transformants containing undigested plasmid DNA grew on the selection plates, the recombinant DNA transformants were considered authentic. Both tetracycline and ampicillin were required for selection, since self-ligated pNS1 also confers tetracycline resistance. Recombinants containing double inserts of mtDNA fragments were occasionally found in the plasmid library.

C. Preparation of Filter Replicas

Plasmid DNA was preserved on Whatman 541 filter paper replicas for colony hybridization experiments, and the E.coli cells containing recombinant DNA plasmids were cryo-preserved in Corning microtitre dishes at -80°C (Gergen et al., 1979). The plasmid DNA library was arranged in 6 X 8 colony arrays on the filters and in the microtitre dishes.

D. Clone Identification

Filter replicas were probed with [^{32}P]-labelled DNA probes (Section 2.27), which contained no homology to pBR322 derived plasmid DNA. Hybridization was in Southern hybridization buffer, essentially as described in Section 2.26 F. Plasmid DNA was prepared from positively hybridizing colonies as described (Section 2.25 C). The plasmid DNA was resuspended in 30 µl of TE80 and 15 µl was removed and digested with EcoRI. The DNA was fractionated by agarose gel electrophoresis, photographed, transferred to nitrocellulose, and re-probed (Section 2.26) to positively identify cloned DNA fragments.

2.30 Bacteriophage M13 Cloning Techniques: Construction of M13 mp Clones

MtDNA fragments where cloned into M13mp strains by 'shotgun' or 'forced' cloning techniques (Messing, 1983). Recombinant DNA clones were identified on minimal agar plates, by an *in situ* color reaction, in the presence of IPTG and Xgal. Infected JM101 cells form blue plaques on minimal plates when the M13mp 'phage expresses *lac* and thereby complements the defective host *lac* gene. Cells transformed with M13mp containing a DNA insert, which interrupts the phage *lac* DNA, are colorless. Plaques were identified by hybridization techniques (Benton and Davis, 1977; Messing, 1983). M13 mp clones were used as hybridization probes, for DNA sequence analysis and as a hybridization template for S_1 transcript mapping studies.

2.31 DNA Sequencing Analysis

A. Sequencing Reaction

DNA sequence analysis was by the chain-termination method using α-[^{32}P]dCTP (Sanger *etal.*, 1978; Sanger *et al*, 1980; Messing, 1983). Sequencing reactions were carried out at RT. To reduce artifacts caused by secondary structure formation reactions were also carried out at 55°C. Sequencing reactions were analyzed by electrophoresis in 6 - 8% acrylamide gels (8M Urea, 6 or

8% (w/v) acrylamide, 0.25 (w/v) bis-acrylamide, 1X TBE, 0.1% (w/v) AMPS, 0.005% TEMED).

B. Computer Analysis of Sequencing Data

DNA sequence analysis was implemented by the programs of the University of Wisconsin Genetics Group (UWGCG), the Wisconsin Genebank and EMBL Database (Release 6). DNA sequences were also assembled and analyzed using sequencing programs on the Apple IIe. Dr. P.G. Isaac provided assistance in the use of computer programs for DNA sequence analysis.

Chapter III

MITOCHONDRIAL GENOMES, GENES AND GENE PRODUCTS IN SORGHUM

3.1 Aims and Rationale

Various cytoplasmic genotypes (cytoplasms) of sorghum have been characterized by restriction endonuclease digestion of mtDNA and examination of mitochondrial translation products (Pring et al., 1982; Conde et al., 1982; Dixon and Leaver, 1982). From these analyses a number of unique cytoplasms have been identified. Differences in mitochondrial genomes, gene location and gene products were critically examined in order to further characterize the interspecific variation and to ultimately determine differences which are specifically associated with expression of the CMS phenotype.

1) The molecular complexity of the sorghum mitochondrial genome was estimated by the summation of mtDNA fragments generated by restriction digestion.

2) Maize mitochondrial gene probes were used to identify the homologous sequence in four sorghum lines. The EcoRI fragment location(s) of COXI, COXII, COB, and ATPA were determined.

3) Specific protein coding genes were isolated from the sorghum line Kafir nucleus in 9E cytoplasm (referred to as 9E). A recombinant plasmid DNA library of EcoRI generated mtDNA fragments was made in the pBR327 derived plasmid pNS1 of E. coli (Nikolnikov et al., 1984). [^{32}P]-labelled DNA probes from the maize genes encoding COI, COII, COB and α-F_1ATPase were used to identify plasmids containing the homologous sequence in the 9E mtDNA library.

4) MtRNA transcripts from each gene were characterized by northern hybridization analysis.

5) Sorghum mitochondria were isolated and allowed to synthesize proteins in the presence of L-[^{35}S]S-methionine. Mitochondrial proteins were fractionated by two-

dimensional isoelectric focussing and SDS-polyacrylamide gel electrophoresis to estimate the number of polypeptides synthesized by Kafir, Milo and 9E cytoplasm.

6) Several mitochondrial proteins were identified using antisera prepared against yeast polypeptides and the western immunolabelling technique.

B. RESULTS

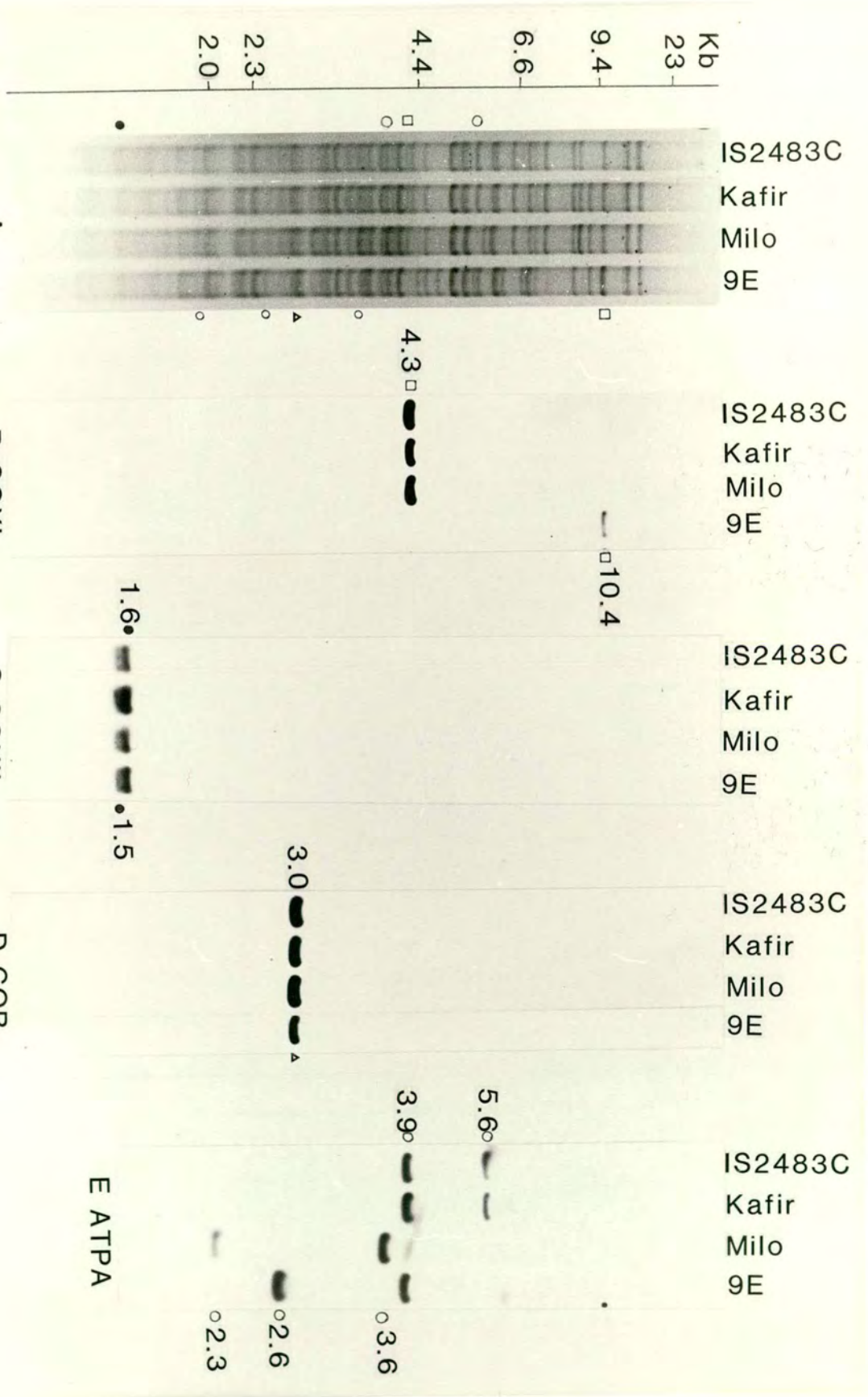
3.2 Estimation of the Minimum Molecular Weight of the Sorghum Mitochondrial Genome

Mitochondria were purified from four lines of sorghum with different cytoplasmic genotypes. MtDNA was isolated, digested with EcoRI, fractionated by electrophoresis in a 0.8% (w/v) agarose gel, stained with EtBr and photographed under UV light (Figure 3.1 A). Upon examination of the gel photograph, it is evident that the EcoRI restriction pattern of IS2483C, Kafir, Milo and 9E cytoplasm mtDNA are different. From the gel photograph and from three additional EcoRI digests (not shown) the minimum molecular weight of the mitochondrial genome of IS2483C, Kafir, Milo and 9E was estimated as >230, >234, >238 and >257 Kb, respectively. The size of each EcoRI generated mtDNA fragment was estimated from its mobility relative to molecular weight markers run in adjacent lanes (Section 2.26 D). A gel densitometer scan of each digest was made so that non-equimolar fragment quantities could be considered. For example, the third largest EcoRI fragment of 9E mtDNA (Figure 3.1; []) was counted as two fragments since the densitometric peak area of this band was approximately twice that of similar sized fragments. The minimum molecular weight of each genome was estimated from the summation of the EcoRI fragments. Since numerous fragments <1Kb could not be resolved, these molecular weight calculations are minimum estimates.

Figure 3.1

Identification of EcoRI Generated MtDNA Fragments Containing COXI, COXII, COB, and ATPA Genes in Sorghum.

MtDNA from IS2483C, Kafir, Milo and 9E cytoplasm was digested with EcoRI, fractionated by electrophoresis in a 0.8% (w/v) agarose gel, stained with EtBr and photographed (A). The mtDNA was transferred to nitrocellulose and probed sequentially with [^{32}P]-labelled clones containing a portion of a maize mitochondrial gene and autoradiographed. The Southern blot was washed before re-probing. The EcoRI fragment(s) (Kb) which hybridized to each gene probe is indicated and the corresponding fragments on the EtBr stained gel are denoted with symbols: COXI ■ (B), COXII ● (C), COB ▲ (D), and ATPA ○ (E). Molecular weight markers were HindIII restriction fragments of lambda phage-DNA.


Kb
23
9.4
6.6
4.4
2.3
2.0
IS2483C
Kafir
Milo
9E
A
4.3
B COXI
10.4
1.6
1.5
C COXII
3.0
D COB
5.6
3.9
2.6
3.6
2.3
E ATPA

3.3 Identification and Characterization of the EcoRI Fragments Containing the Mitochondrial Genes for COXI, COXII, COB and ATPA

Maize mitochondrial gene probes were used to identify the homologous sequence of sorghum mtDNA. EcoRI digested mtDNA was fractionated by agarose gel electrophoresis (Figure 3.1 A), transferred to nitrocellulose (Southern, 1975) and probed under stringent conditions with [^{32}P]-labelled clones containing an internal portion of the maize COXI, COXII, COB, or ATPA mitochondrial gene (Table 3.1) and autoradiographed.

Table 3.1.
Maize Mitochondrial Gene Probes Used to Identify Homologous Sequences in Sorghum MtDNA

GENE	PROBE DESIGNATION	ORIGIN	SIZE (KB)	VECTOR	REFERENCE
COXI	M3B2	internal	0.2	M13	Isaac etal.,1985
COXII	pZmE1	entire ORF	1.9	pBR	Fox and Leaver,1981
COB	ZmEH680 pZmEH680	internal	0.68	M13 pBR	Dawson et al.,1983
ATPA	SalD5	5'-ORF	0.45	M13	Isaac etal., 1985b

COXI: The maize COXI probe hybridized to one of two EcoRI fragments, depending upon the cytoplasm examined: a 4.3 Kb EcoRI mtDNA fragment in Kafir, Milo and IS2483C, and a 10.4 Kb EcoRI fragment in 9E (Figure 3.1 B).

COXII: The maize COXII probe hybridized to two EcoRI mtDNA fragments in all four cytoplasms: a 1.6 Kb fragment and to a lesser extent to a 1.5 Kb fragment (Figure 3.1 C).

COB: The maize COB probe hybridized to a 3.0 Kb EcoRI generated mtDNA fragment in all four sorghum lines (Figure 3.1 D).

ATPA: The maize ATPA probe hybridized with the same relative intensity to two EcoRI mtDNA fragments in each sorghum line (Figure 3.1 E). The identified fragments were: 5.6 and 3.9 Kb in IS2483C and Kafir; 3.6 and 2.3 Kb in Milo and 3.9 and 2.6 Kb in 9E. This hybridization result indicates that at least the 5' end of ATPA gene is present in two genomic locations in the sorghum lines examined. Therefore, sorghum may be similar to B37N maize in which two complete copies of ATPA have been identified and sequenced (Isaac et al., 1985b).

3.4 Cloning of Specific Mitochondrial Genes

3.4 A Construction of a Plasmid Library of EcoRI Digested 9E MtDNA and Gene Clone Identification

Mitochondrial DNA was isolated from the sorghum line 9E, digested to completion with EcoRI, ligated into the EcoRI site of plasmid pNS1 (Nikolnikov, et al., 1984), and used to transform competent E. coli HB101 cells (Section 2.29). Transformants were selected by growth on L Broth agar plates containing 10 ug/ml tetracycline and 25 ug/ml ampicillin. Approximately 600 tet^r, amp^r colonies were obtained. Filter replicas of the mtDNA library were prepared on Whatman 541 filters and clones stored in microtitre dishes at -80°C (Gergen et al.,1979).

The maize mitochondrial probes listed in Table 3.1 were used to identify the homologous sequence in the 9E mtDNA library by colony hybridization, under stringent conditions. Plasmid DNA was isolated from individual E.coli colonies which gave a positive hybridization signal (Birnboim and Doly, 1979). The plasmid DNA was digested with EcoRI, fractionated by agarose gel electrophoresis, transferred to nitrocellulose (Southern, 1975) and re-probed with the specific maize gene probe to confirm the identity of the clone.

COXI: A [^{32}P]-labelled clone containing an internal portion of the maize COXI gene was hybridized to the 9E mtDNA library. Four positively hybridizing clones which contained a 10.4 Kb EcoRI fragment were identified by re-hybridization with the maize probe (Figure 3.2 A). One of these clones was designated pS9E10.4 and further characterized. The plasmid pS9E10.4 was digested with hexanucleotide recognizing restriction endonucleases and a restriction map was constructed (Figure 3.3 A). Restriction fragments of pS9E10.4 with homology to the maize COXI gene were identified by hybridization with a [^{32}P]-labelled M13 clone containing an internal portion of the maize gene (not shown). The 9E COXI gene is located on a 2.2 Kb BamHI - PstI fragment (Figure 3.3 A) and was subsequently sequenced (See Chapter IV).

COXII: The 2.2 Kb EcoRI insert of pZmE1 was purified (Section 2.28), [^{32}P]-labelled by nick translation (Rigby et al., 1977), and hybridized to the replica filters of the 9E mtDNA library. A clone which contained a 1.6 Kb EcoRI fragment insert was identified (Figure 3.2 B). This clone, designated pS9E1.6, hybridized to a [^{32}P]-labelled clone containing a portion of the maize COXII exon I (Figure 3.2 C) (Fox and Leaver, 1981). This result indicates that the cloned 1.6 Kb EcoRI fragment contains at least the 5' portion of the COXII ORF. A clone containing a 1.5 Kb EcoRI fragment insert, a second fragment of sorghum mtDNA which hybridized to pZmE1, was not isolated in this experiment.

A preliminary restriction map of pS9E1.6 was constructed and the sites observed suggest that the sorghum COXII, like wheat and rice, contains an intron which is larger than in maize (data not shown).

COB: A [^{32}P]-labelled M13 clone which contains an internal portion of the maize COB gene was hybridized to the 9E mtDNA and a clone containing a 3.0 Kb EcoRI insert was identified (Figure 3.2 C). This clone has been designated pS9E3.0 and awaits further characterization.

ATPA: A [^{32}P]-labelled M13 clone containing a portion of the maize ATPA gene was used to identify clones containing homologous sequence in the 9E mtDNA library. A single clone which contained a 3.9 Kb EcoRI generated insert was identified (Figure 3.2 D). The 9E ATPA clone candidate, designated pS9E3.9, was subjected to single and double digestion by hexanucleotide recognizing restriction enzymes that are known to digest the maize ATPA gene (Figure 3.4) (Isaac, et al., 1985b). A 2 Kb portion of the pS9E3.9 restriction map is identical to the map of the 2 Kb EcoRI fragment which contains the maize ATPA gene. Restriction site homology between the EcoRI fragment containing the maize ATPA gene and pS9E3.9 is lost after the ClaI site beyond the carboxy terminus of the maize gene (Ibid). This analysis indicates that the 3.9 Kb fragment probably contains a complete copy ATPA.

3.4 B Cloning of the COXI Gene from Milo and Kafir Cytoplasms

The COXI gene of Milo and Kafir cytoplasm is located on a 4.3 Kb EcoRI mtDNA fragment (Figure 3.1 B). A 4.3 Kb EcoRI fragment of Milo mtDNA which hybridizes to the maize COXI gene was cloned into pBR325 in collaboration with Dr. D.K. Hanson. Similarly, the homologous 4.3 Kb EcoRI fragment of Kafir mtDNA was cloned into pNS1 and designated pSK4.3. The restriction map of the 4.3 Kb EcoRI inserts of pSM4.3 and pSK4.3 were identical for the hexanucleotide recognizing endonucleases used (Figure 3.3 B).

Figure 3.3 (pg 90)

Restriction Map of COXI Clones: pS9E10.4, pSM4.3 and pSK4.3

Hexanucleotide-recognizing endonuclease sites were mapped on clones containing homology to the maize COXI gene:

(A) pS9E10.4, 10.4 Kb EcoRI fragment of 9E cytoplasm mtDNA.

The restriction sites of HindIII (H), BamHI, (B), PstI (P), BglII (Bg), and XhoI (X) were mapped on the 10.4 Kb EcoRI (RI) fragment of 9E cytoplasm. No sites for SalI were identified on this fragment. The approximate location of COXI was determined by hybridization of a [^{32}P]-labelled COXI gene probe to a Southern blot of restricted plasmid DNA (not shown).

(B) pSM4.3 and pSK4.3: the clones containing the 4.3 Kb EcoRI fragment of Milo and Kafir cytoplasm mtDNA, respectively.

The restriction sites of HindIII (H) and BamHI were mapped on the clones pSM4.3 and pSK4.3. No sites for PstI, BglII, XhoI, or SalI were found within the 4.3 Kb inserts of these clones. The restriction maps of pSM4.3 and pSK4.3 are identical.

Figure 3.4 (pg 91)

Restriction Map of ATPA Clone: pS9E3.9

Restriction map of hexanucleotide-recognizing endonuclease sites on pS9E3.9, the 3.9 Kb EcoRI mtDNA fragment of 9E cytoplasm which contains homology to the maize ATPA gene.
The restriction sites of ClaI (C), SmaI (Sm), BamHI (B) and SalI (S) on the 3.9 Kb EcoRI (RI) mtDNA fragment were identified. The approximate location of the ATPA gene on this fragment was determined by hybridization of a [^{32}P]-labelled maize ATPA gene probe to a Southern blot of restricted plasmid DNA (not shown).

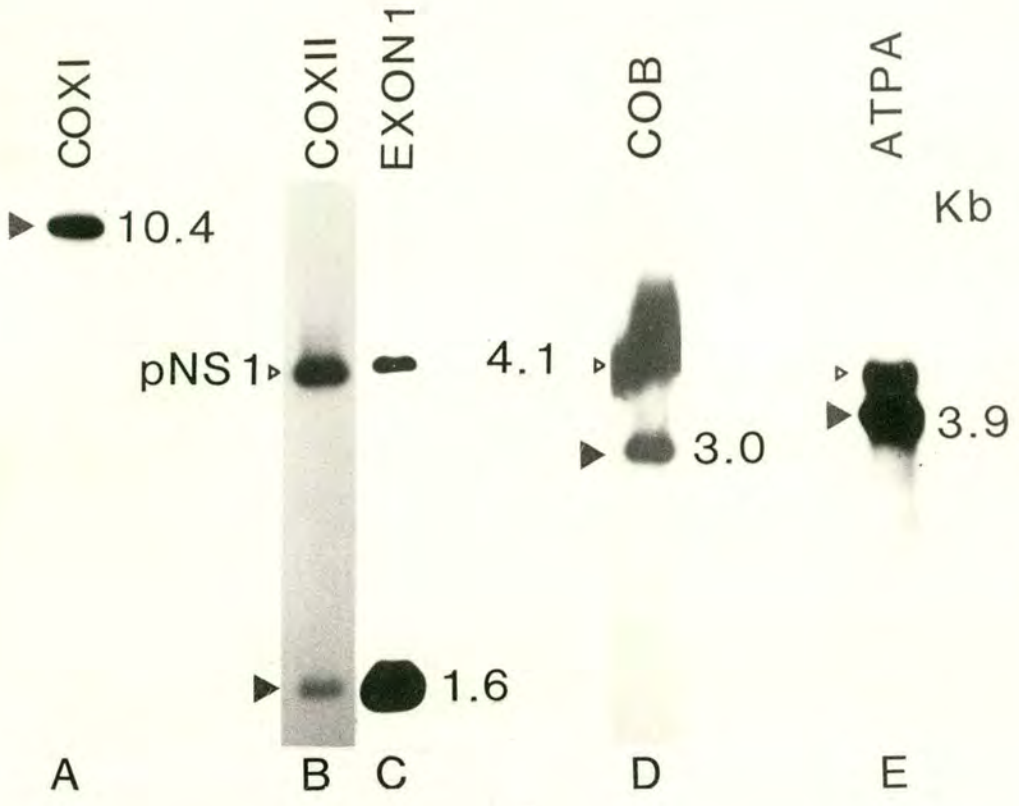


Figure 3.2

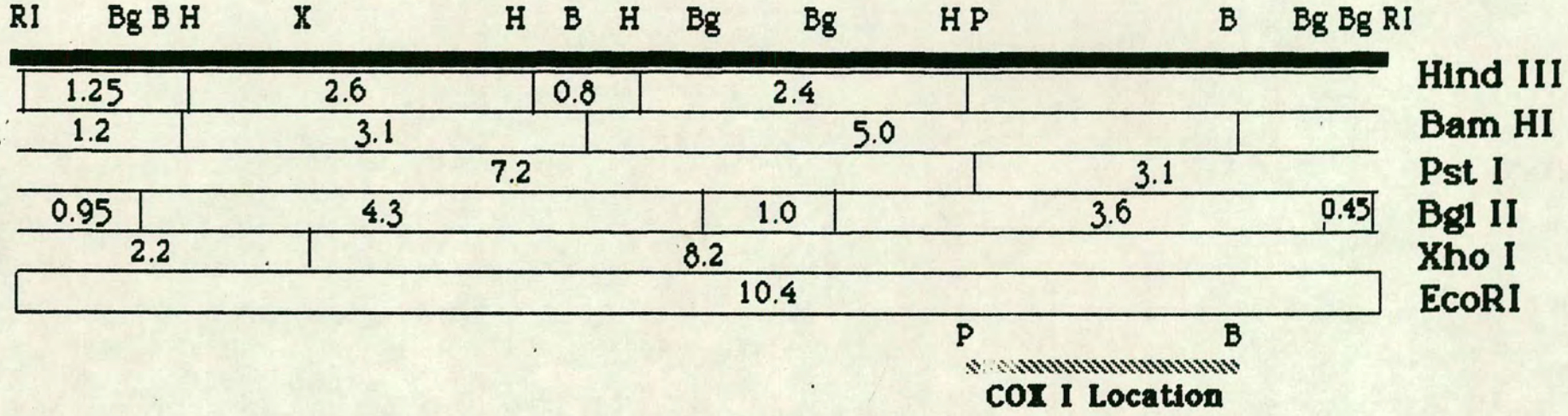
Identification of Clones from the 9E MtDNA Library with Sequence Homology to Maize Genes: COXI (A), COXII (B, C), COB (D), and ATPA (E).

Plasmid DNA was isolated from positively hybridizing clones, digested with EcoRI, stained with EtBr, photographed (not shown), transferred to nitrocellulose, hybridized with a [^{32}P]–labelled maize gene probe and autoradiographed. Representative clones showing positive hybridization:

(A) COXI: 10.4 Kb EcoRI fragment of clone pS9E10.4 (M13 probe).
(B) COXII:1.6 Kb EcoRI fragment of clone pS9E1.6 (plasmid probe).
(C) COXII:1.6 Kb EcoRI fragment of clone pS9E1.6 (plasmid probe containing exon I sequence).
(D) COB: 3.0 Kb EcoRI fragment of clone pS9E3.0 (plasmid probe) (E) ATPA: 3.9 Kb EcoRI fragment of clone pS9E3.9 (plasmid probe).

fig. 3.3

A. pS9E10.4 Restriction Map



B. pSM4.3 Restriction Map

pSK4.3

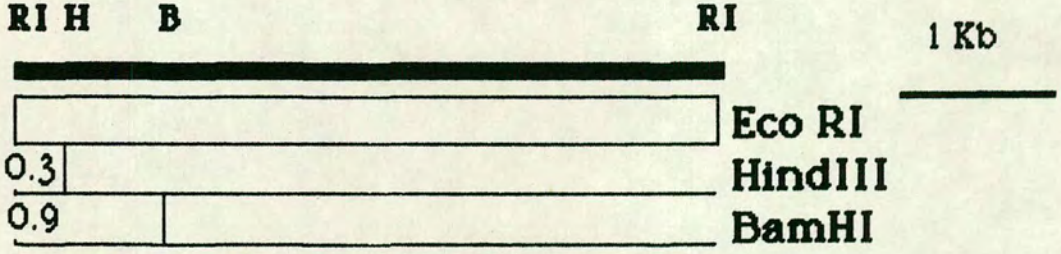
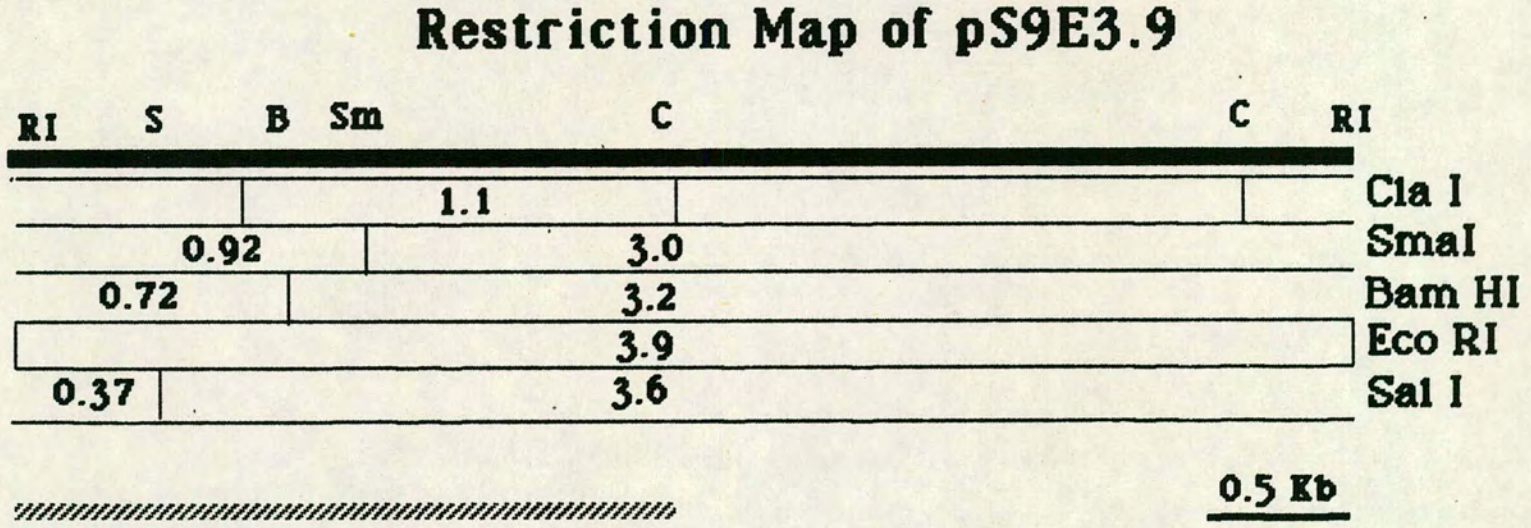


Fig. 3.4


Restriction Map of pS9E3.9
RI
S
B
Sm
C
C
RI
1.1
0.92
3.0
0.72
3.2
3.9
0.37
3.6
Cla I
SmaI
Bam HI
Eco RI
Sal I
0.5 Kb
Region homologous to maize ATPA

3.5 Transcription of Mitochondrial Genes in Sorghum

Mitochondrial transcripts of COXI, COXII, COB and ATPA genes have been detected in maize (Isaac et al., 1984; Fox and Leaver, 1981; Dawson et al., 1983; Jones, 1984). Northern hybridization analysis was performed to determine if the homologous genes are transcribed in sorghum mitochondria (Section 2.22). MtRNA was isolated from purified mitochondria as described (Section 2.20) and fractionated by electrophoresis in a 1.3% (w/v) agarose, 17.3% (v/v) formaldehyde gel. A portion of the gel was stained with EtBr and photographed to visualize the molecular weight markers: E.coli 23S and 16S rRNA and CCMV RNA (Figure 3.5 I). The unstained portion of the gel was transferred to nitrocellulose and probed with [^{32}P]-labelled DNA containing a portion of a maize mitochondrial gene (Table 3.1).

COXI: A northern blot of IS2483C, Kafir, Milo, 9E sorghum and maize B37N mtRNA was used to identify mtRNA with homology to an internal portion of the COXI gene (Figure 3.5 II). The COXI gene probe hybridized to at least three gene specific transcripts of IS2483C, Kafir and Milo (Figure 3.5 II, Lanes A,B,C) and to a single, different transcript of 9E (Figure 3.5 II, Lane D) (See Chapter IV). Thus, COXI transcription appears to be determined by gene location: when the gene is located on a 4.3 Kb EcoRI fragment as in IS2483C, Kafir and Milo the major transcript is 1.8 Kb, whereas when the gene is located on a 10.4 Kb EcoRI fragment the major transcript is 2.2 Kb. The probe hybridized to two maize COXI transcripts of 2.1 and 2.2 Kb (Figure 3.5 II, Lane E) (values are lower than those calculated by Isaac et al., (1985)).

COXII: The blot shown in Figure 3.5 II was washed and probed with [^{32}P]-labelled pZmE1 which contains the maize COXII ORF and intron. The probe hybridized strongly to two sorghum mtRNAs of approximately 1.3 and 0.9 Kb, and at

least five minor, larger transcripts (Figure 3.5 III, Lanes A - D). These less abundant transcripts were similar to the major transcripts of maize COXII (Figure 3.5 III, Lane E). Detailed northern transcription analysis of COXII in maize has revealed that circa 5 large transcripts hybridize strongly to intron probes (and weakly to pZmE1), and circa 5 smaller transcripts hybridize strongly to exon probes and pZmE1 (Jones, 1984). Thus, the transcription of COXII is complex in both maize and sorghum seedling mitochondria. Further analysis of the sorghum COXII transcripts is required to determine if the abundant 1.3 Kb and 0.9 Kb transcripts are homologous to intron or exon sequences.

COB: Transcription of COB appeared to be identical in the four sorghum lines examined (Figure 3.5 IV). A major COB transcript of 1.8 Kb and two less abundant transcripts of 2.4 and 3.8 Kb were detected with the labelled maize gene probe (the 3.8 Kb transcript was only observed after prolonged exposure and is not visible in this photograph).

ATPA: The blot used to identify COXI, COXII and COB mitochondrial transcripts was probed with a [^{32}P]-labelled clone containing an internal portion of the maize ATPA gene and faint hybridization signals were detected. A second northern blot was prepared and a major 1.8 Kb transcript in 9E and at least two transcripts of 2.0 and 1.8 Kb in Kafir were identified (Figure 3.5 V, Lanes A,B). Thus, as with COXI, the transcription of ATPA appears to be influenced by the genomic location of ATPA.

Figure 3.5

Identification of mtRNA Transcripts of COXI, COXII, COB and ATPA.

MtRNA was purified from seedling mitochondria, fractionated on a 1.3% (w/v) agarose 17.3% (v/v) formaldehyde gel, photographed (I) or transferred to nitrocellulose, hybridized with [^{32}P]-labelled clones of maize mitochondrial genes and autoradiographed (II, III, IV, V). The size (Kb) of the major transcript observed for each sample is indicated.

I. EtBr stained gel photograph of RNAs used as molecular weight markers:

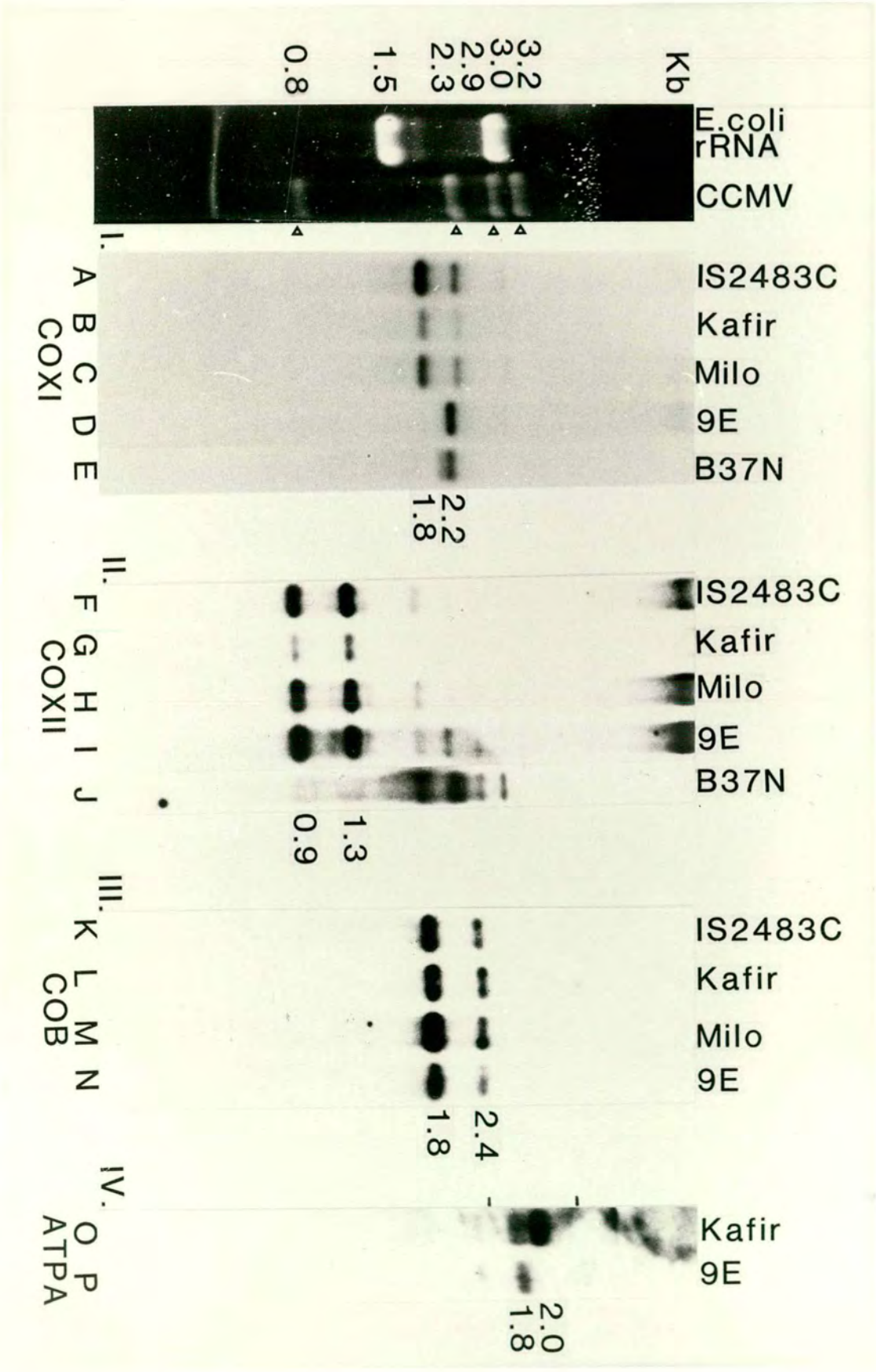
E.coli RNA	23S	2.9 Kb
	16S	1.5 Kb
CCMV	3.2, 2.9, 2.3 and 0.8 Kb	

I. Autoradiograph of IS2483C (A), Kafir (B), Milo (C), 9E (D) and B37-N maize (E) mtRNA hybridized with a clone containing an internal portion of the maize COXI gene.

II. Autoradiograph of IS2483C (F), Kafir (G), Milo (H), 9E (I) and B37-N maize (J) mtRNA hybridized with a clone containing the entire maize COXII gene.

III. Autoradiograph of IS2483C (K), Kafir (L), Milo (M) and 9E (N) mtRNA hybridized with a clone containing a portion of the maize COB gene.

IV. Autoradiograph of 9E (O) and Kafir (P) mtRNA hybridized with a clone containing an internal portion of the maize ATPA gene.


Kb
3.2
3.0
2.9
2.3
1.5
0.8
E.coli rRNA
CCMV
I.
A
B
C
D
E
COXI
IS2483C
Kafir
Milo
9E
B37N
2.2
1.8
II.
F
G
H
I
J
COXII
IS2483C
Kafir
Milo
9E
B37N
0.9
1.3
III.
K
L
M
N
COB
IS2483C
Kafir
Milo
9E
1.8
2.4
IV.
O
P
ATPA
Kafir
9E
2.0
1.8

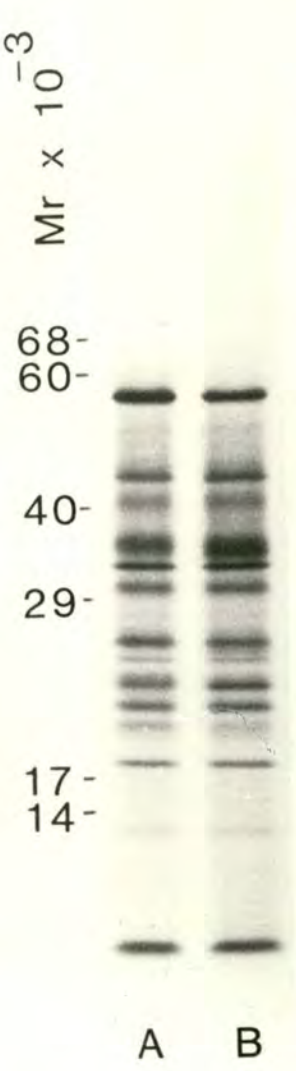
3.6 Translation Products Synthesized by Isolated Sorghum Mitochondria

Sorghum mitochondrial translation products were labelled by incubating mitochondria for 90 min in a medium containing L-[^{35}]S-methionine and an energy generating system (Leaver et al., 1983; Section 2.11). Creatine phosphate and creatine phosphokinase (CP/CPK) or succinate and ADP (succinate) were used to support protein synthesis. The incorporation of [^{35}S]-methionine and the products of mitochondrial translation using the two energy generating systems were compared. The contribution to overall incorporation by contaminating bacteria was assayed by substituting a non-oxidizable substrate, sodium acetate as the energy source.

The incorporation of [^{35}S]-methionine into TCA precipitable material was linear for 90 min with the two energy generating systems. Incorporation of input [^{35}S]-methionine was 8 - 12% with CP/CPK or succinate and only 0.5% with sodium acetate as the energy source (Figure 3.6 I). These values are similar to those observed for maize (Forde et al., 1978). Mitochondrial polypeptides were solubilized in SDS and fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel and autoradiographed (Section 2.15). About 18 similar mitochondrial polypeptides were resolved as products of the two energy generating systems (Figure 3.6.II A,B).

3.7 Two Dimensional Isoelectric Focussing and SDS-Polyacrylamide Gel Electrophoresis of Mitochondrial Proteins and Translation Products

The two-dimensional isoelectric focussing, SDS-polyacrylamide gel electrophoresis profile of mitochondrial proteins and in organello translation products from three sorghum cytoplasms was examined. Isoelectric focussing was carried out in gels ranging from pH 3.5 - 10 or pH 5 - 7, which were loaded at either the anode or cathode end. It



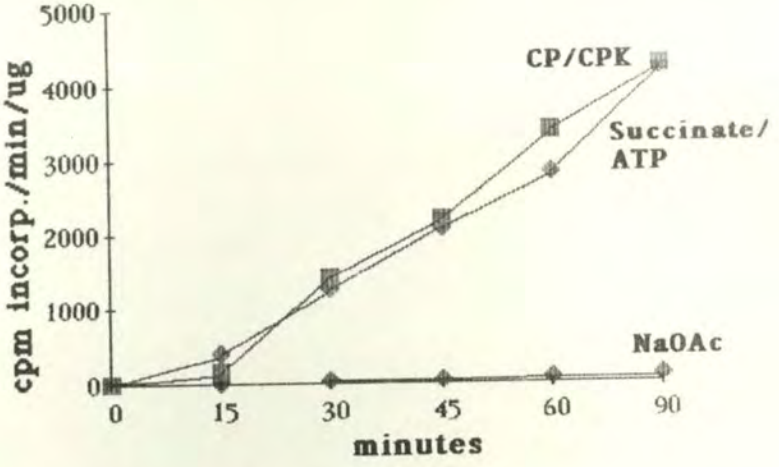


Figure 3.6

In Organello Translation Products of Sorghum

I. Incorporation of [^{35}S]-methionine by isolated 9E cytoplasm mitochondria for 90 min at 25°C using as energy substrate:

1) 10 mM succinate and 2 mM ADP (◆),
2) 8mM creatine phosphate, 25 ug of creatine phosphokinase, and 6 mM ATP (■), or
3) 20 mM sodium acetate (◆).

II. Autoradiograph of 9E cytoplasm mitochondrial proteins labelled in organello using succinate and ATP (A) or CP/CPK and ADP as the energy substrate (B). 0.25x 10^6cpm of TCA insoluble labelled protein was solubilized in 4% (w/v) SDS, heated to 100°C for 2 min and electrophoresed in a 16% (w/v) polyacrylamide gel, and exposed to X-ray film.

was determined that the optimal resolution of proteins was obtained using pH 3.5 - 10, loading gels at their basic end (O' Farrell, 1975; Section 2.18), and by molecular weight fractionation in a 16% (w/v) SDS-polyacrylamide gel (Section 2.15). Gels were stained with Coomassie blue, photographed and autoradiographed.

Approximately 75 polypeptide spots were resolved in the stained gel of Kafir, Milo and 9E mitochondrial proteins (Figure 3.7 A, 3.8 A, 3.9 A). Autoradiography of the fractionated in organello labelled translation products revealed between 80 - 100 polypeptide 'spots'. The spectrum of polypeptides synthesized by Kafir, Milo and 9E mitochondria showed reproducible differences which were quantitative (open arrows) and qualitative (solid arrows) (Figure 3.7 B, 3.8 B, 3.9 B). The significance of these differences are not known. Clusters of spots may be a single polypeptide which has undergone charge modificaions (i.e.: amidation, methylation, phosphorylation or proteolysis) in vivo or during sample preparation. At least 16 groups of apparently related polypeptide were visible in the autoradiographs. It is apparent from the streak of radioactivity at the top of the gels that a portion of the mitochondrial protein sample was not sufficiently solubilized to enter the IEF gel, perhaps because of their hydrophobic nature (eg. COI) (Cabral and Schatz, 1979).

Figures 3.7, 3.8 and 3.9

2-Dimensional Isoelectric Focussing, SDS-Polyacrylamide Gel Electrophoresis of *In Organello* Labelled Mitochondrial Polypeptides

Mitochondrial protein from Kafir (Figure 3.7), Milo (Figure 3.8), and 9E (Figure 3.9) cytoplasm.

1.0×10^6 cpm of [^{35}S]-labelled TCA insoluble mitochondrial protein (250 µg), was solubilized in 10mM K_2CO_3, neutralized with 0.5 M DTT, and electrophoresed on a pH 3.5 - 10 isoelectric focussing gel. The tube gel was equilibrated with 5% (v/v) BME and 2% SDS (w/v) and the proteins were fractionated in a second dimension in a 16% (w/v) SDS-polyacrylamide gel. Gels were stained with Coomassie blue (A), and exposed to fluorography (B).
A few quantitative (open arrows) and qualitative (solid arrows) differences amongst the mitochondrial translation products of the three cytoplasms are indicated. A variant 65K polypeptide is synthesized by Milo cytoplasm and a variant 42K polypeptide by 9E cytoplasm (Dixon and Leaver, 1982). The position of the 38K polypeptide of Milo and Kafir cytoplasms, which is replaced by the 42K polypeptide of 9E cytoplasm is indicated. Proteins of 65, 60, 40, 29, 20, 17 and 14 K dalton were run as markers (right-hand control lane of the Coomassie blue stained gels).

Fig. 3.7

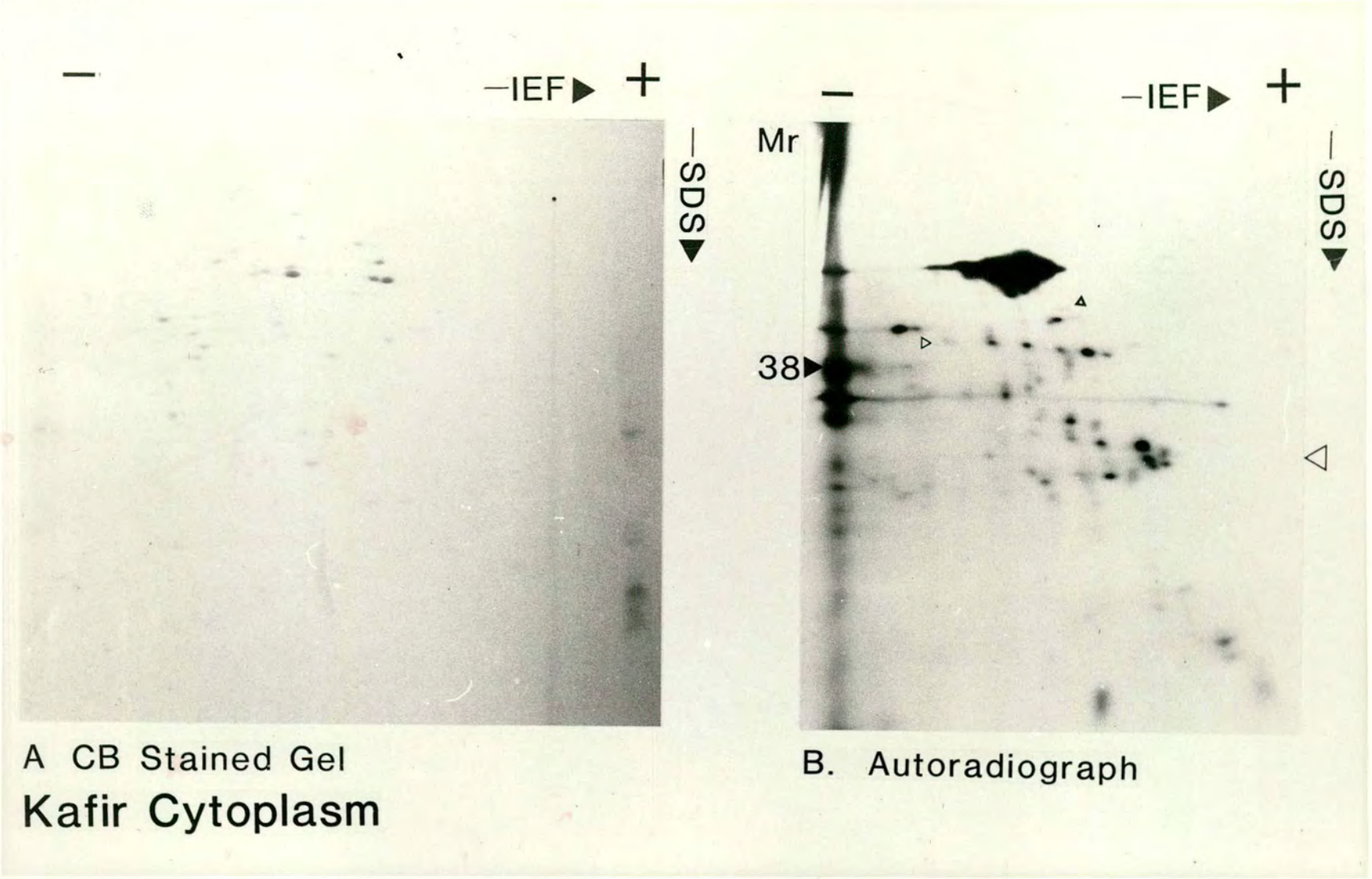
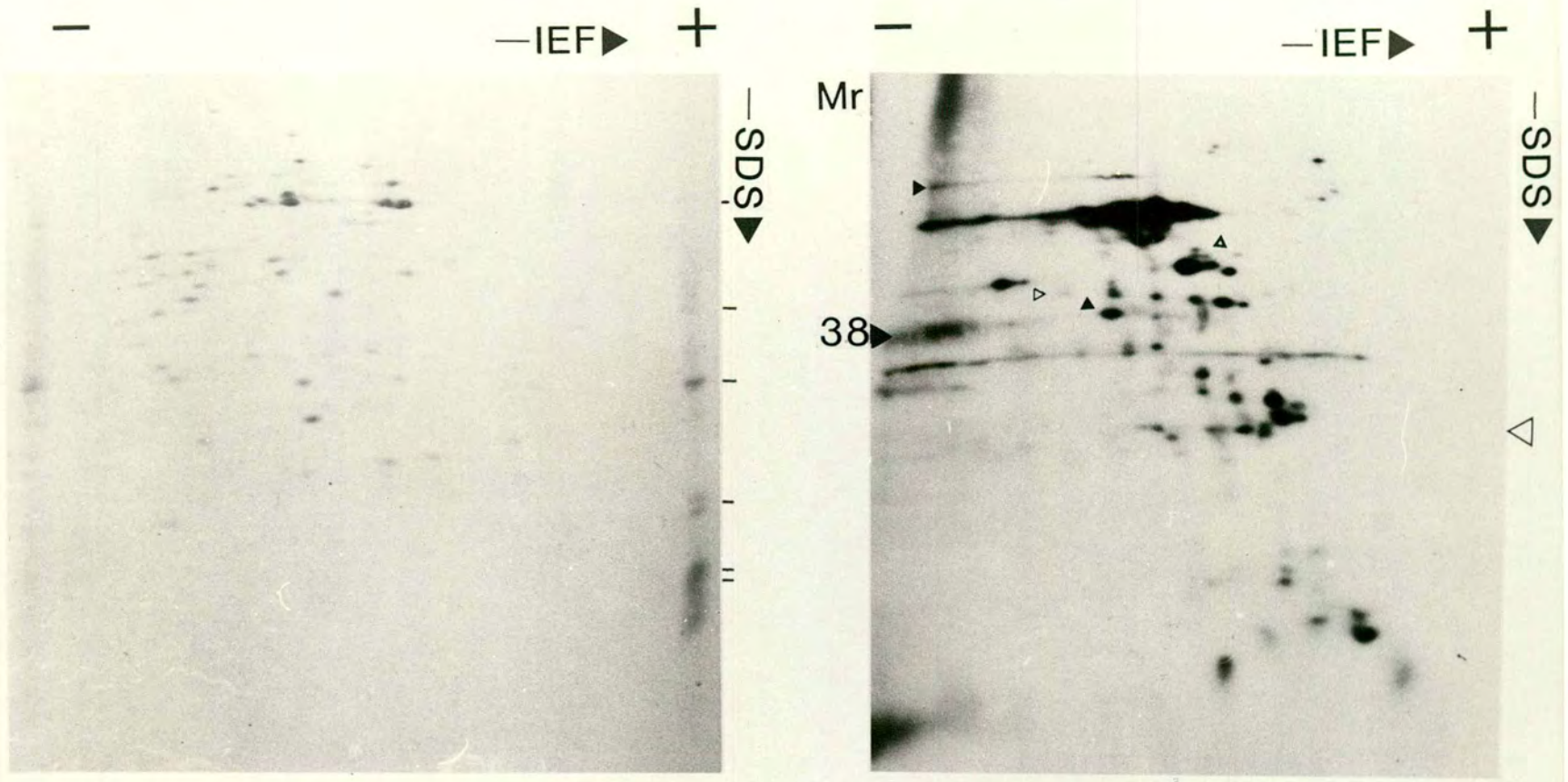
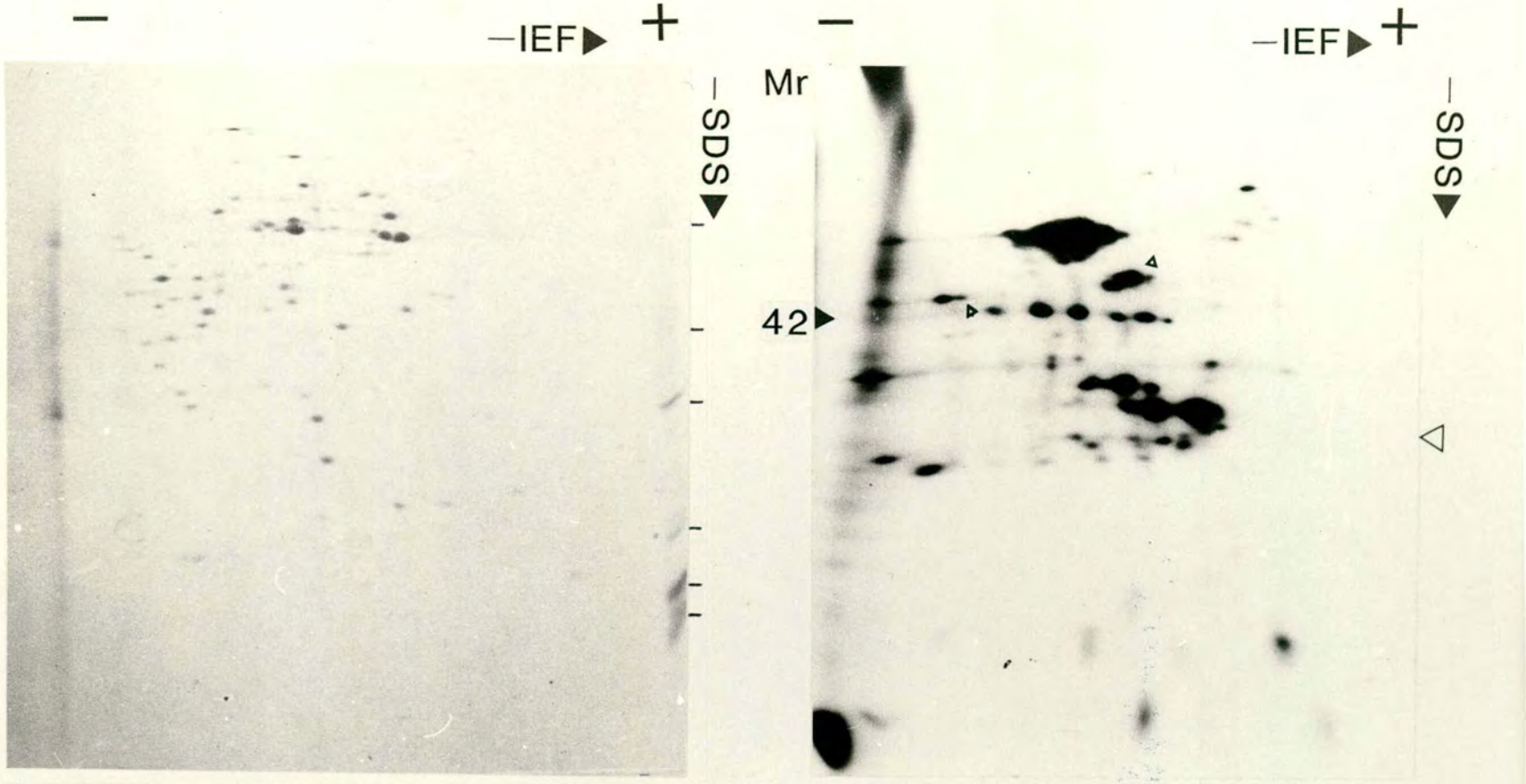

–
–IEF▶
+
–SDS▼
Mr
38▶
A CB Stained Gel
B. Autoradiograph
Kafir Cytoplasm

Fig. 3.8



Milo Cytoplasm

Fig. 3.9


–
–IEF▶
+
–SDS▼
A. CB Stained Gel
9E Cytoplasm
–
Mr
42▶
–IEF▶
+
–SDS▼
B. Autoradiograph

3.8 Immunological Identification of Sorghum Mitochondrial Proteins

Proteins synthesized by maize mitochondria have been identified by the immunoprecipitation of *in organello* labelled proteins (Section 1.3 B). The western technique (Section 2.19 A), an alternative method for immunological identification of mitochondrial proteins was investigated.

Unlabelled mitochondrial polypeptides from Kafir cytoplasm were solubilized with SDS, fractionated in a 16% (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose, incubated with antiserum against yeast COI and labelled with [^{125}I]-Protein A by the western technique, and autoradiographed (Figure 3.10). The immunodetection of COI from increasing amounts of Kafir mitochondrial protein was determined by densitometer tracing of the autoradiograph (Figure 3.10 A). At least 25µg of mitochondrial protein was required to detect labelling of COI after a 72 hr exposure of the western blot (Figure 3.10 B). Identical western blots of 150 µg of Kafir mitochondrial protein were used to identify unlabelled sorghum mitochondrial proteins with homology to yeast COI, COII and α-subunit of F_1ATPase.

α-F_1ATPase: A 58,000 dalton polypeptide was identified with an antiserum prepared against the yeast α-subunit F_1ATPase (Figure 3.11 C). This polypeptide co-migrates with an abundant Coomassie blue stained mitochondrial polypeptide (Figure 3.11 A), and an *in organello* labelled mitochondrial protein (Figure 3.11 B). The immunolabelled 58,000 dalton protein is the same size as the maize mitochondrially synthesized α-subunit F_1ATPase (Hack and Leaver, 1983).

COI: A 38,000 dalton polypeptide was identified with an antiserum prepared against yeast COI using the western technique (Figure 3.11 D). The homologous sorghum polypeptide is the same size as the maize COI polypeptide which has been identified as a product of mitochondrial

protein synthesis (Forde and Leaver, 1980). An antiserum against yeast COI was used to tentatively identify a variant form of COI in 9E cytoplasm sorghum (Dixon and Leaver, 1982) which has led to the characterization of a mutation in the *COXI* gene (See Chapter IV).

COII: Two polypeptides of approximately 34,000 and 43,000 daltons, with similar antigenicity to the yeast COII antiserum, were detected (Figure 3.11 E). The smaller polypeptide has the same apparent molecular weight as maize COII (34,000 daltons) and probably is the homologous sorghum polypeptide. The larger polypeptide could be 1) a precursor of COII, 2) a polypeptide homologous to a contaminating antibody, or 3) a complex of COII and another protein (ie. another subunit of cytochrome *c* oxidase). The third possibility is most likely since the yeast COII precursor is only 15 residues larger than the mature subunit (Prajte *et al.*, 1983) and purified cytochrome *c* oxidase subunits often form aggregates which are visible upon SDS-polyacrylamide fractionation (M. Hawkesford, personal communication).

An attempt to immunolabel sorghum apocytochrome *b* with yeast antiserum was not successful. However, this protein has been identified in a partially purified cytochrome bc_1 complex fraction from maize mitochondria (M. Hawkesford, personal communication).

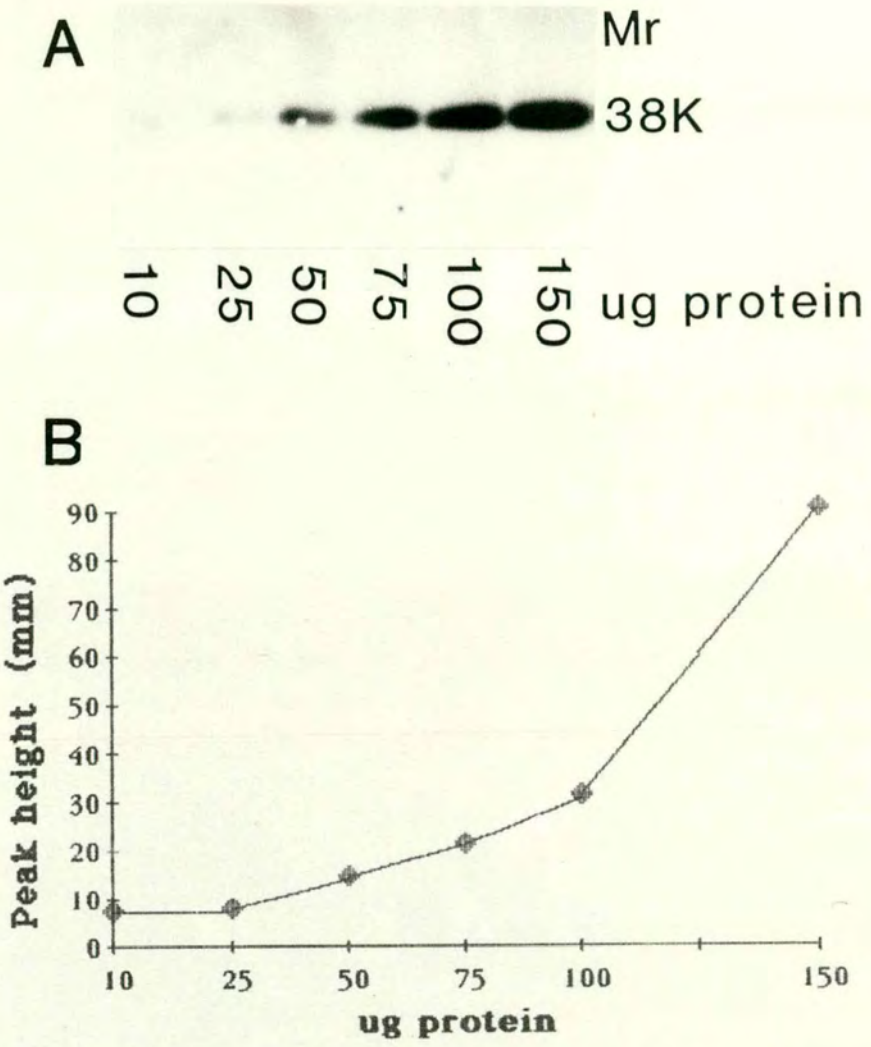


Figure 3.10

Quantitation of Western Immunodetection of COI

Increasing amounts of mitochondrial protein was fractionated on a 16% (w/v) SDS–polyacrylamide gel and transferred to nitrocellulose. The western blot was incubated in an antiserum prepared against yeast COI, labelled with [^{125}I]–Protein A and exposed to X–ray film. Autoradiograph – (A). The level of immunodetection was determined from densitometer tracings of the autoradiograph, and is shown graphically (B).

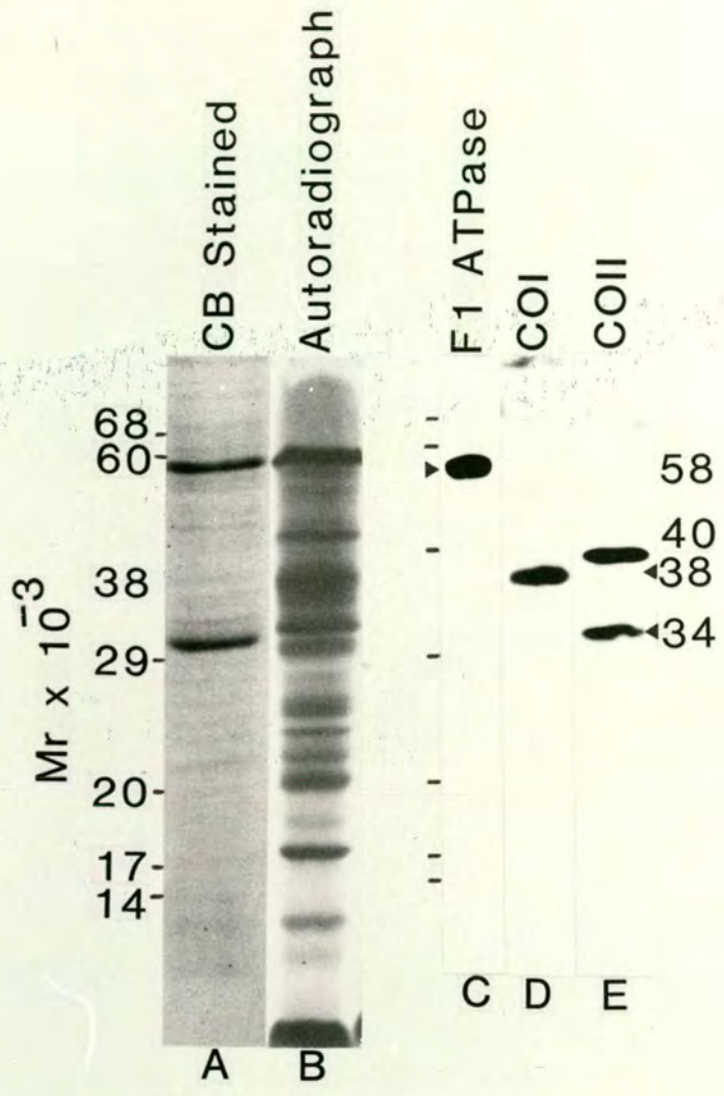


Figure 3.11

Immunodetection of Sorghum Mitochondrial Polypeptides with Antisera Prepared Against Yeast Mitochondrial Proteins

150 ug of Kafir mitochondrial protein were fractionated by electrophoresis in a 16% (w/v) SDS–polyacrylamide gel. A lane of the gel was excised and stained with Coomassie blue (A). The remaining protein was transferred to nitrocellulose. The western blot was cut into strips corresponding to individual lanes of the gel. The gel strips were incubated with antisera prepared aganist yeast F_1ATPase (C), COI (D) and COII (E), labelled with [^{125}I]–Protein A, and exposed to X–ray film. An autoradiograph of in organello labelled mitochondrial proteins fractionated on a similar gel is shown for comparison (B).

3.9 Discussion

The data presented here show that mitochondrial genome structure and expression is variable in Sorghum bicolor. The EcoRI digestion patterns of mtDNA isolated from four sorghum cytoplasms were different, which indicates that, as has been shown in Zea mays (Lonsdale et al., 1984; C. Fauron, personal communication), Sorghum bicolor probably has more than one mitochondrial 'master chromosome'. This genomic heterogeneity is reflected in the EcoRI fragment location and transcript pattern of mitochondrial genes, and quantitative and qualitative differences observed amongst in organello synthesized proteins. Hybridization of maize mitochondrial gene probes to EcoRI digested mtDNA from different sorghum cytoplasms revealed that both COXI and ATPA can be located on different EcoRI fragments in sorghum mtDNA. Northern hybridization analysis showed that the different genomic locations of COXI and ATPA correspond to different patterns of gene transcription. In 9E cytoplasm, the observed difference in the EcoRI fragment location and transcription pattern of COXI, correlates with the expression of an apparently larger COI polypeptide (Dixon and Leaver, 1982). To further investigate this correlation, the mutation leading to synthesis of an larger COI in 9E cytoplasm sorghum was studied in detail (Chapter IV). In addition, to determine if the inter-specific variation is related to the expression of the CMS phenotype, gene clones were used to examine the mtDNA of fertile and CMS nuclear-cytoplasmic combinations of five different cytoplasmic genotypes (Chapter V).

CHAPTER IV

CYTOCHROME c OXIDASE SUBUNIT I IN SORGHUM

4.1 Aims and Rationale

Variability in mitochondrial genome organization and expression in sorghum (Chapter III) may be due to specific deletions, duplications or rearrangements of mtDNA which may have arisen spontaneously or as a result of hybrid seed production. Such alterations in genome structure may be lethal, silent or result in a mutant phenotype (ie. cytoplasmic male sterility). Dixon and Leaver (1982) reported that mitochondria isolated from a CMS line containing 9E cytoplasm synthesize a variant form of cytochrome c oxidase subunit I (COI) with an apparent molecular weight of 42,000 (42K) which appears to replace the normal 38,000 dalton (38K) polypeptide synthesized by other sorghum lines. To characterize variability in mitochondrial genome organization and expression, the mutation resulting in the synthesis of a variant COI in 9E cytoplasm was examined. To this end, the following questions were addressed:

1) Is the variant form of COI synthesized *in vivo* as well as *in organello*? Is the variant COI associated with any variation in cytochrome c oxidase activity or reduced cytochrome spectrum?
2) Is the 42K polypeptide synthesized by mitochondria isolated from 9E cytoplasm related to the 38K COI synthesized by other sorghum lines?
3) Is there a precursor-product relationship between the 42K and 38K polypeptides?
4) What is the molecular basis for the synthesis of the variant form of COI in 9E cytoplasm?
5) Is expression of the variant COI related to the the CMS phenotype?

4.2 Identification of the Normal and Variant Forms of Cytochrome c Oxidase Subunit I

Mitochondria were isolated from CMS Milo (Kafir/Milo) and 9E (Kafir/9E) cytoplasms and allowed to synthesize protein in the presence of L-[^{35}S]-methionine (Leaver et al., 1983). The labelled mitochondrial proteins were fractionated in a 16% (w/v) SDS-polyacrylamide gel and autoradiographed (Figure 4.1, Lanes A and B). As previously reported, Milo cytoplasm synthesized a variant 65 K polypeptide and 9E cytoplasm a variant 42K polypeptide (Dixon and Leaver, 1982). To confirm the tentative identification of the variant 42K polypeptide of 9E cytoplasm as cytochrome c oxidase subunit I (COI), labelled mitochondrial translation products were immunoprecipitated by a rabbit antiserum prepared against yeast COI, followed by SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 4.1 Lanes C and D). As shown previously (Dixon and Leaver, 1982), mitochondria isolated from 9E cytoplasm synthesized a COI polypeptide with an estimated molecular weight of 42K, which appears to replace the 38K COI of Milo cytoplasm.

To rule out the possibility that the larger form of the COI polypeptide was an artifact of the in organello protein synthesis system, COI was identified by western blotting. 100 µg of unlabelled mitochondrial protein, was fractionated by 16% (w/v) SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with an antibody against yeast COI and [^{125}I]-Protein A. The autoradiograph in Figure 4.1 (Lanes E and F) shows that 9E mitochondria contained a 42K polypeptide and Milo mitochondria a 38K polypeptide. Therefore, the variant 42K COI appears to be a structural component of cytochrome c oxidase in 9E cytoplasm.

Mitochondria were isolated from fourteen additional nuclear-cytoplasmic combinations containing 9E cytoplasm, and fifteen non-9E cytoplasms and the apparent molecular weight of COI in each line was determined by the western

Figure 4.1

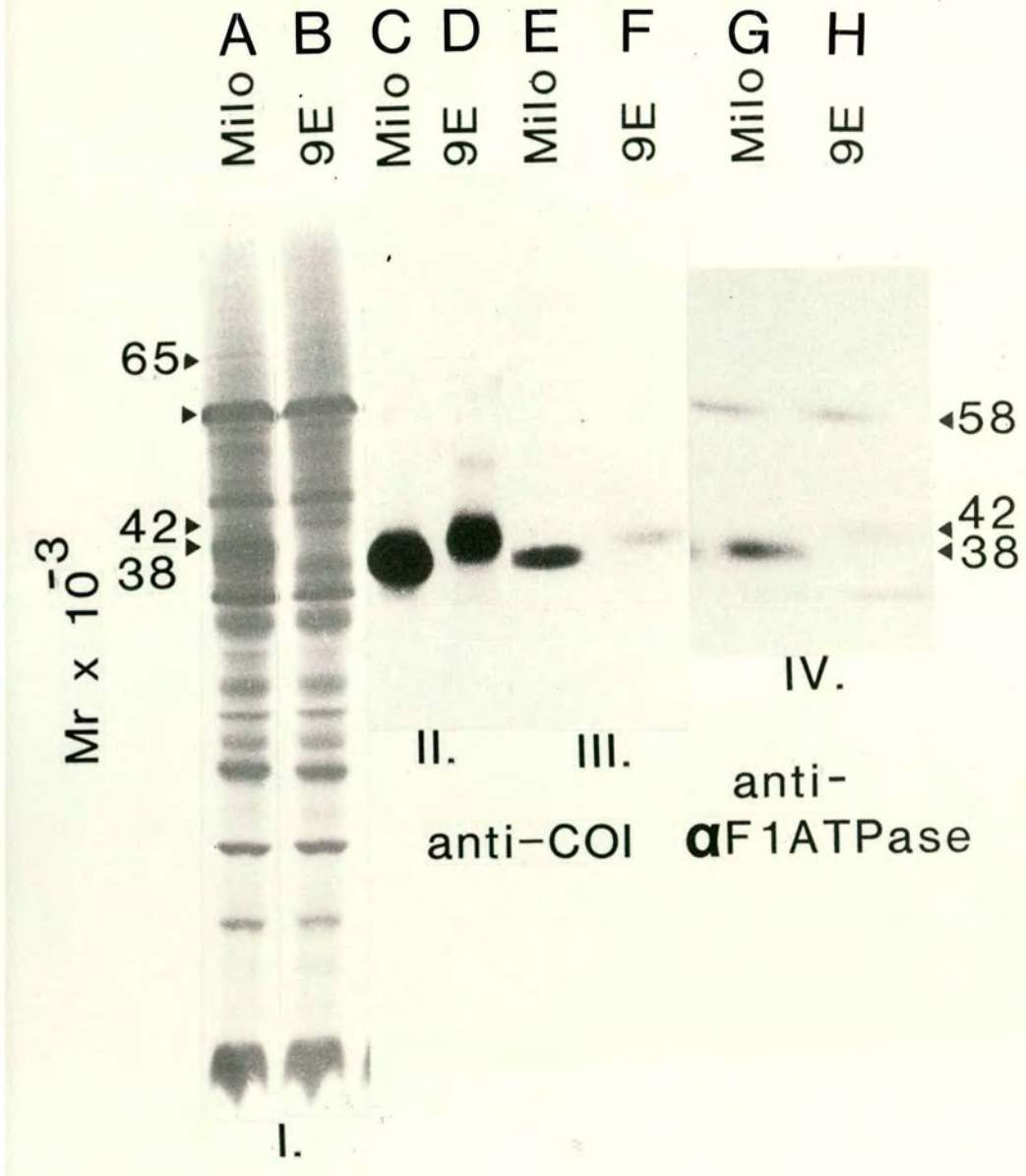
Identification of the Normal and Variant Cytochrome c Oxidase Subunit I (COI) Polypeptide in Sorghum

I. Mitochondrial translation products were labelled with [^{35}S]-methionine *in organello*. 0.25 x 10^6 cpm of labelled proteins were fractionated by 16% SDS-polyacrylamide gel electrophoresis and exposed to X-ray film. Autoradiograph of Milo (A) and 9E (B) translation products.

II. Mitochondrial polypeptides were immunoprecipitated with antiserum against yeast COI, fractionated by electrophoresis on a 16% (w/v) SDS-polyacrylamide gel and fluorographed. Immunoprecipitate from Milo mitochondria (c) and 9E mitochondria (D).

III. Western blot of sorghum mitochondrial polypeptides immunolabelled with an antiserum against yeast COI. 100 µg of mitochondrial protein were fractionated by electrophoresis in each lane of a 16% (w/v) SDS-polyacrylamide gel. The polypeptides were immobilized on nitrocellulose by electrophoretic transfer, incubated with antiserum prepared against yeast COI, washed and labelled with [^{125}I]-Protein A by the method of Batteiger *et al*., 1982). Autoradiograph of Milo mitochondrial protein (E), 9E mitochondrial protein (F).

IV. The western blot shown in E and F was incubated with an antiserum against yeast α-F_1ATPase, washed and labelled with [^{125}I]-Protein A. Autoradiograph of Milo mitochondrial protein (G), 9E mitochondrial protein (H).


A B C D E F G H
Milo 9E Milo 9E Milo 9E Milo 9E
65
42
38
Mr x 10-3
58
42
38
I.
II.
III.
IV.
anti-COI
anti-αF1ATPase

technique. The variant 42K COI was synthesized by lines containing 9E and A4 (IS7920C) cytoplasm, the 38K COI was synthesized by all other cytoplasms examined (Appendix I).

The variant 42K COI of 9E mitochondrial appears as a more diffuse band in autoradiographs of *in organello* labelled mitochondria proteins than the 38K polypeptide of Milo and other lines (Figure 4.1, Lanes A and B). Immunolabelling of equal amounts (100 ug) of mitochondrial proteins suggests that the 38K polypeptide is more abundant than the 42K polypeptide (Figure 4.1, Lanes E and F). This observation has three interpretations: 1) the protein samples were not equivalent weights, 2) the 42K polypeptide is present in relatively lower amounts or 3) is less antigenic to the antiserum than the 38K polypeptide.

To ensure that the protein samples were equivalent weights, the immunodetection of COI was compared to the immunodetection of a second protein, the α-subunit of F_1ATPase. The western blot shown in Figure 4.1 (Lanes E and F) was incubated with an antiserum prepared against yeast α-F_1ATPase, labelled with [^{125}-I]-Protein A and autoradiographed (Figure 4.1 , Lanes G and H). A 58,000 dalton polypeptide, which corresponds to the apparent molecular weight of the maize α-F_1-ATPase was labelled (Hack and Leaver, 1983). The level of immunodetection of the two mitochondrial polypeptides was determined by densitometeric measurement of the two labelled bands in each track and calculation of the area (mm^2) under each peak. Assuming that the amount of α-F_1ATPase present in Milo and 9E mitochondria is equal, the variant 42K COI of 9E was immunodetected at 28% of the level of the normal 38K COI of Milo mitochondria.

To assess whether the low level of immunodetection of the 42K polypeptide arises from reduced antigenicity or a amount of subunit I, the cytochrome *c* oxidase and ATPase activity of Milo and 9E mitochondria was assayed (Table 4.1). It was found that the ratio of cytochrome *c* oxidase to ATPase activity was identical in Milo and 9E mitochondria. In the experiment presented, the enzymatic

activities of the samples were significantly different: a discrepency which could be due to a difference in the amount of these enzyme complexes per μg of total mitochondrial protein in the samples examined. Although it is not certain from this investigation that 9E mitochondria contain the normal amount of COI, the relative activity of cytochrome oxidase to ATPase is similar in lines possessing the normal or variant COI. Thus, it seems likely that the variant COI is less antigenic to the COI antibody. Interestingly, the 45K dalton precursor of N. crassa COI is more antigenic than the mature COI to an antiserum prepared against normal N. crassa COI polypeptide (van't Sant, 1982).

Additional evidence that 9E cytoplasm mitochondria possess a functional cytochrome c oxidase despite a variant 42K polypeptide was obtained by examining the spectra of the reduced cytochromes in Milo, Kafir and 9E mitochondria. Mitochodria were isolated, cytochromes reduced with an excess of dithionite and absorbtion spectra from 500 to 650 nm were measured at liquid nitrogen temperature by P. Pajot (Center of Molecular Genetics, Gif Sur Yuette, France) (Figure 4.2). No difference in the cytochrome b, cytochrome c and cytochrome aa_3 (cytochrome c oxidase) peaks of Kafir, Milo or 9E cytoplasm was observed.

Table 4.1 Cytochrome c Oxidase and ATPase Activity of Isolated Mitochondria

Cytoplasm -->	Milo	9E
moles cyt. c oxidized/ min/μg	4.2×10^{-8}	1.9×10^{-8}
mole Pi liberated/min/μg	32.7×10^{-12}	14.6×10^{-12}
Ratio CO activity/ ATPase activity	1.3×10^{-3}	1.3×10^{-3}

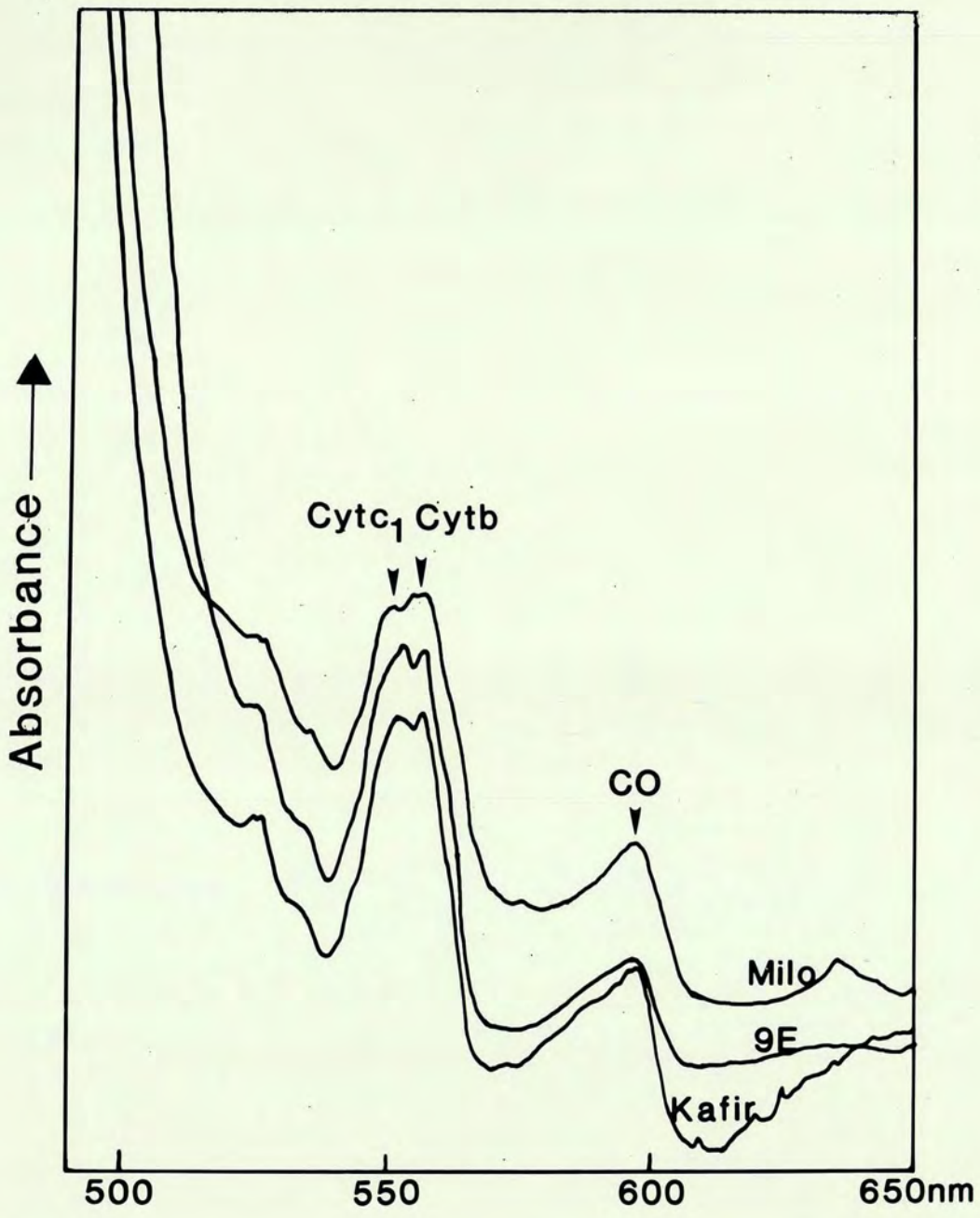


Figure 4.2

Reduced Cytochrome Spectrum of Sorghum Mitochondria

Liquid nitrogen temperature absorbtion spectra from 500 to 650 nm of dithionite reduced mitochondria from Kafir, Milo and 9E cytoplasm. Resolved peaks: cyt b – cytochrome b; cyt c1 – cytochrome c, CO – cytochrome oxidase.

4.3 Is the 42K Polypeptide Synthesized by Mitochondria from 9E Cytoplasm Related to the 38K COI of Other Lines?

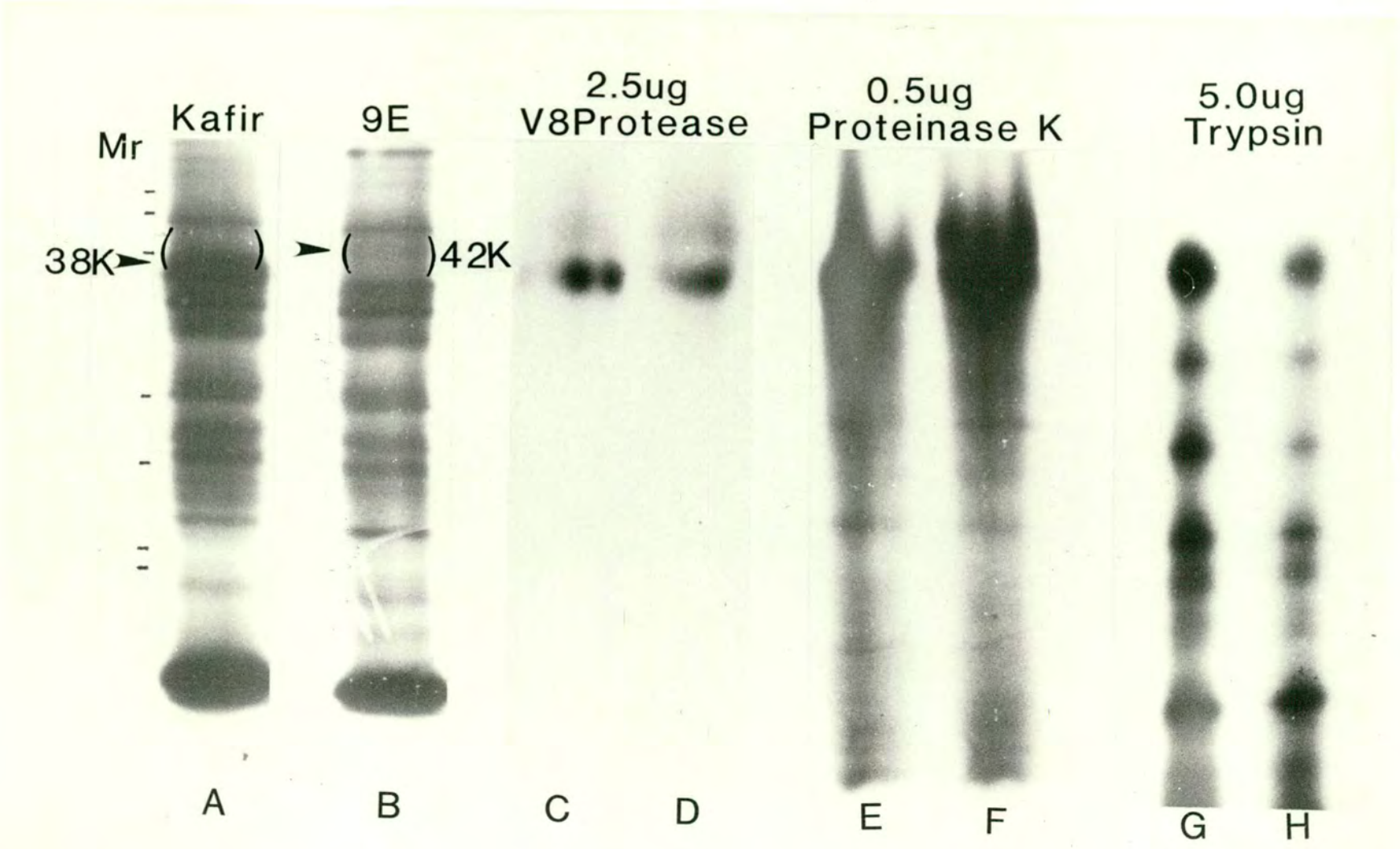
To determine if the variant 42K COI from 9E cytoplasm contains an amino acid sequence which is similar to the 38K polypeptide synthesized by other sorghum lines, limited partial proteolytic digestion of the 42K and 38K polypeptides was performed and the 'fingerprint patterns' of the proteolytic fragments were compared. Mitochondrial translation products from 9E cytoplasm and from a line with a normal 38K COI (Kafir cytoplasm) were labelled *in organello* with [^{35}S]-methionine, fractionated by electrophoresis and autoradiographed. The autoradiograph was aligned with the dried gel and polypeptides in the region corresponding to 38K to 42K daltons were excised (Figure 4.3 A and B). Gel slices containing approximately equal counts of [^{35}S]-labelled protein were placed into the wells of a 15 - 20% (w/v) SDS-polyacrylamide gradient gel and digested with varying amounts of either *Staphylococcus aureus* V8 protease, *Tritriachium album* Proteinase K or soybean trypsin (Cleveland *et al.*, 1977).

V8 protease hydrolyzes polypeptides at the carboxy side of aspartic and glutamic acid residues (Ma *et al.*, 1980). 2.5 µg of protease V8 digested the 42K polypepetide to an apparent molecular weight of 38,000 (Figure 4.3, Lane D). This amount of protease V8 did not appear to digest the 38K polypeptide (Lane C). Upon closer examiniation of the autoradiograph very faint bands at circa 8,000 - 10,000 daltons were visible in both digests (data not shown). Proteinase K hydrolyzes polypeptides at the carboxy side of available aromatic and alipahatic amino acids (Ma *et al.*, 1980). 0.5 µg of Proteinase K generated a similar but complicated fingerprint pattern for the 38K and 42K polypeptides (Figure 4.3, Lanes E and F). Trypsin promotes enzymatic cleavage at the carboxy side of lysine and arginine residues. 5.0 µg of Trypsin also generated a similar fingerprint pattern of the two polypeptides (Figure 4.3, Lanes G and H).

Figure 4.3

Partial Proteolytic Digestion of the Putative COI Polypeptide of Kafir and 9E Cytoplasm

Mitochondrial polypeptides were labelled *in organello* with [^{35}S]-methionine and fractionated by electrophoresis on an SDS-polyacrylamide gel. Following autoradiography, polypeptides of molecular weight 38 to 42K were excised (A and B) and digested in a 15 - 20% (w/v) SDS-polyacrylamide gel according to the protocol of Cleveland *et al*. (1977). Fluorograph of proteolytic digestion of the Kafir and 9E samples with 2.5 µg V8 protease (C and D), 0.5 µg Proteinase K (E and F), and 5 µg Trypsin (G and H).


Mr
Kafir
9E
2.5ug
V8Protease
0.5ug
Proteinase K
5.0ug
Trypsin
38K
42K
A
B
C
D
E
F
G
H

In conclusion, partial proteolytic digestion of the 38K and 42K polypeptides with protease V8, Proteinase K or Trypsin, followed by SDS-polyacrylamide gel electrophoresis, yielded similar fingerprint patterns. These data confirm that the two proteins share a similar amino acid sequence and the 42K polypeptide is an structurally related to the 38K polypeptide. The partial digestion of the 42K polypeptide to 38,000 daltons by protease V8 presents the possibility that the 42K polypeptide differs from the 38K polypepetide by an extension at the amino or carboxy terminus.

4.4 Is There Any Precursor-Product Relationship Between the 42K and 38K Polypeptides?

It is not known whether the mitochondrially encoded components of the inner mitochondrial membrane are synthesized as higher molecular weight precursors in higher plants. Larger precursors of mitochondrial translation products have been identified in fungi: COI (N. crassa, van't Sant et al, 1981) and COII (S.cerevisiae, Sevarino and Poyton, 1980). In both cases the precursor is membrane bound and is presumably larger because of an extension at the carboxy or amino terminus. A number of cytoplasmically synthesized mitochondrial proteins are made as higher molecular weight precursors and have an amino terminal prepiece which is removed during post-translational import (Reid, 1984; Hay et al., 1984).

COI of N. crassa is synthesized as a short lived precursor with an apparent molecular weight of 45,000 daltons which is postranslationally processed to 41,000 daltons. In the N. crassa mi3 mutant, a 45,000 dalton polypeptide cross reacts with an antiserum against COI and appears to replace the normal 41,000 dalton COI (van't Sant et al., 1981). Available data suggest that the 45,000 dalton precursor of COI accumulates in mi3 because of failure to cleave an amino terminal extension.

Under normal growth conditions and at normal growth

temperature (30°C), precursor polypeptides are rapidly processed in yeast and N. crassa. Precursor polypeptides accumulate when cell cultures are incubated with cyclohexamide, aurintricarboxyilic acid or grown at low tempertatures (van't Sant, 1982; van't Sant et al., 1981; Sevarino and Poyton, 1980).

Since, partial proteolysis by protease V8 reduced the COI from 9E cytoplasm to 38,000 daltons, the possibility that the 42K polypeptide is precursor of the 38K polypeptide was considered. To obtain direct evidence for a precursor-product relationship between the 42K and 38K polypeptides, the following questions were asked:

A) Is the 42K polypeptide a soluble or membrane bound protein and is any 42K polypeptide present in lines which synthesize a 38K COI?

B) By varying the in organello translation conditions,

   a) can the 42K polypeptide of 9E cytoplasm be processed to 38K?

   b) can mitochondria which normally synthesize a 38K form of COI be induced to accumulate the 42K polypeptide?

Mitochondria from Milo and 9E cytoplasm were labelled in organello with [^{35}S]-methionine, separated into soluble and membrane bound proteins, fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel and autoradiographed (Figure 4.4, Lanes A - D). Examination of the fractionated mitochondrial translation products indicated that, in support of their identification as COI, the 38K and 42K polypeptides are membrane bound (Figure 4.4, Lanes A, B) (Schatz and Mason, 1974). In addition, the soluble in organello labelled mitochondrial translation products of both lines were similar (Figure 4.4, Lanes C, D).

Unlabelled mitochondria were fractionated into soluble and membrane bound proteins, separated by electrophoresis on a 16% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose. COI was was identified as a membrane bound protein in both lines using the western blotting

technique (Figure 4.4, Lanes E, F). No evidence for the presence of the 38K or 42K form of COI in the soluble fraction was observed even after prolonged exposure of the filter (Figure 4.4, Lanes G, H).

To determine if the 42K polypeptide of 9E cytoplasm is slowly processed to 38K, a 'pulse-chase' expreriment was performed. 9E cytoplasm mitochondria were isolated and allowed to synthesize protein in the presence of [^{35}S]-methionine for 30, 60 or 90 min. A excess of unlabelled methionine was then added and *in organello* protein synthesis was allowed to continue for 60 min. The labelled mitochondrial proteins were fractionated by SDS-polyacrylamide gel electrophoresis and autoradiographed. In these 'pulse-chase' experiments the 42K polypeptide was not apparently reduced in size (data not shown).

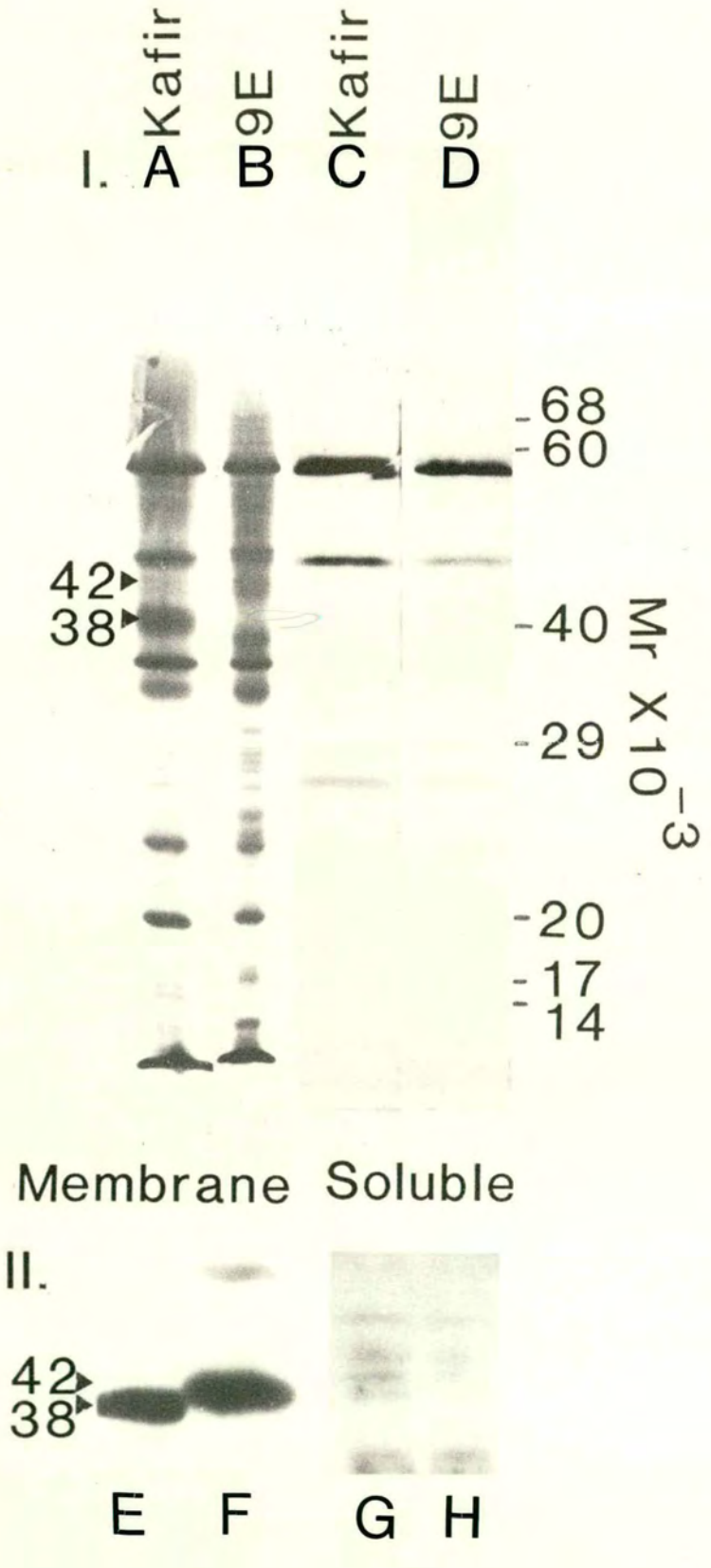
The synthesis of sorghum mitochondrial translation products was examined at three temperatures. Mitochondria were isolated at 4°C and incubated in the translation system at 10, 25 or 30°C. To rule out the possiblity that precursors are rapidly processed during the chase with unlabelled methionine (Section 2.11), *in organello* translation was stopped by rapid mitochondrial lysis. The efficiency of incorporation of TCA insoluble [^{35}S]-methionine and the products of *in organello* translation at the three temperatures was compared. Incorporation of [^{35}S]- methionine was 8% at 25°C, 6% at 30°C and 0.1% at 10°C. Mitochondrial translation products labelled at 10°C were similar to those synthesized at 25°C and 30°C (Figure 4.5). The 42K COI of 9E and the 38K COI of Milo and Kafir was synthesized at these temperatures. An additional 60,000 dalton polypeptide was synthesized by 9E cytoplasm mitochondria at 10°C, and not by Kafir or Milo mitochondria. Variant mitochondrial translation products were not observed in Kafir or Milo cytoplasm.

In conclusion, manipulation of the *in organello* translation system gave no evidence for a precursor-product relationship between the 42K and 38K form of COI.

Figure 4.4

SDS-Polyacrylamide Gel Electrophoresis of Soluble and Membrane Bound Mitochondrial Proteins and Immunological Identification of COI

Mitochondrial proteins were fractionated into soluble and membrane bound proteins, solubilized in 4% (w/v) SDS and fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel. Autoradiograph of [^{35}S]-labelled mitochondrial translation products (I).
Unlabelled mitochondrial proteins were transferred to nitrocellulose and the western blot was incubated in an antiserum prepared against yeast COI, labelled with [^{125}I]-Protein A and exposed to X-ray film. 72 hr exposure of membrane bound proteins, 1 week exposure of soluble proteins (II).


I.
A Kafir
B 9E
C Kafir
D 9E
68
60
42
38
40
29
20
17
14
Mr X10⁻³
Membrane
Soluble
II.
42
38
E
F
G
H

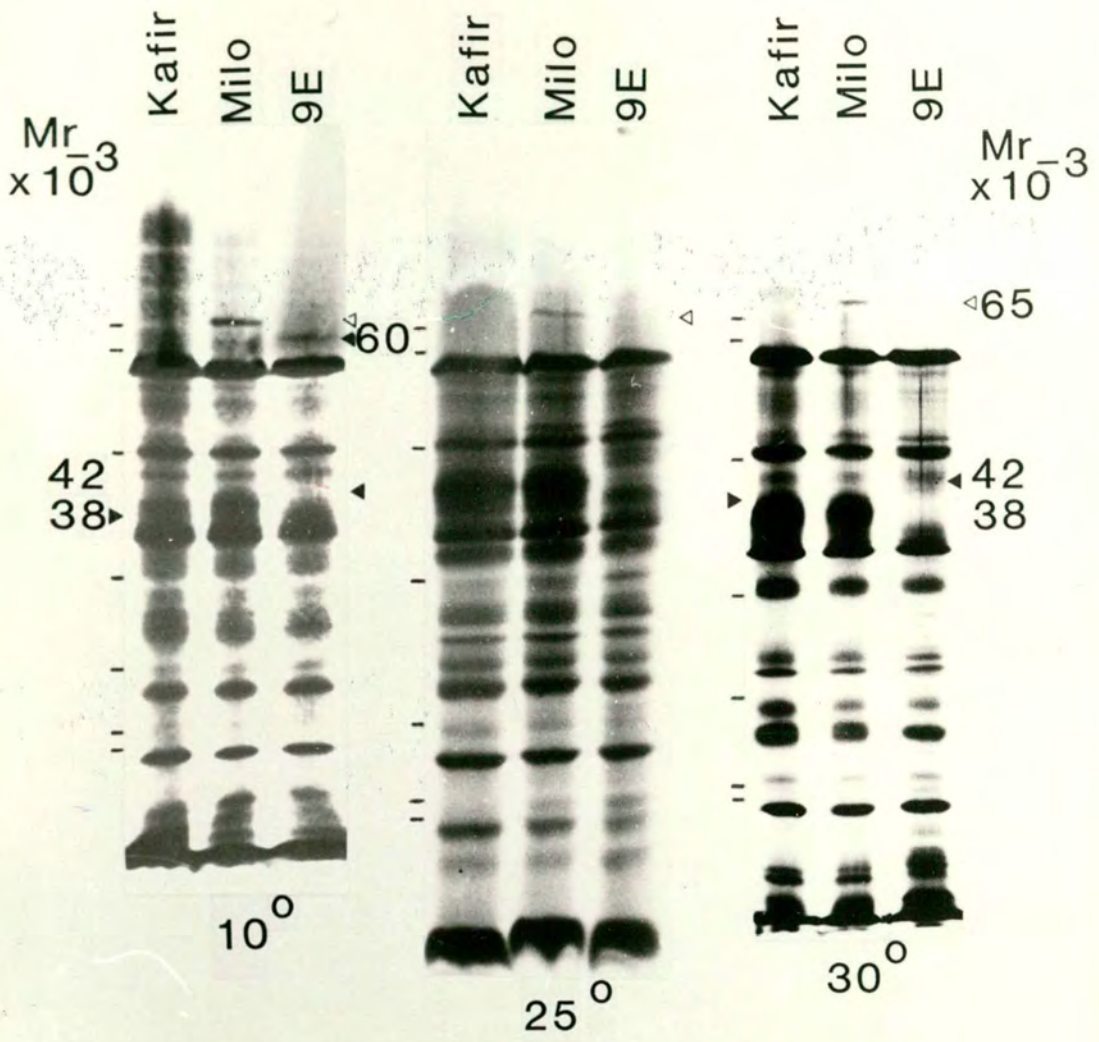


Figure 4.5

Mitochondrial Translation Products Synthesized at 10°, 25° and 30°C

Mitochondria were isolated from Kafir, Milo and 9E cytoplasms and allowed to synthesize proteins in organello, in the presence of [^{35}S]-methionine at 10°, 25° or 30°C. Mitochondria were lysed rapidly in 4.5% (w/v) SDS, 2.25 mM EDTA at 100°C for 2 min, precipitated with 10% (w/v) TCA at 4°C, washed with acetone and fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel. Autoradiographs of Kafir and 9E mitochondrial translation products at three temperatures. Variant translation products are indicated with arrows.

4.5 What is the Molecular Basis of Synthesis of the Variant COI in 9E Cytoplasm?

In an attempt to determine if the variant COI is due to a mutation in the coding region of the COXI gene, an alteration in post-transcriptional or post-translational processing, the gene encoding cytochrome c oxidase subunit I in mtDNA from Milo and 9E cytoplasm was identified, cloned and sequenced.

4.5 A Identification and Cloning of the Genes Encoding the Two Forms of Cytochrome c Oxidase Subunit I

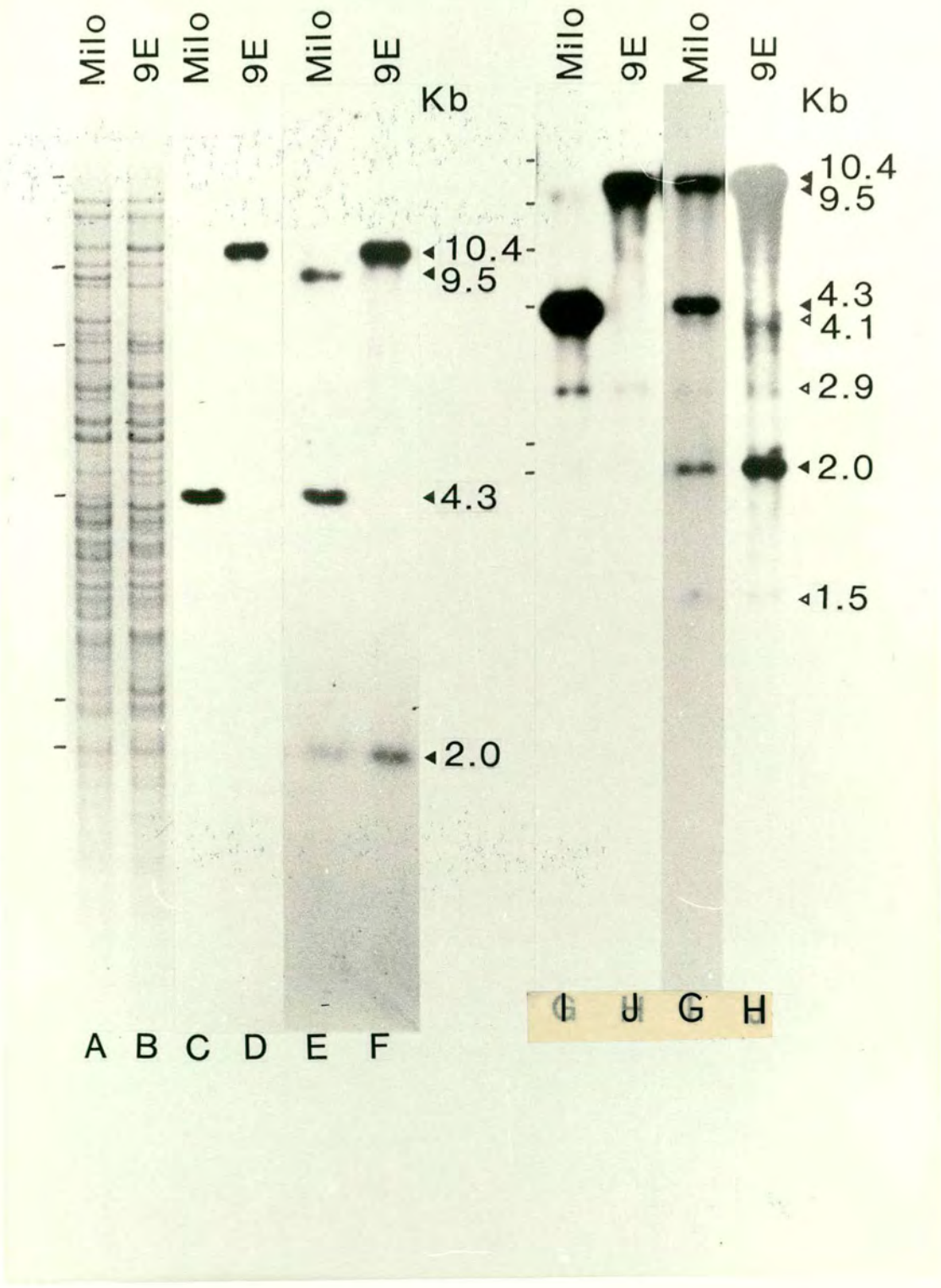
MtDNA was isolated from Milo and 9E seedling mitochondria, digested with EcoRI, fractionated by agarose gel electrophoresis (Figure 4.6, A and B) and probed with a [^{32}P]-labelled DNA fragment containing a portion of the maize COXI gene (Isaac et al., 1985). A 4.3 Kb EcoRI generated mtDNA fragment from Milo and a 10.4 Kb EcoRI generated mtDNA fragment from 9E hybridized to the probe (Figure 4.6, C and D). It was found that the gene is located on a 4.3Kb EcoRI generated fragment in lines which synthesize the 38K form of COI, and on a 10.4 Kb fragment in lines which synthesize the 42K form of COI (Appendix I).

The 4.3 Kb EcoRI fragment from Milo mtDNA was cloned into pBR325 (Bolivar et al., 1978) by Dr. D. K. Hanson and the clone was designated pSM4.3. The 10.4 Kb EcoRI fragment from 9E mtDNA and the 4.3 Kb EcoRI fragment from Kafir mtDNA were cloned into the plasmid vector pNS1 (Nikolnikov et al., 1984) and designated pS9E10.4 and pKM4.3, respectively (see Section 3.3 for details).

Figure 4.6

Identification of the EcoRI Generated MtDNA Fragment Containing the Gene for Cytochrome c Oxidase Subunit I (COXI) in Sorghum.

MtDNA from Milo (A) and 9E (B) was digested with EcoRI, fractionated by electrophoresis in a 0.8% (w/v) agarose gel, stained with EtBr and photographed. The mtDNA was transferred to nitrocellulose, hybridized with a [^{32}P]-labelled clone containing an internal portion of the maize COXI gene and autoradiographed. A 4.3 Kb fragment in Milo (C) and a 10.4 Kb fragment in 9E (D) mtDNA was identified. The blot was then washed and reprobed with pS9E10.4. Additional 9.5 and 2.0 Kb fragments hybridized to Milo mtDNA (E) and a 2.0 Kb in 9E mtDNA (F). In a second experiment mtDNA from Milo and 9E cytoplasms was digested with EcoRI, fractionated in a 0.8% (w/v) agarose gel, transferred to nitrocellulose and probed with pS9E10.4. Autoradiograph of the Southern blot: Milo mtDNA (G), 9E mtDNA (H). The blot was washed, reprobed with [^{32}P]-labelled pSM4.3, and exposed to X-ray film. Milo mtDNA (I), 9E mtDNA (J).


Milo
9E
Milo
9E
Milo
9E
Kb
10.4
9.5
4.3
2.0
A
B
C
D
E
F
Milo
9E
Milo
9E
Kb
10.4
9.5
4.3
4.1
2.9
2.0
1.5
I
J
G
H

4.5 B Heteroduplex Analysis of the Cloned Genes and Restriction Mapping of Flanking Sequences

DNA heteroduplex analysis was performed to determine the extent of sequence homology between the mtDNA fragments in pSM4.3 and pS9E10.4 (Davis et al., 1971). A 1.75 +/- 0.09 Kb heteroduplex region was identified (Figure 4.7 A) which indicated that the two COXI genes share a 1.75 Kb region of homology and are surrounded by non-homologous flanking sequences (Figure 4.7 B).

To characterize further the non-homologous sequences flanking the COXI gene in the 10.4 and 4.3 Kb EcoRI fragment clones, Milo and 9E mtDNA was digested with HindIII (Figure 4.8 A and B) and probed with [^{32}P]-labelled pSM4.3 and pS9E10.4. The hybridization results were used to determine the HindIII sites surrounding COXI in these cytoplasms. Both pSM4.3 and pS9E10.4 hybridized to a 4.5 HindIII fragment of Milo mtDNA indicating that the Milo COXI gene is located on a 4.5 Kb HindIII fragment (Figure 4.8 C). Since a HindIII site was mapped within the cloned 4.3 Kb EcoRI fragment (Figure 4.9) it was deduced that a HindIII site is located 1.8 Kb from this site (Figure 4.8 G). The clone pS9E10.4 hybridized to 10, 2.6, and 2.4 HindIII mtDNA fragments of 9E mtDNA (Figure 4.8 F; the 1.25 and 0.8 Kb HindIII fragment within the 10.4 Kb EcoRI fragment are not visible on this Southern autoradiograph). From this hybridization result and the restriction map of the 10.4 Kb EcoRI fragment (Figure 4.9), it was deduced that the COXI gene is located on a 10 Kb HindIII fragment as shown in the line drawing (Figure 4.8 G). Thus, COXI is in a different genomic location in Milo and 9E mtDNA.

Figure 4.7

Heteroduplex Analysis of the 10.4 and 4.3 Kb EcoRI Fragments Containing the COXI Gene From Milo and 9E MtDNA.

The cloned DNAs were digested with EcoRI to separate the cloned insert from vector DNA. (A) Electronmicrograph of a representative heteroduplex molecule. THe 1.75 +/- 0.09 Kb heteroduplex region is shown. M13 -single stranded DNA marker (7.13 Kb); pAT153 - double stranded DNA marker (3.6 Kb). (B) Diagramatic repersentaion of the heteroduplex.

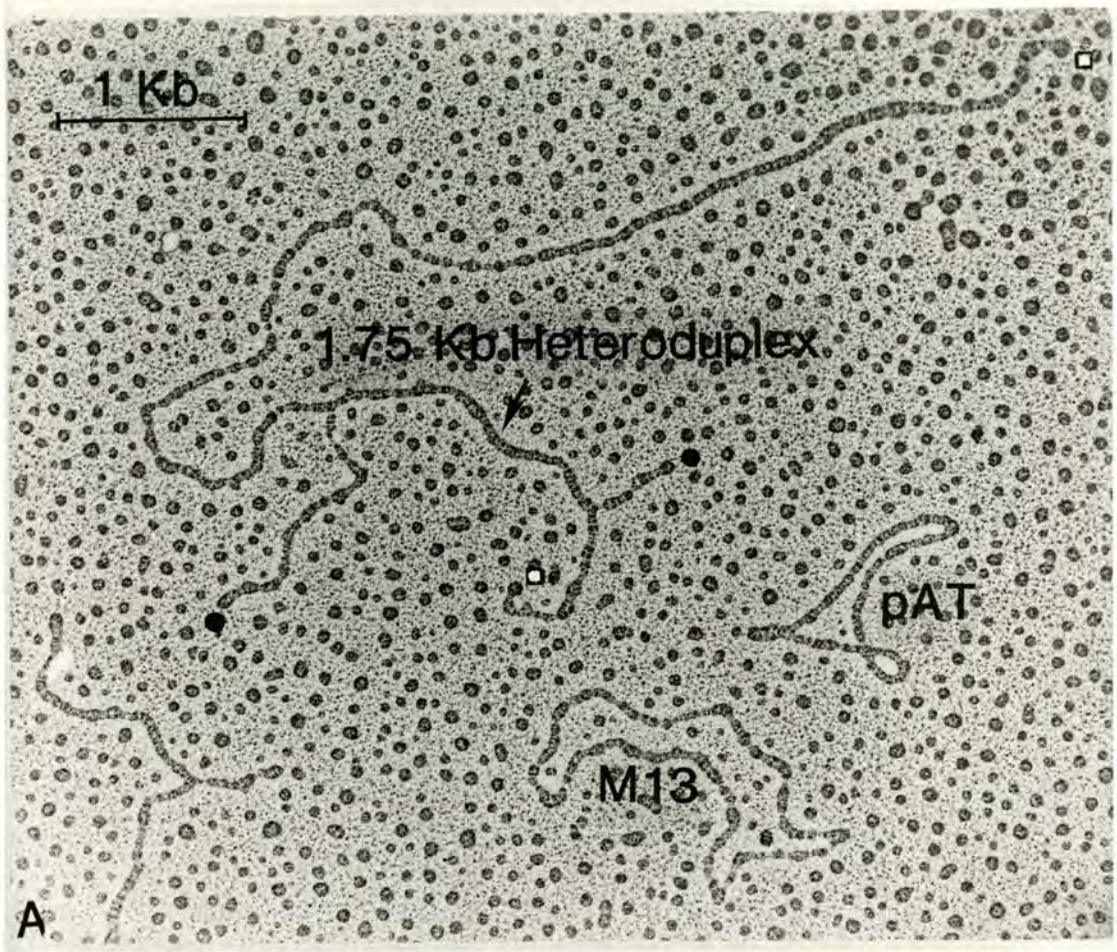
Figure 4.8 (pg. 130)

HindIII Fragment Location of COXI in Milo and 9E MtDNA

(I). MtDNA of Milo (A) and 9E (B) cytoplasms was digested with HindIII, fractionated by electrophoresis in 0.8% (w/v) agarose, stained with EtBr and photographed. The mtDNA was transferred to nitrocellulose and probed with pSM4.3. A 4.5 and 1.9 Kb fragment of Milo (C) and a 10 Kb fragment of 9E (D) mtDNA was identified. The blot was washed and reprobed with pS9E10.4. A 4.5 and 2.0 Kb fragment of Milo (E) and a 10, 2.6, and 2.4 Kb fragment of 9E (F) were identified. The 1.6 and 0.8 Kb fragments of 9E mtDNA which are contained within the 10.4 Kb mtDNA fragment (Figure 3.3) are not visible on this Southern.

HindIII generated fragments of lambda-phage DNA were used as a molecular weight markers.

(II). Line drawing of the HindIII (H) and EcoRI (RI) sites surrounding COXI in Milo and 9E cytoplasm.


1 Kb
1.75 Kb Heteroduplex
pAT
M13
A

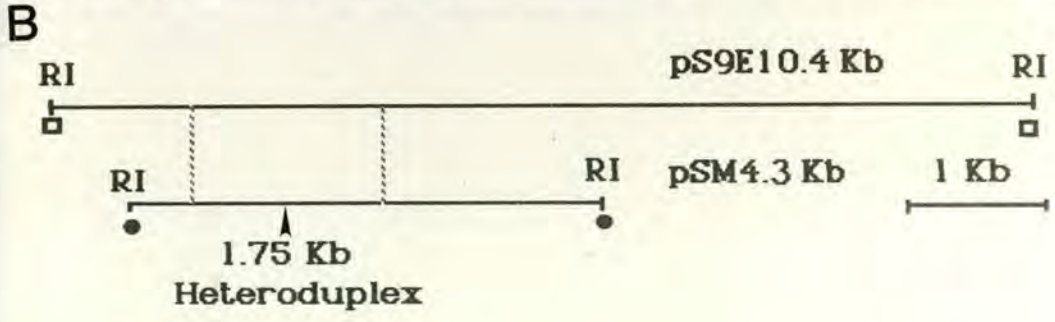
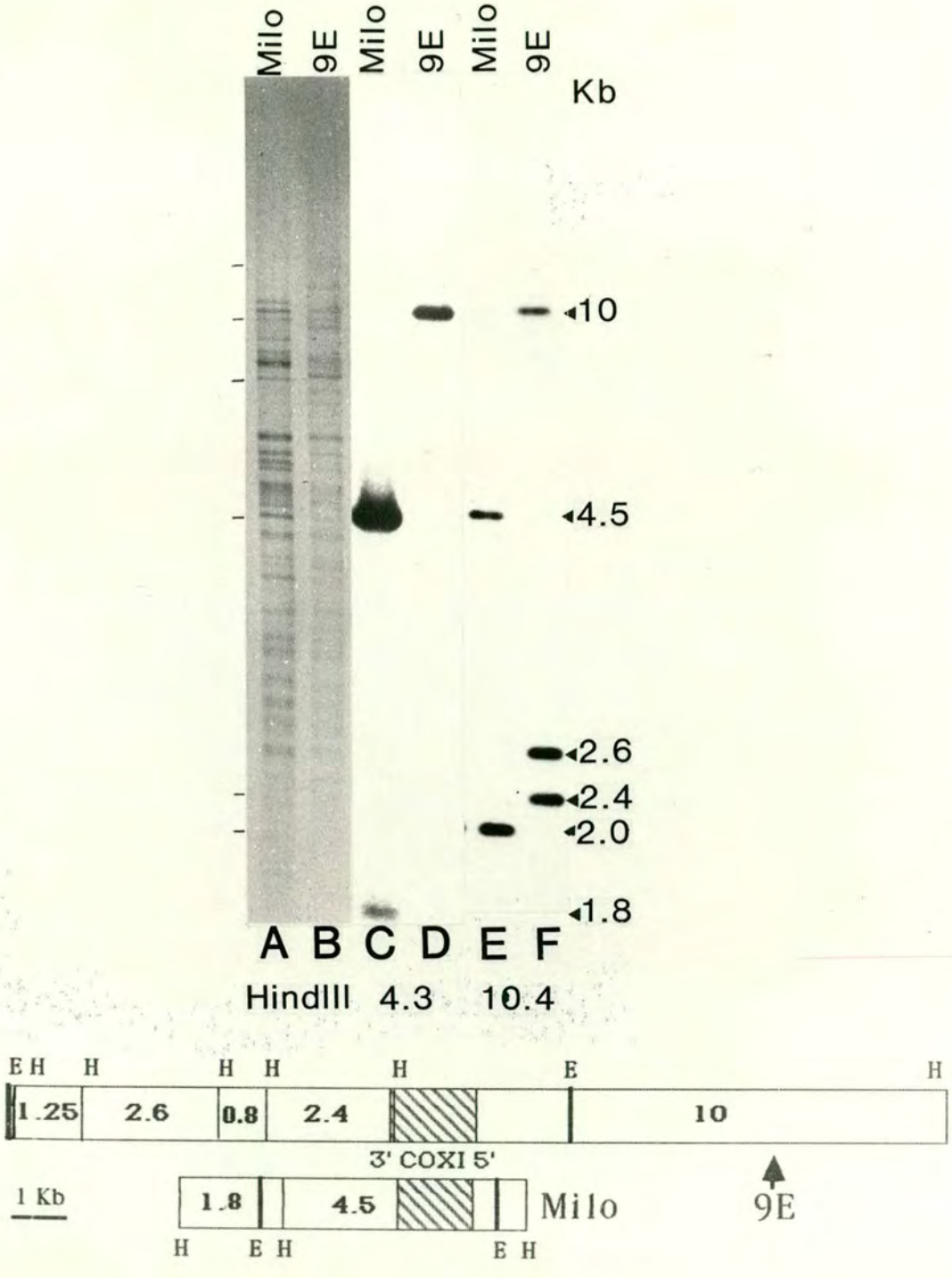

B
RI
pS9E10.4 Kb
RI
RI
RI
pSM4.3 Kb
1 Kb
1.75 Kb
Heteroduplex

Fig. 4.8


Milo
9E
Milo
9E
Milo
9E
Kb
10
4.5
2.6
2.4
2.0
1.8
A B C D E F
HindIII 4.3 10.4
E H H H H H E H
1.25 2.6 0.8 2.4 10
3' COXI 5'
1 Kb
1.8 4.5 Milo
9E
H E H E H

4.5 C Detection of COXI and Flanking Sequences Elsewhere in the Mitochondrial Genome of Milo and 9E

To determine whether the unique sequences flanking COXI in pS9E10.4 are located elsewhere in the Milo mitochondrial genome, the mtDNA blot shown in Figure 4.6 (A and B) was washed and probed with [^{32}P]-labelled pS9E10.4. In addition to the 4.3Kb fragment identified above, a 9.5 Kb EcoRI fragment in Milo mtDNA hybridized strongly to this 9E fragment (Figure 4.6 E and F). Longer exposure of a similar autoradiograph revealed four additional EcoRI fragments (4.1, 2.9, 2.0 and 1.5 Kb) from both Milo and 9E which hybridized weakly to pS9E10.4 (Figure 4.6 G and H). The Southern blot (Figure 4.6 E and F) was washed and probed with the Milo pSM4.3 probe. This clone hybridized to the Milo (4.3 Kb) and 9E (10.4 Kb) EcoRI fragments which contain COXI, and to a lesser extent to a 2.9 Kb EcoRI fragment (Lanes I and J). These weakly hybridizing bands could represent homologous sequences present in sub-stoichiometric amounts, regions of partial homology, or homologous sequences in contaminating cpDNA or nuclear DNA.

4.5 D DNA Sequence Analysis of Milo COXI and Comparison with Other COXI Genes

The sequence of the Milo COXI gene was determined by Dr. D.K. Hanson as part of our collaboration. The Milo COXI clone pSM4.3 was digested with hexanucleotide recognizing restriction enzymes and restriction sites were mapped (Figure 4.9 A). Sau3A, MspI and AluI restriction fragments from pSM4.3 were subcloned into M13 and those which hybridized with the maize COXI gene were subjected to dideoxy chain-termination sequence analysis (Sanger et al.,1980). The sequence was analysed by computer assistance.

The Milo COXI gene, encoding the 38K form of COI, contains an uninterrupted open reading frame of 530 codons (1590 bp) which encodes a polypeptide of 58.5K daltons

(Figure 4.10). The Milo COXI gene shares 98% nucleotide sequence homology with the COXI gene of maize (Isaac et al., 1985). The Milo and maize COXI gene sequences are essentially identical from a position -49 bp upstream of the putative initiation codon, through the ORF to position +1566 bp (Figure 4.10). However, from position -16 to -20 bp the sorghum COXI gene has an additional 4 bp. This insertion in sorghum (or deletion in maize) is at the position of the putative ribosome binding site in maize (Dawson et al., 1983). The first 522 codons of the Milo and maize COXI ORF are identical: homology between the two genes ends 8 and 6 amino acids (24 and 18 bp) prior to the termination codon (Figure 4.10). Thus, the Milo COXI ORF is two codons larger than that of maize (Table 4.3, pg 148).

The Milo COXI gene shares amino acid sequence homology with COI in yeast (60%) (Bonitz, et al., 1980), Neurospora (62%) (Burger et al., 1982), man (68%) (Anderson, et al., 1981) and Drosophila (65%) (de Bruijn, 1983; Clary and Wolstenholme, 1984).

4.5 E DNA Sequence Analysis of the 9E COXI Gene and Comparison with the Milo Gene

Heteroduplex analysis showed that a 1.75 Kb region of homology exists between the EcoRI clones of Milo and 9E mtDNA which contain COXI (Figure 4.7). A 2.2 Kb BamHI - PstI fragment from pS9E10.4 which contained the heteroduplex region and showed homology to the maize COXI gene was subcloned into M13mp10 (Figure 4.9). This clone, designated 9EBP2.2, was digested with Sau3A or MspI, subcloned into M13 and sequenced in collaboration with Dr. D.K. Hanson (Figure 4.10).

A ORF homologous to the first 526 codons of the Milo COXI gene, but encoding a 18% larger polypeptide was identified. The presumptive COXI ORF from 9E cytoplasm is 631 codons (1893 bp) in length and encodes a protein with a predicted molecular weight of 70K (Table 4.3, pg 148).

Fig. 4.9

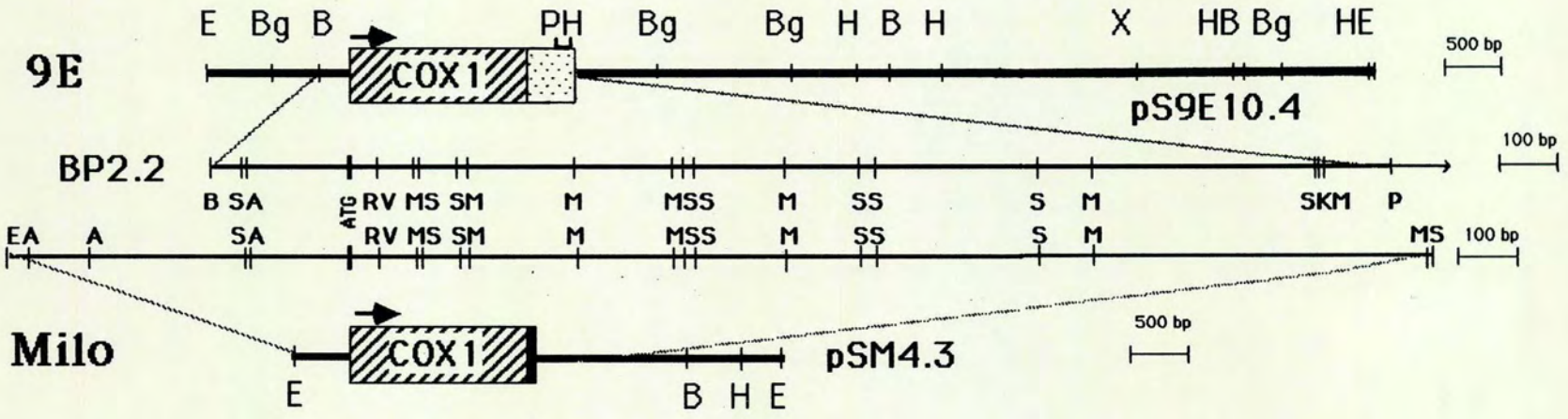

E Bg B
PH
Bg
Bg H B H
X
HB Bg HE
9E
COX1
500 bp
pS9E10.4
BP2.2
100 bp
B SA
ATG
RV MS SM
M
MSS
M
SS
S M
SKM
P
EA
A
SA
RV MS SM
M
MSS
M
SS
S M
MS
100 bp
Milo
COX1
500 bp
pSM4.3
E
B H E

Figure 4.9 (pg 133)

Location of the COXI Gene in Milo and 9E MtDNA.

(A). Restriction map of pS9E10.4 and pSM4.3 (E: EcoRI; B: BamHI; H: HindIII; Bg: BglII; X: XhoI; P: PstI), location of COXI and restriction map of the region sequenced (S: Sau3A; M: MspI; A: AluI; K: KpnI; P:PstI; B: BamHI; H: HindIII). There are no sites for SalI within the 10.4Kb fragment, or for SalI, PstI, or XhoI within the 4.3Kb fragment.

Figure 4.10

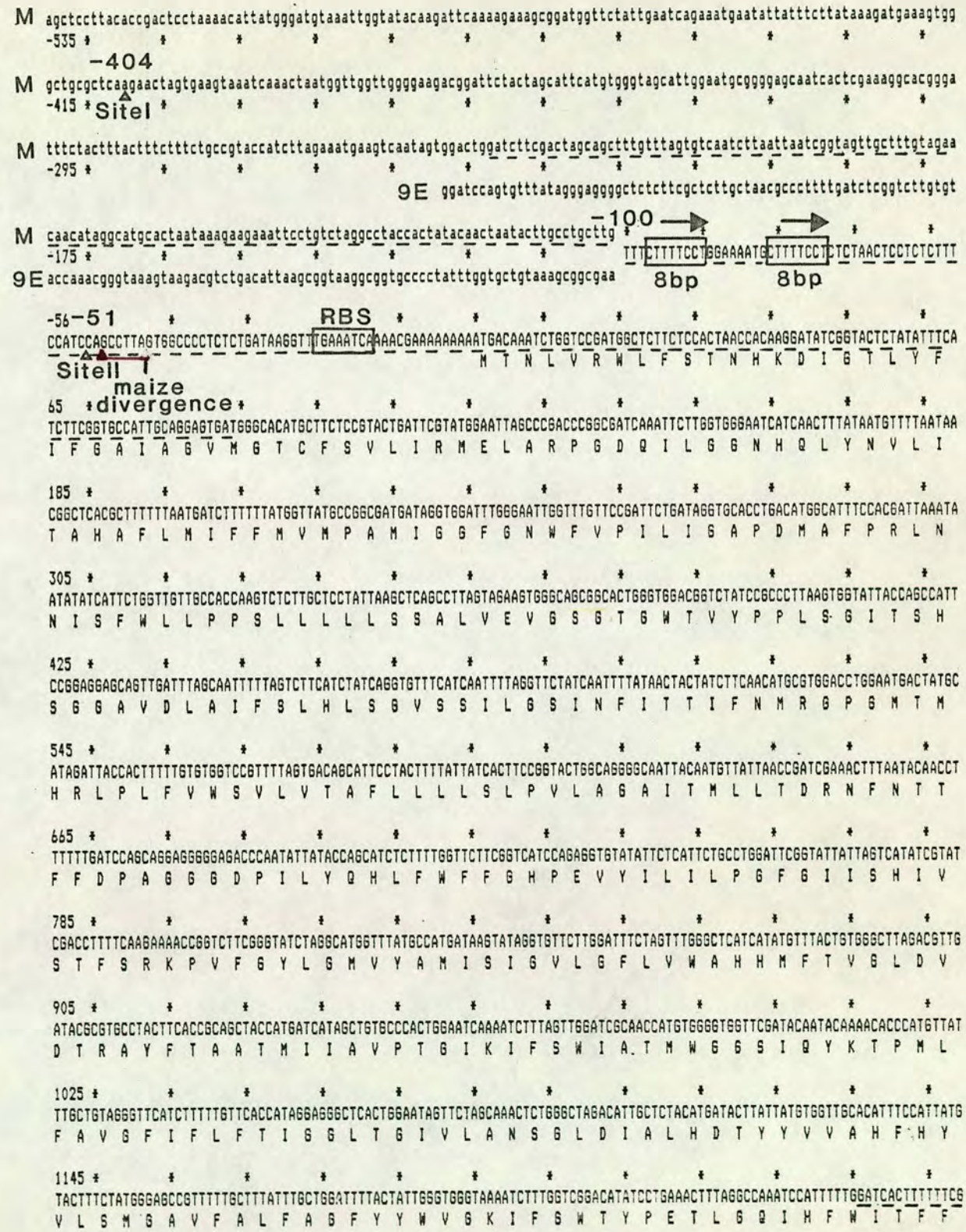
DNA and Amino Acid Sequence of COXI from Milo and 9E MtDNA.

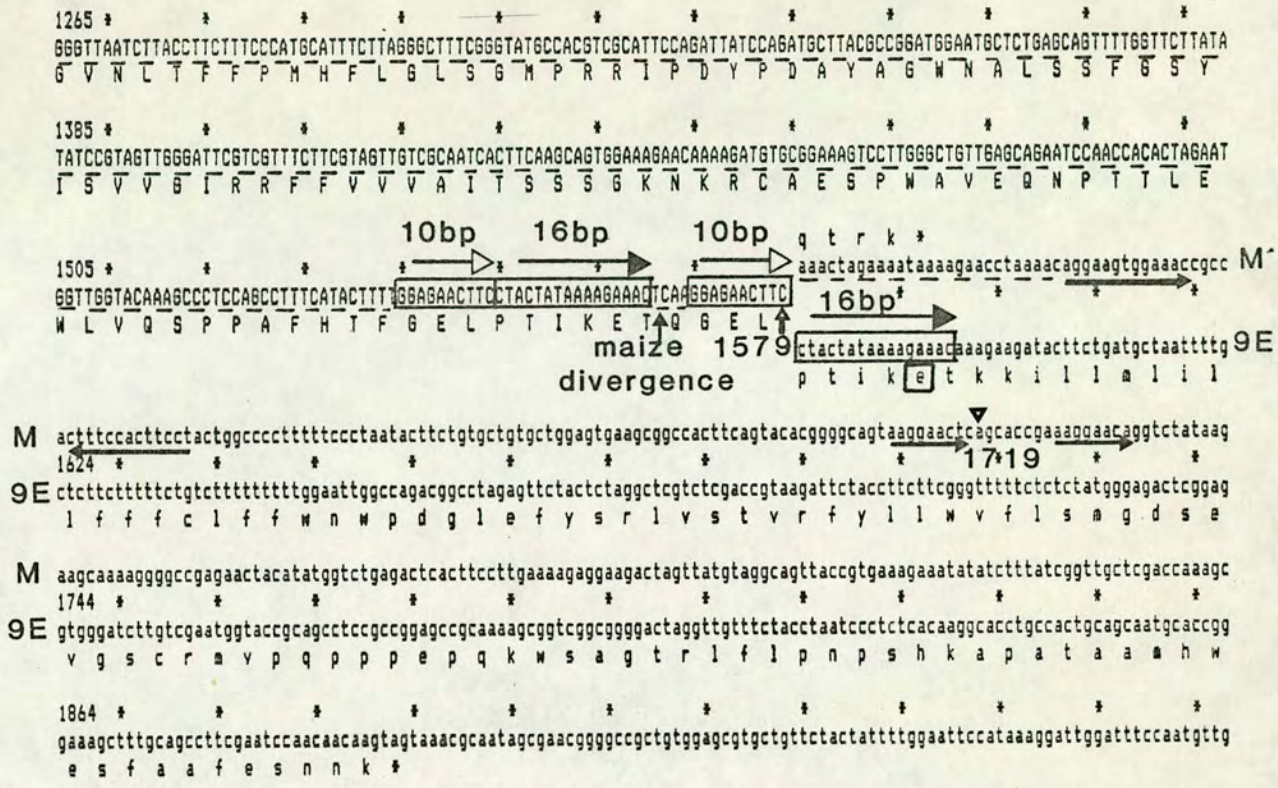
The DNA sequence of the COXI gene from Milo and 9E mtDNA was determined by dideoxy-chain termination sequence analysis. A portion of the COXI sequence of Kafir mtDNA was identified and is indicated as a dashed line under the Milo sequence (- - - - -). Numbering of the nucleotide sequence begins at the A of the putative initiator methionine codon.

The entire COXI gene sequence of Milo is virtually identical to the COXI gene of maize (Isaac, et al., 1985). In comparison, in sorghum the sequence GAAA is inserted between position -17 to -16 bp of maize. This insertion is at the putative ribosome binding site (RBS) of maize (Dawson et al., 1983). Six conservative nucleotide differences between positions +1 and +1565 bp were identified (maize -> sorghum): position 426 bp, T -> C; 981 bp, T -> A; 993 bp A -> T; 1374 bp, C -> T; 1551 bp A -> T; 1557 bp C -> A. The 5' (-49 bp) and 3' (+1566 bp) points of divergence between sorghum and maize are indicated.

The 5' (-100 bp) and 3' (+1579 bp) points of divergence between Milo and 9E are indicated. An 8 bp repeated DNA sequence at the 5' divergence point and a 10 bp repeated sequence in Milo and a 10 bp + 16 bp repeated sequence in 9E at the 3' divergence point are boxed.
The 5' ends of the major RNA transcripts at position -404 bp (Site I) in Milo and at position -51 bp (Site II) in Milo and 9E are marked (*). The 3' end of the major Milo transcript at position +1866 bp is marked (*).
A 14 bp inverted repeat located just 3' of the Milo COXI stop codon is indicated by two arrows.

The glutamic residue (E) at position +1791 - 93 (the 597th codon) is boxed. This residue could be the site where V8 protease cleaves the 42 K polypeptide to 38K.


M
9E
-404
SitEI
-100
8bp
8bp
RBS
-56
-51
SitEII
maize
divergence


10bp
16bp
10bp
16bp
maize
divergence
1579
M
9E
1824
1719
1744
1864

Homology between the Milo and 9E COXI genes begins at position -100 bp from the putative initiation codon and extends to position +1579 bp within the protein coding sequence (Figure 4.10). There is no detectable sequence homology 5' to position -100 bp or 3' to position +1579 bp (within the 527th codon). Termination of the ORF encoding the 38K (Milo and Kafir) form of COI occurs 11 bp (4 amino acids) beyond the 3' point of divergence. In contrast, the ORF encoding the 42K (9E) form of COI appears to continue for a further 314 bp (105 amino acids). So, the presumptive COI of 9E is 101 amino acids, or 12K daltons larger than the corresponding protein in Milo. The 9E COXI coding sequence 3' to position +1579 bp bears no obvious homology to any known portion of the maize, wheat, yeast, Aspergillus or human mitochondrial genomes or any sequences in the EMBL (Release 6) and Genbank databases.

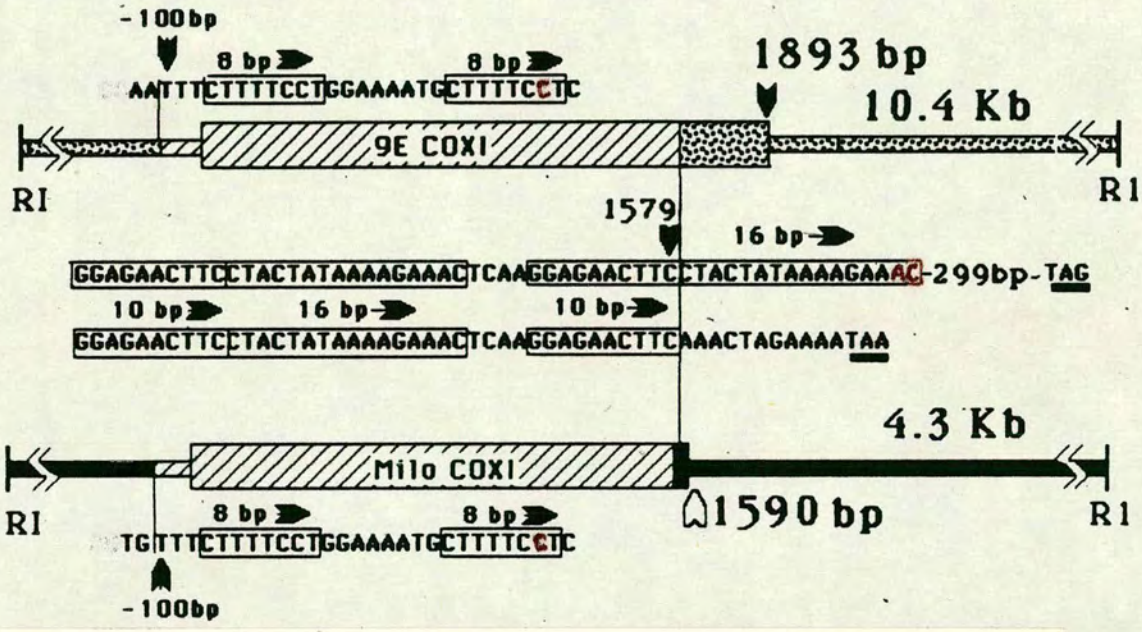
4.5 F Analysis of the Points of divergence between the Milo, Kafir and 9E COXI Genes

Directly repeated DNA sequences are located at the 5' and 3' points of divergence between the Milo and 9E COXI genes (Figure 4.11). An 8 bp direct repeat is found within the homologous region, 3 bp from the 5' divergence point. A 10 bp direct repeat is found within COXI at the 3' point of divergence. This repeat is actually part of a 26 bp direct repeat in 9E cytoplasm, since the following 16bp are repeated both 5' and 3' to the divergence point.

To determine if the points at which the Milo and 9E COXI gene diverge were identical in another line which synthesizes the normal 38K COI, the COXI gene of Kafir mtDNA was examined. The plasmid pSK4.3 (Section 3.3) was digested with Sau3A and subcloned into M13. Clones with homology to the 5' and 3'points of COXI divergence were identified by hybridization to [^{32}P]-labelled probes of Milo COXI and sequenced. The sequence of Kafir COXI at the 5' and 3' points of divergence from the 9E gene was identical to that of Milo (Figure 4.10).

Figure 4.11

DIRECT REPEATS AT THE COXI DIVERGENCE POINTS



4.5 G Codon Usage in the COXI Genes of Sorghum

The codon usage in the Milo and maize COXI genes is similar to that of other plant mitochondrial genes (with the exception of ATPA (Isaac et al., 1985b)). In particular, there is a strong bias towards the use of T in the third position (35%). Codon usage in Milo COXI and the carboxy extension of 9E COXI was compared and significant differences were found (Table 4.2 A; Milo - column 1; 9E carboxy terminus - column 2). Firstly, there is a bias for G in the thrid position in the carboxy extension of the 9E COXI, although the G + C content is 47% in both the novel portion of 9E COXI and the Milo COXI gene. Secondly, the codon CGG occurs three times in the unique portion of the 9E COXI ORF and only once in the Milo COXI. It has been proposed that this codon may encode tryptophan in plant mitochondria and not arginine as in other mitochondrial systems (Fox and Leaver, 1981).

Table 4.2 Codon Usage in the Milo COXI gene and in the Carboxy Terminal Extension of the 9E COXI Gene

	T			C			A			G			
	F	28	6	S	5	4	Y	13	0	C	1	2	T
T	F	21	5	S	5	1	Y	5	2	C	2	0	C
	L	21	0	S	8	0	*	0	0	*	0	0	A
	L	5	2	S	3	3	*	1	1	W	14	2	G
	L	10	5	P	6	5	H	15	0	R	6	0	T
C	L	6	3	P	5	1	H	2	2	R	1	0	C
	L	10	3	P	13	1	Q	6	2	R	4	1	A
	L	7	1	P	5	4	Q	2	1	W	1	3	G
	I	15	1	T	16	5	N	11	2	S	10	0	T
A	I	16	0	T	10	1	N	6	2	S	6	1	C
	I	13	3	T	8	1	K	6	2	R	4	1	A
	M	21	4	T	3	0	K	2	5	R	0	2	G
	V	17	1	A	13	0	D	9	0	G	16	0	T
G	V	4	1	A	8	2	D	4	2	G	7	1	C
	V	9	2	A	13	4	E	7	5	G	19	3	A
	V	9	1	A	2	0	E	2	3	G	12	1	G

Milo COXI ORF - column 1: 9E COXI gene positon +1581 to +1893 bp - column 2.

4.5 H Identification of COXI Transcripts

Northern hybridization analysis confirmed that both forms of COXI are transcribed. MtRNA was isolated from Milo and 9E cytoplasms, fractionated by electrophoresis, blotted to nitrocellulose and probed with a [^{32}P]-labelled M13 clone containing a common portion of the COXI gene (Figure 4.12). In Milo, a major COXI transcript of 1.8 Kb and three minor transcripts (3.4, 2.6 and 2.3 Kb) were identified (Figure 4.12, Lane A). In contrast, in 9E a major COXI transcript of 2.2 Kb was observed (Figure 4.12, Lane B). Two 9E COXI specific transcripts (3.3 and 3.2 Kb) present in very low abundance were also detected after extensive exposure of the autoradiograph (not shown).

Transcription of COXI was examined in numerous sorghum lines. It was found that when the gene is located on a 4.3 Kb EcoRI generated mtDNA fragment the transcription pattern is identical to that of Milo and the apparent molecular weight of COI is 38K. In contrast, lines which contain the COXI gene on a 10.4 Kb EcoRI fragment synthesize a major 2.2 Kb gene transcript and the 42K form of COI (data not shown). It appears that pattern of transcription is determined by the non-homologous sequences flanking the Milo and 9E COXI genes. In order to understand the importance of the COXI genomic environment on gene expression the 5' termini of the Milo and 9E COXI transcripts were mapped.

4.5 I Mapping of the 5' Termini of COXI Transcripts from Milo and 9E Mitochondria

To map the origins of the multiple COXI transcripts in Milo, duplicate RNA blots were probed with specific [^{32}P]-labelled M13 clones (Figure 4.12, Lanes C,D,E). This analysis indicated that the minor 3.4 Kb transcript initiates within the AluI fragment located between positions -531 and -218 bp (Figure 4.12, Lane D). The 2.6, 2.3 and 1.8 Kb transcripts hybridized to the probe

used in Lane E and therefore initiate within the Sau3A fragment located between positions -235 bp to +139 bp.

To define the 5' ends of the Milo transcripts more precisely, S_1 nuclease analysis was performed using a [^{32}P]-labelled, 810 bp EcoRI - MspI fragment (Figure 4.13, B) which spans the 5' ends of the Milo COXI transcripts. The DNA fragment was hybridized to mtRNA, treated with S_1 nuclease and the protected hybrids analyzed by electrophoresis on an 8% (w/v) polyacrylamide-urea gel. Two S_1 nuclease protected fragments of 540 +/- 5 b. (Site I) and 187 +/- 6 b. (Site II) were identified (Figure 4.13 Lane 1). Therefore, these termini are approximately located at positions -404 and -51 bp 5' to the presumptive initiation codon (Figure 4.10). From the northern blot transcript analysis above it can be predicted that the 5' end of the 3.4 Kb transcript maps from position -404 bp. Since only one site is found 3' to this postion, the S_1-nuclease protected fragment(s) initiating at position -51 bp probably corresponds to the 5' termini of the 2.6, 2.3 and 1.8 Kb transcripts (Figure 4.13 B). The varied lengths of these transcripts may arise from multiple termination or processing sites.

The 2.2 Kb COXI transcript of 9E mtRNA hybridized to a 330 bp Sau 3A fragment extending from position -191 bp to +139 bp (Figure 4.12, Lane F) and not to the preceeding 5' Sau3A fragment (data not shown). The 330 bp Sau3A fragment was used to determine the 5' terminus of the 9E COXI transcript by S_1-nuclease protection. A S_1-nuclease protected fragment of approximately 190 +/- 6 b. was generated (Figure 4.13 A), which corresponds to a 5' transcript terminus at position -51 bp 5' to the initiation codon, within the region common to both the Milo and 9E COXI genes (Figure 4.13 C; Figure 4.10).

Figure 4.12

COXI Transcript Analysis from Milo and 9E

I. Mitochondrial RNA prepared from seedling mitochondria was fractionated under partially denaturing conditions in a 1.25% (w/v) agarose 17.3 (v/v) formaldehyde gel, transferred to nitrocellulose and probed with a [^{32}P]-labelled M13 DNA probe containing an internal portion of the Milo COXI gene (A,B). (A) Milo mtRNA; (B). 9E mtRNA.

II. Hybridization of Specific [^{32}P]-Labelled COXI Gene Clones to Milo and 9E mtRNA. Parallel tracks of Milo and 9E mtRNA were transferred to nitrocellulose, cut into strips and hybridized to specific M13 DNA probes (C, D, E, F). The origin of each probe is shown in the line diagram of the Milo and 9E genes.

A - AluI. S - Sau3A. M - MspI. P - PstI. B - BamHI. RI - EcoRI

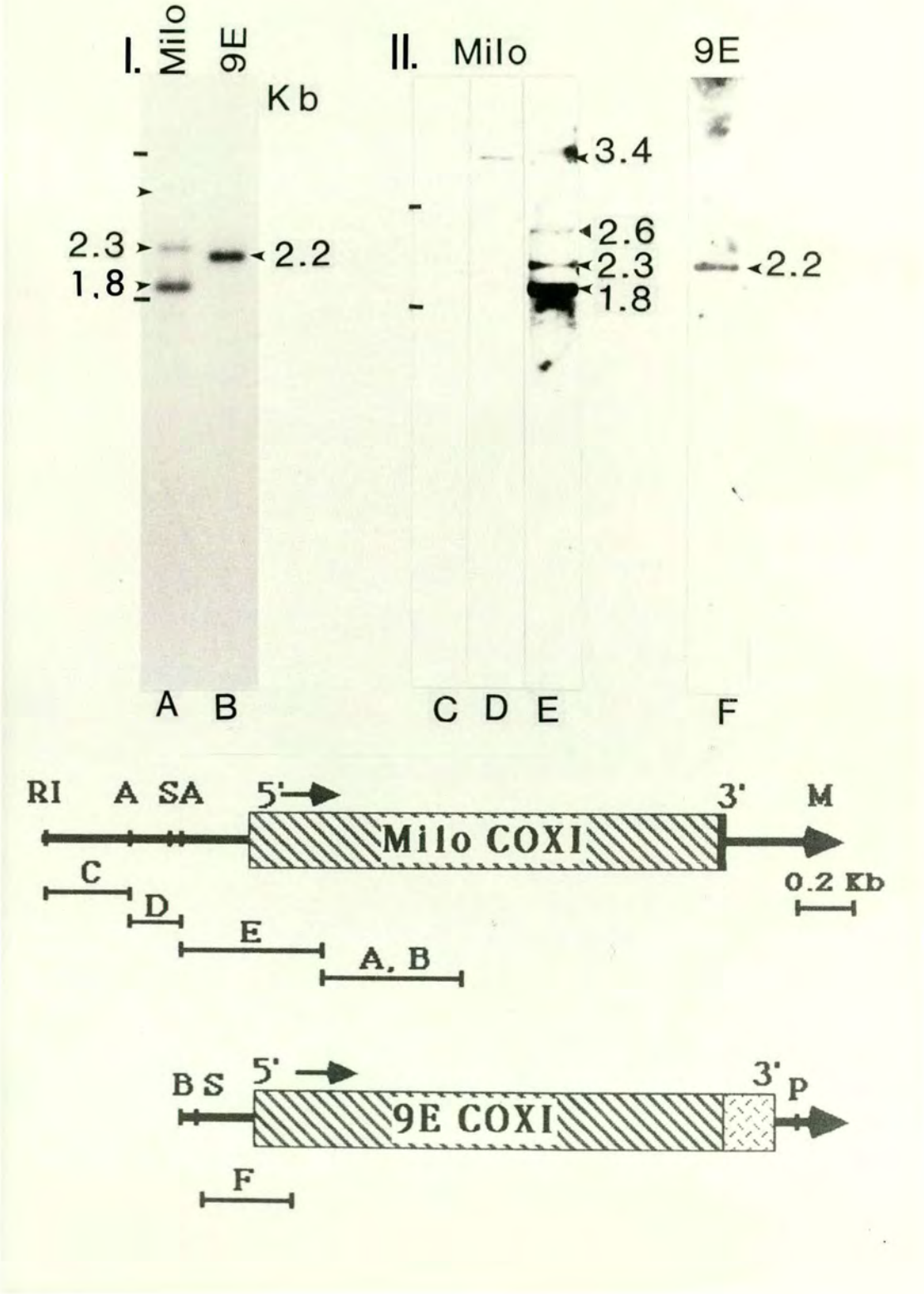

I. Milo
9E
Kb
2.3
1.8
2.2
II. Milo
9E
3.4
2.6
2.3
1.8
2.2
A B C D E F
RI A SA 5' 3' M
Milo COXI
0.2 Kb
C
D
E
A, B
BS 5' 3' P
9E COXI
F

Figure 4.13

S_1 Nuclease Transcript Mapping of the Major Ends of the COXI Transcripts from Milo and 9E Mitochondria

I. Transcript Mapping of the Major 5' Ends

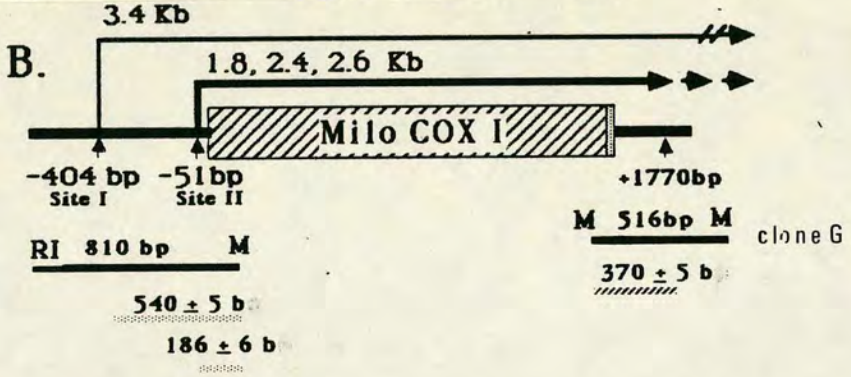
(1) Milo COXI: Hybrids were formed with a cloned, [^{32}P]-labelled 810 bp MspI fragment and Milo mtRNA. Hybrids were treated with 400u/ml S1 nuclease and the protected hybrids were fractionated in an 8% (w/v) sequencing gel. Fragments of approximately 540 +/- 5 b. and 186 +/- 6 b. were generated (Lane 1). The 5' termini of these fragments correspond to positions -404 bp (Site I) and -51 bp (Site II). See diagram B.

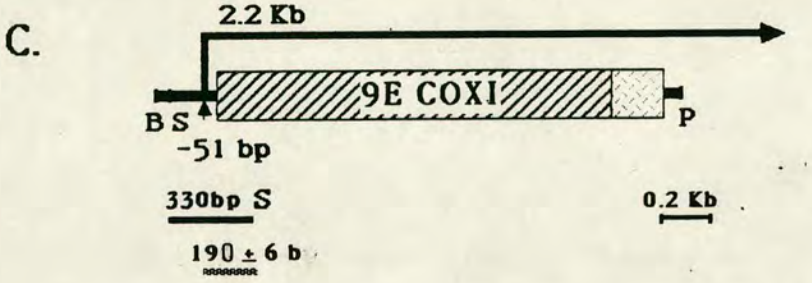
(2) 9E COXI: Hybrids were formed with a [^{32}P]-labelled fragment (containing the 330 bp Sau3A fragment located at the 5' end of the 9E COXI gene) and 9E mtRNA, digested with 400u/ml S_1-nuclease and fractionated by gel electrophoresis. Fragments of approximately 190 +/- 6 b. . In addition a fragment of 330 bp (the size of the cloned insert) was observed (Lane 2).
(3 and 4) As a control reaction, the Milo (Lane 3) or 9E (Lane 4) input DNA was hybridized to tRNA and digested with S_1-nuclease. See diagram C.

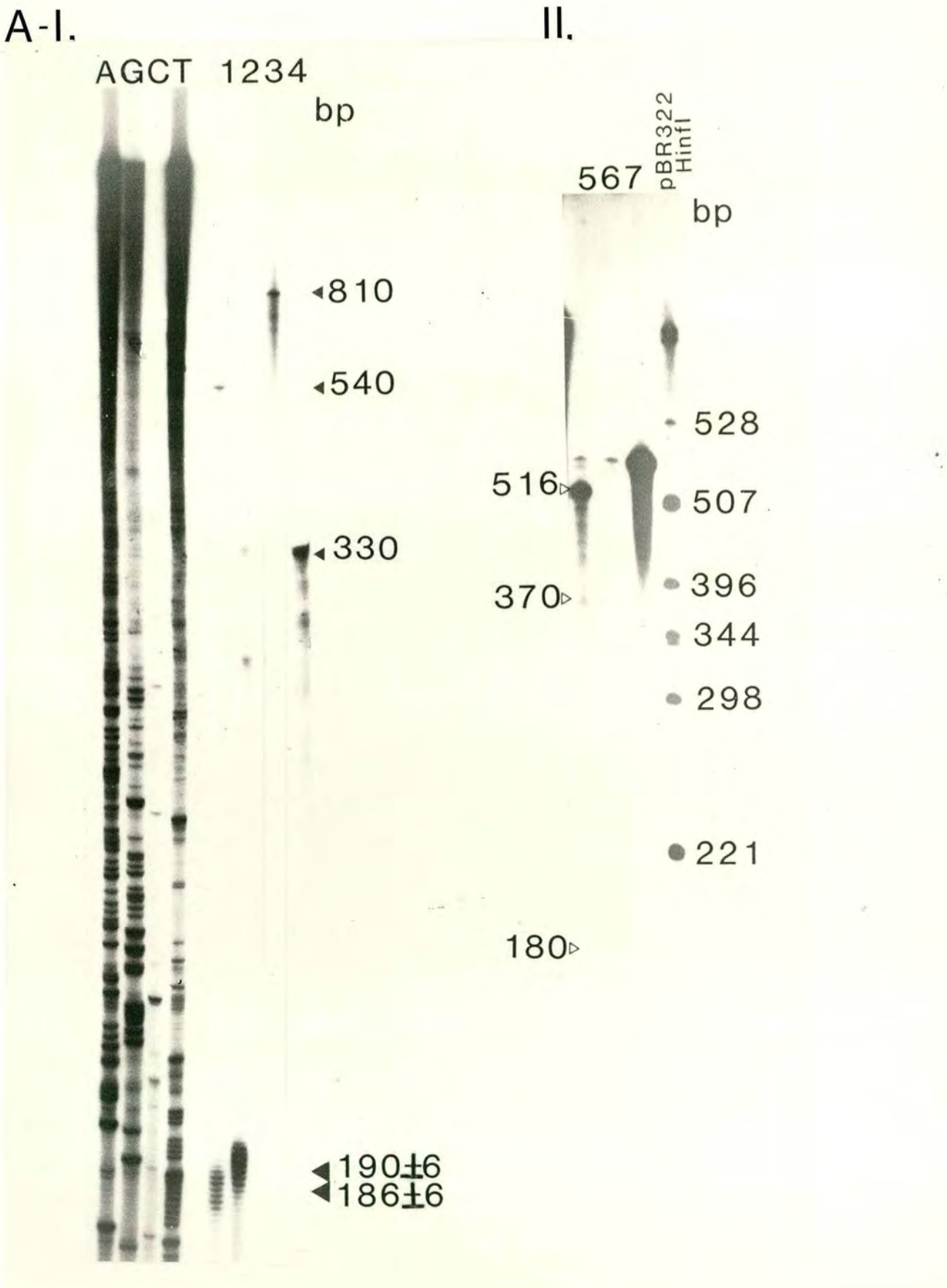
II. Transcript Mapping of the Major 3' End of Milo COXI

(5) DNA-RNA hybrids were formed with clone G (a 516 bp MspI fragment) and Milo mtRNA and treated as described above. 3 S_1 nuclease protected fragments of 180 +/- 5 b. , 370 +/- 5 b. and circa 520 b. were generated. See B. below.
(6) As a control reaction the input DNA was hybridized to tRNA and digested with S_1-nuclease. (7) Input DNA.

A - AluI. S - Sau3A. M - MspI. P - PstI. B - BamHI. RI - EcoRI






A-I.
AGCT 1234
bp
810
540
330
190±6
186±6
II.
567 pBR322 HinfI
bp
528
516
507
396
370
344
298
221
180

An attempt was made to determine if any of the COXI transcripts represent primary transcripts. This was done by labelling mtRNA using guanylyl transferase from vaccina virons and ɣ-[^{32}P]GTP which label 5'-diphosphate, non-capped termini of primary fungal transcripts. Sorghum mtRNA was [^{32}P]-labelled as described by Levens et al. (1981), and transcripts with homology to COXI were selected by hybirid release (Riccardi et al., 1979). As a control, yeast mtRNA was labelled and transcripts homologous to COXIII were released. No labelled transcripts with homology to Milo or 9E COXI were identified (data not shown). However, at least 6 sorghum mtRNA species were specifically labelled with guanylyl transferase (data not shown). This preliminary result indicates that higher plant mtRNA may have 5' di or triphosphate transcript termini. Whether these termini correspond to the 5' ends of primary transcripts as in fungi has yet to be determined.

4.5 J Mapping the 3' Terminus of the Major Milo COXI Transcript

The results of the S_1-nuclease transcript mapping of the 5' termini of the Milo and 9E COXI genes suggests that the major Milo and 9E COXI transcripts terminate in 3', non-homologous sequence.

S_1-nuclease transcript mapping analysis was performed to determine the 3' terminus of the major 1.8 Kb Milo COXI transcript. Since the 1.8 Kb Milo transcript maps from position -51 bp, it was predicted that the transcript would terminate within the 516 bp MspI M13 clone which extends 274 bp beyond the TAA stop codon, to position +1866 bp (Figure 4.13, B). This clone was labelled with α-[^{32}P]dCTP, hybridized to Milo mtRNA and digested with S_1-nuclease. The protected hybrids were analyzed by electrophoresis on an 8% (w/v) polyacrylamide-urea gel and autoradiography. Three S_1-nuclease protected fragments of 180 +/-5, 370 +/- 5, and circa 520 b. were generated (Figure 4.13 5). From the northern blot

transcript analysis (data not shown), it is apparent that this 516 bp clone hybridizes to a 1.3 Kb transcript in addition to the COXI specific transcripts of Milo (1.8, 2.3, 2.6 and 3.4 Kb). Thus, a logical interpretation of this S_1 nuclease mapping experiment is:

1) The 370 +/- 5 b. S_1-nuclease protected fragment, which maps the 1.8 Kb transcript to position +1719 bp, corresponds to the hybridization of the 1.8 Kb transcript to the labelled MspI fragment. If so, the size of the 1.8 Kb transcript is 1770 +/- 8 b. and transcript termination or processing occurs 126 +/- 3 bp downstream of the TAA stop codon within two 7 bp direct repeats (Figure 4.10).

2) The circa 520 bp fragment, which is the size of the labelled MspI fragment, corresponds to hybridization of the larger COXI transcripts to the labelled DNA.

3) The 180 +/- 5 b. S_1 nuclease protected fragment which maps the 1.8 Kb COXI transcript terminus within the ORF of the Milo COXI gene represents a hybrid between the MspI clone and the novel 1.3 Kb transcript.

4.5 K Summary and Discussion

Two forms of COXI have been identified in sorghum (Figure 4.14). The cytochrome c oxidase gene in Milo cytoplasm (and Kafir cytoplasm) is located on a 4.3 Kb EcoRI mtDNA fragment and encodes a 530 amino acid polypeptide (58,484 daltons) which correlates with the synthesis of the normal form of COI (apparent molecular weight 38K). The COXI gene in Milo sorghum and maize share 98% sequence homology and encode a polypeptide of similar length (Table 4.3). In contrast, in 9E sorghum the COXI gene is located on a 10.4 Kb EcoRI mtDNA fragment and encodes a 631 amino acid protein (70,358 daltons), which correlates with the synthesis of a variant COI (apparent molecular weight 42K). A rearrangement within 9E COXI gene resulted in a 101 codon continuation of the ORF, altered gene transcription and presumably the synthesis of a 11,874 dalton larger polypeptide. The

presumptive 9E COI is by far the largest reported to date (Table 4.3). In conclusion, the variant COI of 9E cytoplasm is probably not a precursor of the normal COI of other lines, but arises from the expression of a variant 9E COXI gene. The consequence of the relocation of COXI in 9E cytoplasm on the gene, transcript and protein are discussed below. Whether this mutation is correlated with expression of the CMS phenotype is examined in Section 4.6.

Figure 4.14

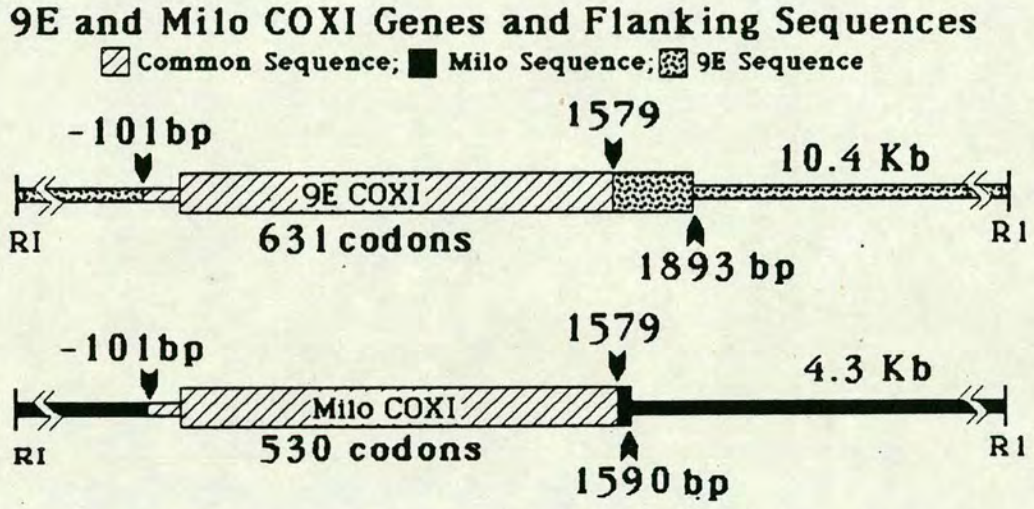


Table 4.3 Cytochrome c Oxidase Subunit I

Organism	ORF bp	ORF amino acids	Protein size predicted from DNA sequence (K daltons)	Protein size estimated from SDS-PAGE (K daltons)
Sorghum				
Milo	1590	530	58.5	38
9E	1893	631	70.0	42
Zea mays				
B37 N	1584	528	58.2	38
S.cerevisae	1530	510	55.9	40
N.crassa	1665	555	61.0	40
Bovine	1542	514	57.0	41.5

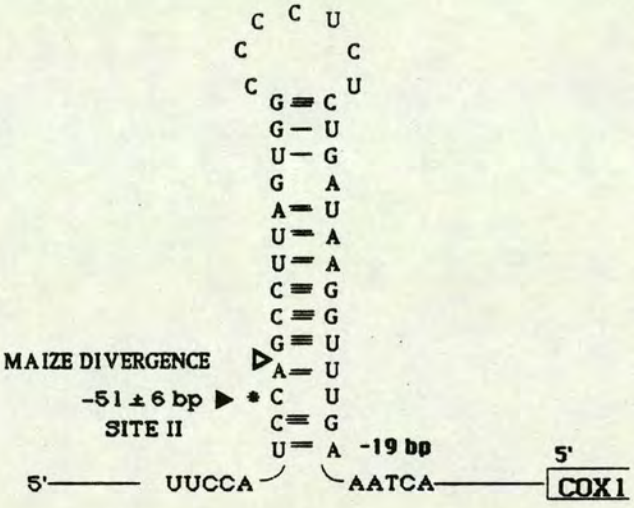
References: Isaac et al., 1985; Dixon and Leaver, 1982; Bonitz et al., 1981; Burger et al., 1982; Anderson et al., 1982; Azzi, 1980

Gene: The Milo and 9E COXI genes are identical from position -100 bp 5' to the initiation codon, to a position +1579 bp within the COXI ORF. Directly repeated DNA sequences are found at the points of divergence between the 9E COXI and Milo and Kafir COXI genes. Further evidence is required to determine if the variant COXI gene arose from recombination events across repeated DNA sequences, such as those thought to be responsible for the generation of sub-genomic plant mtDNA molecules (Lonsdale et al., 1984; Palmer and Shields, 1984; Chapter VI). Evidence that DNA with homology to the 3' unique sequence of the 9E COXI gene is located elsewhere in these genomes has been revealed by hybridization of a clone containing 3' 9E COXI unique sequence to EcoRI digested Milo and 9E mtDNA. This probe hybridizes to a 2.0 Kb EcoRI fragment. This fragment was identified previously in both Milo and 9E cytoplasm by hybridization with the 10.4 Kb EcoRI fragment containing the 9E COXI gene (Figure 4.6 E and F). DNA sequence analysis of this 2.0 Kb mtDNA fragment may determine if the final 101 codons of the 9E COXI gene are a portion of another gene of mitochondrial, chloroplast or nuclear origin and may reveal the importance of the repeated DNA sequences at the point of divergence.

Transcript initiation: The relocation of the COXI ORF is associated with altered transcription of the 9E COXI gene. Northern hybridization analysis has revealed that the major COXI transcript of Milo cytoplasm is 1.8 Kb, whereas the major COXI transcript of 9E cytoplasm is 2.2 Kb. Despite the apparent difference in transcription, S_1 nuclease transcript mapping studies have shown that the 5' end of the major transcript from both the Milo and 9E COXI genes maps to position -51 bp within the 5' homologous region (Figure 4.10). The 5' terminus of the major maize COXI transcript (position -57 bp) maps to a site near to that of sorghum (Isaac et al., 1985). This site in sorghum and maize COXI has dyad symmetry and therefore the transcript may form a structure which could have a

role in transcript processing (Figure 4.15). The observation that the S_1 nuclease protected fragments which map to position -51 bp are not identical in size suggests that the DNA-RNA hybrid formed was not perfectly stable or the size of the RNA varied +/- 5 bp. This could be due to secondary structure formation at this site.

Figure 4.15



The 5' terminus of a large (3.4 Kb) and relatively less abundant Milo COXI transcript mapped to position -404 bp (Site I) in the region which is non-homologous to the 9E gene. It is not known whether transcript initiation or processing occurs at the 5' transcript termini at -51 bp, common to both genes, or at the Milo specific 5' end at -404 bp. The Milo sequence at position -404 bp does not show striking homology to the nonanucleotide consensus sequence of the yeast promoter (Osinga et al., 1984a). Alignment of the DNA sequence around position -404 bp and the 5' transcript terminus of maize COXI, pea, Oenothera and wheat COXII and sugarbeet $P_{\odot}$ mtDNA plasmid transcript reveals no consensus characteristics amongst higher plants (Table 4.4). Perhaps the mitochondrial promoter sequence differs amongst plants and possibly individual genes.

Large 9E COXI transcripts were barely detectable and a single 5' terminus was mapped at position -51bp. If 9E COXI transcript initiation occurs in the 5' non-homologous region, then the presumed DNA rearrangement has placed the 9E COXI ORF downstream of a functional promoter sequence.

Table 4.4
Alignment of Yeast and Plant 5' Transcript Termini (*)

```
YEAST NONANUCLEOTIDE SEQUENCE
                                5'- ATATAAGTA -3'
                                          *
                                                          TERMINUS
SORGHUM COXI (SITE II)
 TATTTCTTATAAA-GAT-GAAAGTGGGCTGCGCTCAAGAACTAGTGA (-404bp)
                                        *
MAIZE COXI
 ACTTGCCTACTTTTGCACC GAAGAAA---CTCATAAGTAATCCAA  (-152bp)
                                          *
PEA COXII (SITE II)
 TTATTC TATAAAA(15bp)GAAGAAATC--ACGTAAGTGATAGAAA (-285bp)
          (SITE I)                  *
 TTATTC TATAAA<------------>TTTAC TAAGAGAAGAAAT (-302bp)
                                    *
OENOTHERA COXII
CGCGTCTTGGGCTCTTTTACCTCTAACTAAAAATCT-CGTATGAGAAT (-210/11)
                                          **
WHEAT COXII
TTTCCGTTGTTGCTCTTCAGAAAACGCGTATAGTAAAGTAGTCTTC   (-172bp)
                                          ?
Sugarbeet plasmid mtDNA
  CAAGAGAATAGCAGCTAATTAGCTAAAA TCATAAGTGATATCC
                                        **
```

References: Isaac et al., 1985; Moon et al., 1985; Hiesel and Brennicke, 1985; L. Bonen pesonal communication; Munk-Hansen et al., 1984.

Transcript termination: Termination of the major 9E COXI transcript occurs approximately 0.1 Kb downstream of the predicted stop codon. The Milo COXI gene transcripts terminate at four positions, approximately 0.1, 0.7, 0.9 and 1.4 Kb downstream of the termination codon. S_1--nuclease transcript mapping analysis revealed that the major, 1.8 Kb transcript of Milo COXI terminates 126 +/- 3 bp downstream from the stop codon within a 7 bp direct repeat (Figure 4.10). The 3' terminus of the pea COXII mRNA has also been mapped. This transcript also ends within a 6 bp direct repeat, 193 - 195 bp downstream of the termination codon (Moon etal., 1985). Milo COXI transcripts may have extensive 3' untranslated sequences, in common with the maize COXI and wheat COXII transcripts, (Isaac et al., 1985; Bonen et al., 1984).

Protein: The data presented here suggest that the variant 42K polypeptide is the product of the modified 9E COXI gene. However, a discrepancy is observed between the molecular weight of COI estimated by SDS-polyacrylamide gel electrophoresis and predicted by DNA sequence analysis. This discrepancy is most likely due to the anomalous mobility of COI in SDS-polyacrylamide gels (Bonitz et al., 1981; Table 4.3 above), as observed for other hydrophobic mitochondrial proteins, including COIII (Thalenfeld and Tzagoloff, 1980) and apocytochrome b (Nobrega and Tzagoloff, 1980). Partial proteolytic digestion of the normal and variant COI support the conclusion that the 9E COXI gene encodes a COI with an extended carboxy terminus. The variant 42K polypeptide was digested to 38,000 daltons by V8 protease which cleaves at the carboxy side of aspartic and glutamic acid residues. Upon examination of the sequence of the 9E polypeptide it is evident that there is one site within the unique portion of the 9E COI where this protease could cleave and reduce the larger COI to approximately the same size as the normal COI. This site is the glutamic residue encoded by the 597th codon (position +1591 - 1593 bp), near the 3' point of divergence (Figure 4.10).

Evidence that the large COI of 9E cytoplasm is processed was not obtained from pulse-chase analysis or by manipulation of the in organello mitochondrial translation system. To completely rule out the possiblity that the variant 9E COI is processed in vivo and to confirm that this polypeptide is indeed 101 amino acids larger than that of Milo, the carboxy terminus of this protein must be sequenced.

The extension of the 9E COXI ORF does not appear to have any affect on cytochrome c oxidase activity or the reduced cytochrome spectra of seedling mitochondria. This is surprising since COI is thought to be important in the assembly of the enzyme complex and is probably the site of the haem a_3 and Cu_B binding center (Stephens and Buse, 1979; Wikstrom et al., 1985). The structure of COI has

been predicted from the hydrophobic domains of the protein, determined by the analysis of Kyte and Doolittle (1982). COI is a very hydrophobic protein which may be composed of 12 transmembraneous segments with the carboxy terminus of the protein located on the matrix side of the inner membrane (Wikstrom et al., 1985; Figure 4.16). The secondary structure of 9E COI (and the 3' end of the Milo COI), based on Chou and Fasman (1978) measurements of alpha-helicity and Garnier (et al., 1978) predictions for local secondary structure is shown in Figure 4.17. From this plot it can be seen that within the conserved portion of COI (to residue 530) there are about 12 hydrophobic (solid) regions, which correspond to the 12 predicted transmembrane segments. Two additional hydrophobic domains of 9E COI are predicted beyond the 3' point of divergence. Whether these hydrophobic regions form additional transmembrane domain(s) or reside solely on the matrix side inner membrane has yet to be determined. From its predicted hydrophobic nature 9E COI could have an anamolous electrophoretic mobility.

Figure 4.16

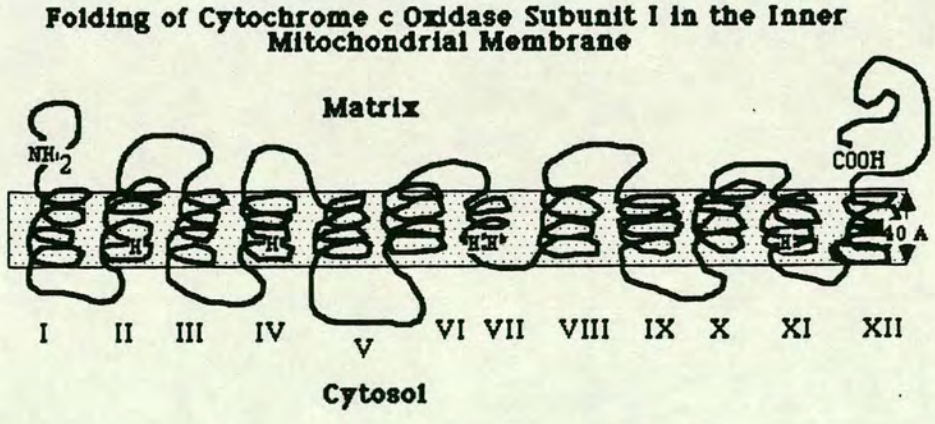


Figure 4.17

Predicted Structural Domains of 9E and Milo COI

The amino acid sequence of the 9E and Milo COI polypeptides was used to generate a plot of predicted secondary structure. This plot was generated using the CHOUFAS and PLOTCHOU programs of UWGCG. CHOUFAS program is designed to predict the secondary structure of globular proteins. The state of each amino acid is determined from a "window" of 7 amino acids (a given residue and the three residues located on either side). The residue state are:

α-helix
ß-pleated sheet
Reverse turn

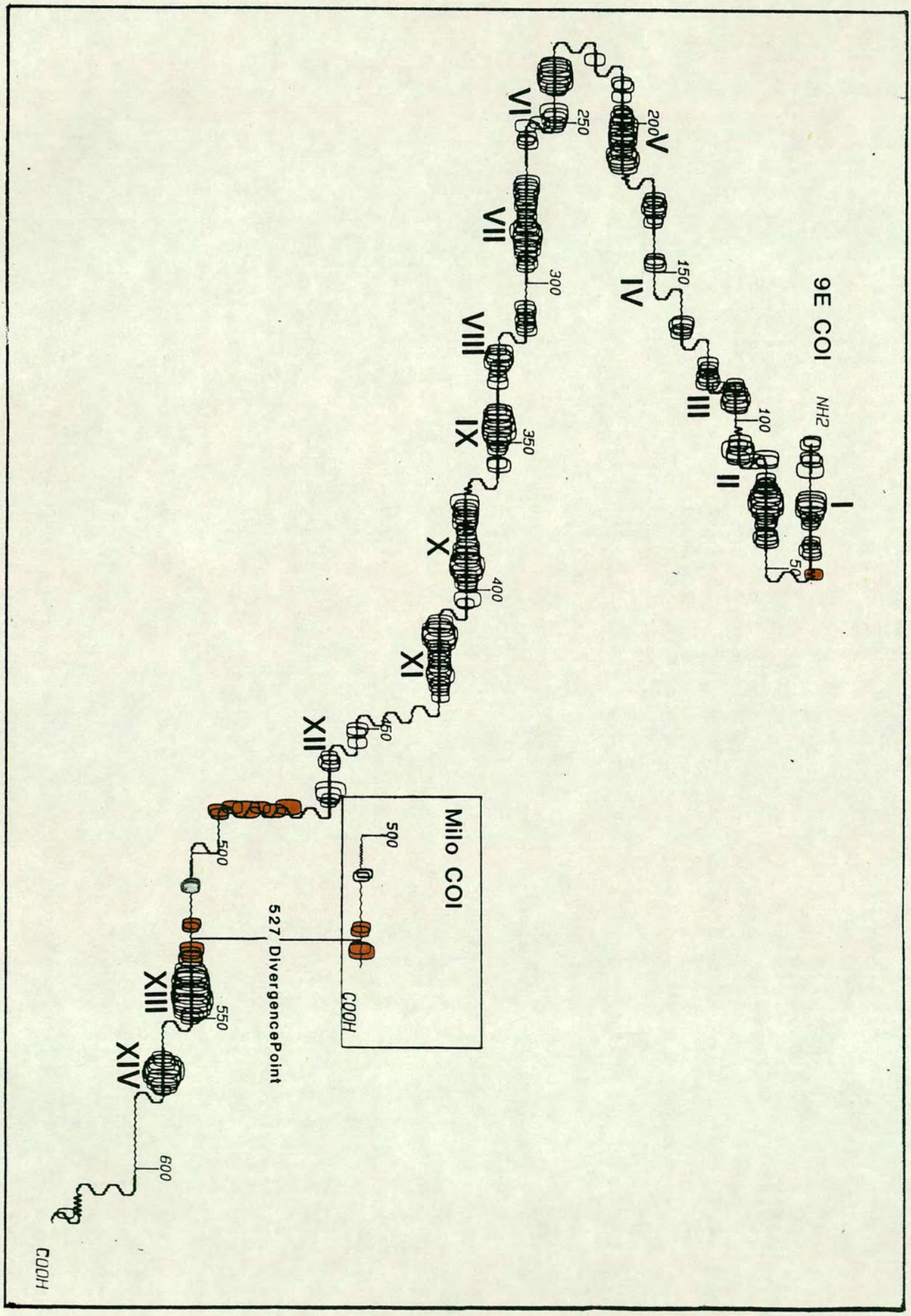
Regions which could form a coil structure are also predicted:

hydrophobic

hydrophilic

The output file of CHOUFAS is converted into visual representation by PLOTCHOU. For globular proteins appoximately 68% of the residue states are correctly assigned.

COI is a highly hydrophobic membrane polypeptide and therefore this plot does not present an extremely accurate representation of secondary structure. However, the 12 hydrophobic domains of COI (I - XII) which were predicted by Wi kstrom et al., (1985) on the basis of hydropathy profile (Kyte and Doolittle (1892)) are evident as hydrophobic coiled regions. Beyond the 3' divergence point of 9E COI two additional hydrophobic regions (XIII, XIV) are predicted.


9E COI
NH2
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
50
100
150
200
250
300
350
400
450
500
550
600
COOH
Milo COI
500
COOH
527 DivergencePoint
COOH

4.6 Is Expression of the Variant COXI Gene Associated with the CMS Phenotype?

The 42K variant COI was identified in the CMS line Kafir nucleus in 9E cytoplasm (Kafir/9E). Preliminary results indicated that the variant polypeptide was not synthesized by the male fertile line containing 9E nucleus in 9E cytoplasm (9E/9E) (Dixon and Leaver, 1982; Leaver et al., 1983a). A line referred to as 9ED/9ED was obtained from Pioneer Hi-bred International. MtDNA was isolated from 9ED/9ED, digested with EcoRI and probed with a [^{32}P]-labelled mtDNA fragment containing a portion of the Milo COXI gene. The mtDNA restriction pattern and EcoRI fragment location of COXI in 9ED/9ED differed from that of Kafir/9E. The COXI gene probe hybridized to a 4.3 Kb EcoRI fragment of 9ED/9ED mtDNA and a 10.4 Kb EcoRI fragment of Kafir/9E mtDNA (Figure 4.18 A, C, E, G). If the cytoplasm of 9ED/9ED and Kafir/9E are indeed the same, then 9E nuclear genes dramatically influence mitochondrial genome structure, COXI location and male fertility status. To confirm these results, additional sources of 9E/9E seed were examined.

A request for 9E/9E seed was to made Dr. O. Webster, who produced the CMS-9E line, and to other sorghum breeders. The 9E/9E seed used by Webster as the female parent of the CMS-9E line was no longer available, however seed with this genotype was obtained by Dr. K. Schertz from the collection of Dr. Sotomayor, Mayaguez, Puerto Rico. This seed (referred to as 9ES/9ES), 9ED/9ED, and Kafir/9E were compared by molecular and genetic analysis (Table 4.5). The seed was grown and classified taxanomically by Dr. K. Schertz. MtDNA was isolated, digested with EcoRI, stained with EtBr, photographed, transferred to nitrocellulose, probed with a COXI probe (pSM4.3), and exposed to X-Ray film (Figure 4.18). In addition, mitochondria were used for in organello protein synthesis in the presence of [^{35}S]-methionine, and for immunological detection of COI (Figure 4.19). It was

determined that 9ES/9ES and Kafir/9E appear identical except in fertility status. In contrast, 9ED/9ED was unlike the other 9E lines and resembled the line IS2483C which has a unique EcoRI restriction pattern (Figure 4.18 Lanes A, B and E, F; See also Table 5.3). It was subsequently found that, a Sudanese race bicolor line (IS2483C) and Kafir/9E, were sent to Pioneer Hi-bred on 1 March 1977 by Schertz. Pioneer reportedly obtained 9ED/9ED through Schertz, although Schertz has no record of sending this seed to Pioneer (G.Dalton and K.Schertz personal communication). From this analysis it seems likely that the 9ED/9ED seed was mislabelled and is actually IS2483C.

It was found that the variant form of COI is also synthesized by CMS and male fertile lines containing A4 (IS7920C) cytoplasm, another race Guinea sorghum (Appendix I). Both 9ES and A4 CMS lines develop pollen and exert anthers but are male sterile because of a failure in normal tapetal cell degeneration (Webster and Singh, 1961; Schertz, 1977). Synthesis of a variant COI in 9E cytoplasm is therefore not correlated with expression of the CMS phenotype. However, it cannot be ruled out that specific nuclear genes of 9ES and IS7920C maintain fertility in the presence of a variant COI, or another mutation associated with a mtDNA rearangement (See Section 5.3).

Table 4.5 Molecular and Genetic Analysis of 9E and IS2483C Cytoplasm Sorghum

LINE	TAXANOMIC CLASSIFICATION (Race)	MTDNA TYPE	COXI FRAGMENT LOCATION	COI SIZE (Dalton)
9ED/9ED	Bicolor	IS2483C	4.3 Kb	38K
9ES/9ES	Guinea	9E	10.4	42
Kafir/9E	Guinea	9E	10.4	42
IS2483C	Bicolor	IS2483C	4.3	38

Figure 4.18

EcoRI Restriction Pattern of 9ED/9ED, IS2483C/IS2483C, 9ES/9ES and Kafir/9E MtDNA and Identification of the Fragment Containing COXI.

MtDNA from the nuclear-cytoplasmic combinations 9ED/9ED (A) IS2483C/IS2483C (B), 9ES/9ES (D) and Kafir/9E (C) was digested with EcoRI, fractionated by electrophoresis in a 0.8% (w/v) agarose gel, stained with EtBr and photographed. The restriction patterns of 9ED/9ED and IS2483C/IS2483C are similar, as are those of 9ES and Kafir/9E. The mtDNA was transferred to nitrocellulose, hybridized with a [^{32}P]-labelled clone containing an internal portion of the Milo COXI gene and autoradiographed. A 4.3 Kb fragment in 9E/9ED (E) and IS2483C/IS2483C (F), and a 10.4 Kb fragment in 9ES/9ES (H) and Kafir/9E (G) mtDNA was identified.

Figure 4.19 (pg 160)

Identification of the COI in the Nuclear-cytoplasmic Combinations 9ED/9ED, Kafir/Kafir, 9ES/9ES and Kafir/9E

I. Mitochondrial translation products of the nuclear cytoplasmic combinations Kafir/9E (B), 9ES/9ES and Kafir/Kafir were labelled with [^{35}S]-methionine *in organello*. 0.25 x 10^6 cpm of labelled proteins were fractionated by 16% SDS-polyacrylamide gel electrophoresis and exposed to X-ray film. The variant 42K polypeptide is visible in Kafir/9E and 9ES/9ES.

II. Western blot of 9ED/9ED (a), 9ES/9ES (b), Kafir/9E (c) and Kafir/Kafir (d) mitochondrial polypeptides immunolabelled with an antiserum against yeast COI.
100 ug of mitochondrial protein were fractionated by electrophoresis in each lane of a 16% (w/v) SDS-polyacrylamide gel. The polypeptides were immobilized on nitrocellulose by electrophoretic transfer, incubated with antiserum prepared against yeast COI, washed and labelled with [^{125}I]-Protein A. A 42K COI polypeptide was identified in 9ES/9ES and Kafir/9E, and a 38K polypeptide in 9ED/9ED and Kafir/Kafir.

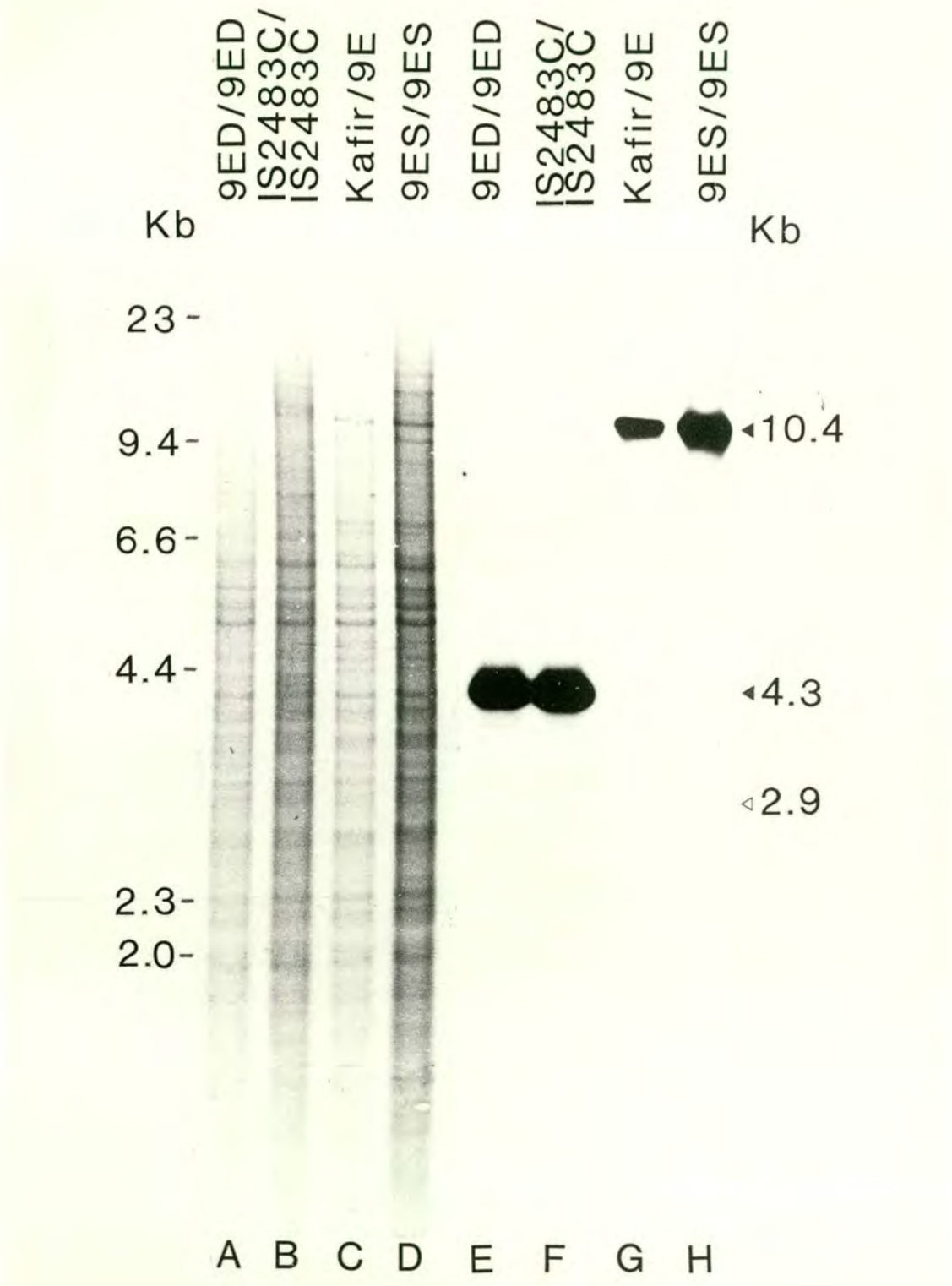
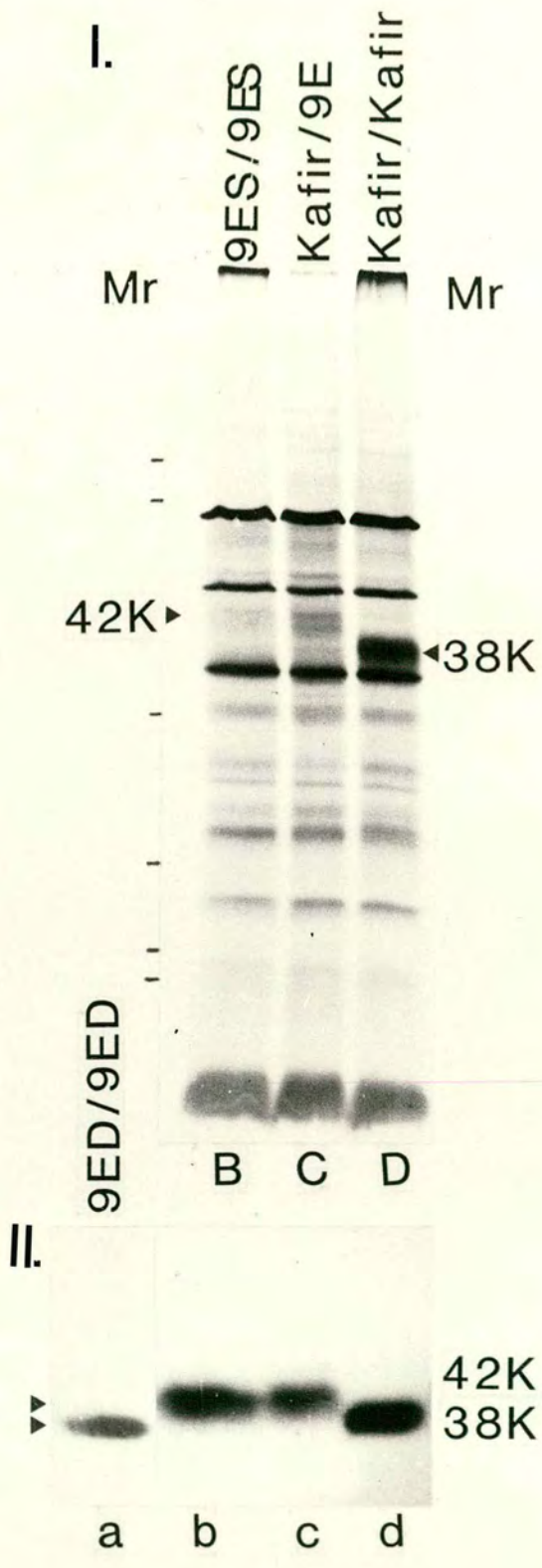

9ED/9ED
IS2483C/IS2483C
Kafir/9E
9ES/9ES
9ED/9ED
IS2483C/IS2483C
Kafir/9E
9ES/9ES
Kb
Kb
23-
9.4-
6.6-
4.4-
2.3-
2.0-
10.4
4.3
2.9
A B C D E F G H

Fig. 4.19


I.
9ES/9ES
Kafir/9E
Kafir/Kafir
Mr
Mr
42K
38K
9ED/9ED
B
C
D
II.
42K
38K
a
b
c
d

4.7 Conclusion

The investigation into the molecular basis of a variant COI synthesized by mitochondria from 9E cytoplasm offers a specific example of the way in which mitochondrial genome rearrangements can lead to altered gene expression. Specifically, genome rearrangements 5' to and within the 9E COXI gene led to the relocation of the gene, altered transcription and the synthesis of a significantly larger COI polypeptide. There is no evidence that the genome rearrangements which led to the relocation of the 9E COXI gene are causally related to a mutant phenotype, however this event may resemble the type of molecular events which underlie the CMS phenotype. Male fertile lines which synthesize the variant COI may possess nuclear gene(s) which have co-evolved with the mitochondrial genome rearrangements and compensate for alterations in mitochondrial gene expression or synthesis of a variant polypeptide. These may include the nuclear fertility restoring genes which are absent in the nuclear-cytoplasmic combinations which express the CMS phenotype.

CHAPTER V

MITOCHONDRIAL GENOME ORGANIZATION, EXPRESSION AND THE CYTOPLASMIC MALE STERILE PHENOTYPE

5.1 Introduction, Aims and Rationale

Cytoplasmic male sterility is a maternally inherited phenotype in which pollen development or normal anther dehiscence is impaired but female fertility is normal (Duvick, 1965, Edwardson, 1970). In maize, sorghum, and numerous other plants the CMS phenotype appears reflect a nuclear-cytoplasmic incompatibility which results when a cytoplasm is transferred into a foreign nuclear background (Hanson and Conde, 1985).

In maize several lines of evidence suggest that the CMS phenotype results from a mitochondrial mutation that can be suppressed by specific nuclear alleles (for review: Leaver and Gray, 1982; Laughnan and Gabay-Laughnan, 1983) or rearrangement of mtDNA (Kemble et al., 1983; Schardl et al., 1985). Three CMS cytoplasms (S, T, and C) can be distinguished from normal fertile N cytoplasm on the basis of nuclear genes which restore pollen fertility (Beckett, 1971), variations in mtDNA restriction pattern and expression of variant mitochondrial translation products.

Since nuclear genes restore pollen fertility, the effect of nuclear genotype on mitochondrial genome organization and expression has been examined. In general, the mtDNA restriction pattern of a CMS or nuclear restored fertile line with a given cytoplasmic genotype is the same as the original female parent (Laughnan and Gabay-Laughnan, 1983; Kemble et al., 1980). However, diversity of mtDNA restriction pattern and restriction fragment stoichiometry has been observed when fertile N cytoplasm maize is crossed into different nuclear backgrounds (Levings and Pring, 1977; Borck and Walbot, 1982; Oro et al., 1985). A nuclear effect on the

mitochondrial genome has been observed in S but not T cytoplasm (Pring and Levings, 1978; Laughnan and Gabay-Laughnan, 1983). In S cytoplasm, the abundance of plasmid mtDNA molecules and the expression of variant mitochondrial polypeptides varies with nuclear genotype (Laughnan and Gabay-Laughnan, 1983; C.J. Leaver, personal communication: See Section 1.6 C). In T cytoplasm, nuclear restorer genes apparently suppress the CMS phenotype and concomitantly the synthesis of a variant 13K polypeptide by isolated mitochondria (Forde and Leaver, 1980). Changes in mitochondrial genome organization have been observed when T and S cytoplasm is passed through a tissue culture cycle (Gengenbach et al., 1981, Brettell et al., 1980; McNay et al., 1984). These alterations may be related to nuclear variation arising during tissue culture (McNay et al., 1984).

As in maize, the various cytoplasms of sorghum can be distinguished by restriction endonuclease digestion of mtDNA, presence of 'plasmid-like' mtDNA molecules, and synthesis of variant mitochondrial translation products (Pring et al., 1982; Conde et al., 1982; Dixon and Leaver, 1982). To extend earlier observations, the effect of varying nuclear genotype on mitochondrial genome organization and expression was examined. It was found that when cytoplasms are transferred from their original nuclear background (in which the phenotype is male fertile), to a foreign nuclear background (in which the phenotype is CMS) changes in mitochondrial genome organization or expression may occur.

5.2 CMS and Male Fertile Lines with the Same Cytoplasmic Genotype: The Effect of Nuclear Genotype on Mitochondrial Genome Organization and Expression

1) Five cytoplasms in different nuclear backgrounds were examined (Table 5.1); each group included the following nuclear-cytoplasmic combinations:

1) a CMS line with Kafir nucleus,
2) the female parent of the CMS line, and
3) progeny of the CMS line crossed with an unrelated male fertile line, ie, CMS ♀ X 9E/9ED ♂ .

2) MtDNA was isolated from seedling mitochondria, fractionated by electrophoresis in an 0.8% (w/v) agarose gel, stained with EtBr and photographed under UV light to visualize any plasmid mtDNA molecules.

3) MtDNA was digested with EcoRI or HindIII, fractionated by electrophoresis in 0.8% (w/v) agarose, stained with EtBR, photographed, transferred to nitrocellulose and probed with [^{32}P]-labelled clones (Section 3.6):

pSK4.3: a 4.3Kb EcoRI mtDNA fragment which contains the COXI gene from Kafir cytoplasm.

pS9E10.4: a 10.4 Kb EcoRI mtDNA fragment which contains the COXI gene from 9E cytoplasm.

pS9E3.9: a 3.9 Kb EcoRI mtDNA fragment which contains the ATPA gene of 9E cytoplasm.

4) If not previously examined by Dixon and Leaver (1982), or to confirm their results, mitochondria were isolated and allowed to synthesize [^{35}S]-methionine labelled polypeptides in organello. Proteins were fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel and visualized by autoradiography.

The results obtained are summarized in Tables 5.2 (A and B) according to male fertility status, type of mtDNA restriction pattern, EcoRI fragment(s) which hybridized to the specific gene probes, presence of 'plasmid-like' mtDNAs and synthesis of variant polypeptides. A characteristic mtDNA restriction digestion pattern was observed for male fertile and CMS lines with Kafir, 9ED, A3, Milo, A2 and A4 cytoplasm. However, a different mtDNA restriction pattern was observed for male fertile and CMS M35-1 cytoplasm (Table 5.2 A). The EcoRI mtDNA fragments identified with the COXI and ATPA gene probes were similar for Kafir and 9E/9ED; M35-1 and A3; Milo and A2 (Table 5.2 A). A single copy of COXI was observed in each of these

cytoplasms. Two EcoRI fragments hybridized to the ATPA probe in each cytoplasm except M35-1 and A3: in these lines a single EcoRI fragment was identified.

From these results and those of others, sorghum cytoplasms can be classified by molecular analyses (Table 5.3). These lines can be placed into 3 groups from restriction endonuclease digestion of cpDNA (Pring et al., 1982), 11 groups from restriction digestion of mtDNA (Pring et al., 1982; Conde et al., 1982; Bailey-Serres, presented here) and 5 groups based on synthesis of variant polypeptides by isolated mitochondria (Dixon and Leaver, 1982; Bailey-Serres, presented here). M35-1 and A3 (IS1112C) cytoplasm have in addition, two linear plasmid mtDNA molecules of 5.7 and 5.3 Kb (Dixon and Leaver, 1982; Pring et al., 1982b).

The effect of nuclear genotype on mitochondrial genome organization, location mtDNA sequence homologous to COXI and ATPA probes, presence of plasmid mtDNAs and the expression of variant mitochondrial translation products has currently been examined and is summarized below.

1) M35-1 cytoplasm: The mtDNA restriction pattern of male fertile and CMS lines containing M35-1 cytoplasm is not identical. The change in fertility status is correlated with mitochondrial genome rearrangement(s) which was evidenced by differences in the hybridization of the 9E cytoplasm COXI and ATPA probes to EcoRI digested mtDNA (Table 5.2 A). The most striking difference is that a only one fragment of Kafir/M35-1 hybridized to the ATPA gene probe, which indicates that this cytoplasm contains a single complete copy of the ATPA gene (Table 5.2 A).

Two plasmid mtDNA molecules of 5.7 and 5.3 Kb are present in reduced amounts in the male fertile line containing M35-1 cytoplasm and are highly abundant in the CMS line Kafir/M35-1 (data not shown). The presence of these mtDNA plasmids can be correlated with the synthesis of a variant 82K dalton polypeptide by M35-1 mitochondria and the expression of the CMS phenotype (L. Dixon, personal communication).

2) A3 (IS1112C) cytoplasm: The male fertile and CMS lines containing A3 cytoplasm were not distinguished by mtDNA digestion or presence of plasmid mtDNAs: these lines were distinguished by *in organello* protein synthesis since CMS lines containing A3 cytoplasm synthesize variant 12K dalton polypeptide. This result suggests that there may be nuclear control over the transcription or translation of the mitochondrial gene encoding the 12K polypeptide.

3) Milo cytoplasm: The synthesis of an additional 65K dalton polypeptide by Milo cytoplasm is correlated with the expression of the CMS phenotype. The 65K polypeptide is synthesized by the fertile line Milo nucleus in Milo cytoplasm at less than 1% of the level observed when Milo cytoplasm is crossed into a foreign nucleus. It appears that nuclear genes may regulate the level of transcription or translation of the 65K polypeptide.

4) A2 (IS12662C) Cytoplasm: As in the closely related Milo cytoplasm, the introduction of Kafir nucleus into A2 cytoplasm did not appear to affect the mitochondrial genome organization of A2 mtDNA, but can be correlated with the synthesis of a variant 65K polypeptide and the expression of the CMS phenotype.

5) A4 (IS7920C) Cytoplasm: The *Eco*RI mtDNA digestion pattern of CMS and male fertile lines containing A4 cytoplasm appeared similar. However, a restriction fragment polymorphism was observed between the CMS and male fertile lines containing A4 cytoplasm when *Hind*III digested mtDNA was probed with the 4.3 Kb fragment containing the Kafir *COX*I gene (Figure 5.1 I). This fragment hybridized with a 1.9 Kb *Hind*III fragment of A4/A4 which was not observed in Kafir/A4 or in the nearly identical Kafir/9E mtDNA. So, some portion of the mitochondrial genome of the CMS A4 line is absent, in respect to the male fertile line.

The *COX*I gene of A4 cytoplasm is located on a 10.4 Kb *Eco*RI mtDNA fragment (Table 5.2 A) which correlates with the synthesis of a variant COI (apparent molecular weight 42K) (Figure 5.1 II.). The expression of this variant COI

by fertile and CMS lines containing A4 cytoplasm suggests that this variant polypeptide is not directly correlated with the CMS phenotype. Possibly, A4 nucleus may compensate for the altered form of COI, alternatively, mtDNA sequence within the 1.9 Kb HindIII fragment of A4/A4 may correct a mitochondrial mutation.

6) The effect of 9ED (IS2483C) nucleus on mitochondrial genome organization: The introduction of 9ED (IS2483C) nuclear genes by backcrossing apparently resulted in mitochondrial genome reorganization in Milo, A2 and A3 cytoplasms and not in M35-1 and A4 cytoplasms. It is apparent from hybridization of the ATPA and COXI gene probes to EcoRI digested mtDNA (Table 5.2 A) that the mitochondrial genome rearrangements observed in the presence of 9ED nuclear genes were not identical, but the mtDNA of each cytoplasm was converted to another known cytoplasm:

A3 ----------> Milo (identical to A2)
Milo --------> 9ES
A2 ----------> A3

In light of the fact that the lines which underwent mitochondrial genome reorganization (A2, A3 and Milo) were produced by Pioneer Hi-bred during 1982 and the lines which did not (M35-1 and A4) were produced during 1983, these data must be interpreted with caution. One interpretation is that the seed resulting from the backcross of the CMS females (A2, A3 and Milo) with the 9ED/9ED male in 1982 was mislabelled. Another interpretation is that 9ED nucleus dramatically influences mitochondrial genome organization in some, but not all sorghum cytoplasms. In conclusion, it is imperative that these crosses (ie. Kafir/Milo ♀ X 9E/9ED ♂) be repeated and the new genetic material examined before these results are presented publicly. If 9E nuclear genes do affect mitochondrial genome organization it will be extremely interesting to examine the events which cause the COXI gene to move from a 4.3 Kb EcoRI fragment in Kafir/Milo to a 10.4 Kb EcoRI fragment in Kafir + 9ED/Milo!

Figure 5.1

HindIII MtDNA Digestion Pattern and Mitochondrial Translation Products of A4 Cytoplasm

I. Identification of HindIII generated mtDNA fragments which hybridize to pSM4.3, the Milo COXI gene probe.
Kafir/9E, Kafir/A4 and A4/A4 mtDNA was digested with HindIII, fractionated by electrophoresis in a 0.8% (w/v) agarose gel, stained with EtBr and photographed (A). The gel was transferred to nitrocellulose and hybridized with the [^{32}P]-labelled pSM4.3, which contains the 4.3 Kb EcoRI fragment of Milo mtDNA, and exposed to X-ray film. Autoradiograph (B).

II. *In organello* labelled mitochondrial translation products of A4/A4 and Kafir/A4 and Immunological identification of COI.
Mitochondria were isolated from the nuclear-cytoplasmic combinations A4/A4 and Kafir/A4 and allowed to synthesize proteins *in organello*. 0.25 x 10^6cpm labelled proteins were solubilized in 4% (w/v) SDS, fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel and autoradiographed (C).
Unlabelled mitochondrial proteins from these lines were fractionated similarly, transferred to nitrocellulose, incubated with an antiserum prepared against yeast COI, and labelled with [^{125}]-Protein A. Autoradiograph of western blot (D).

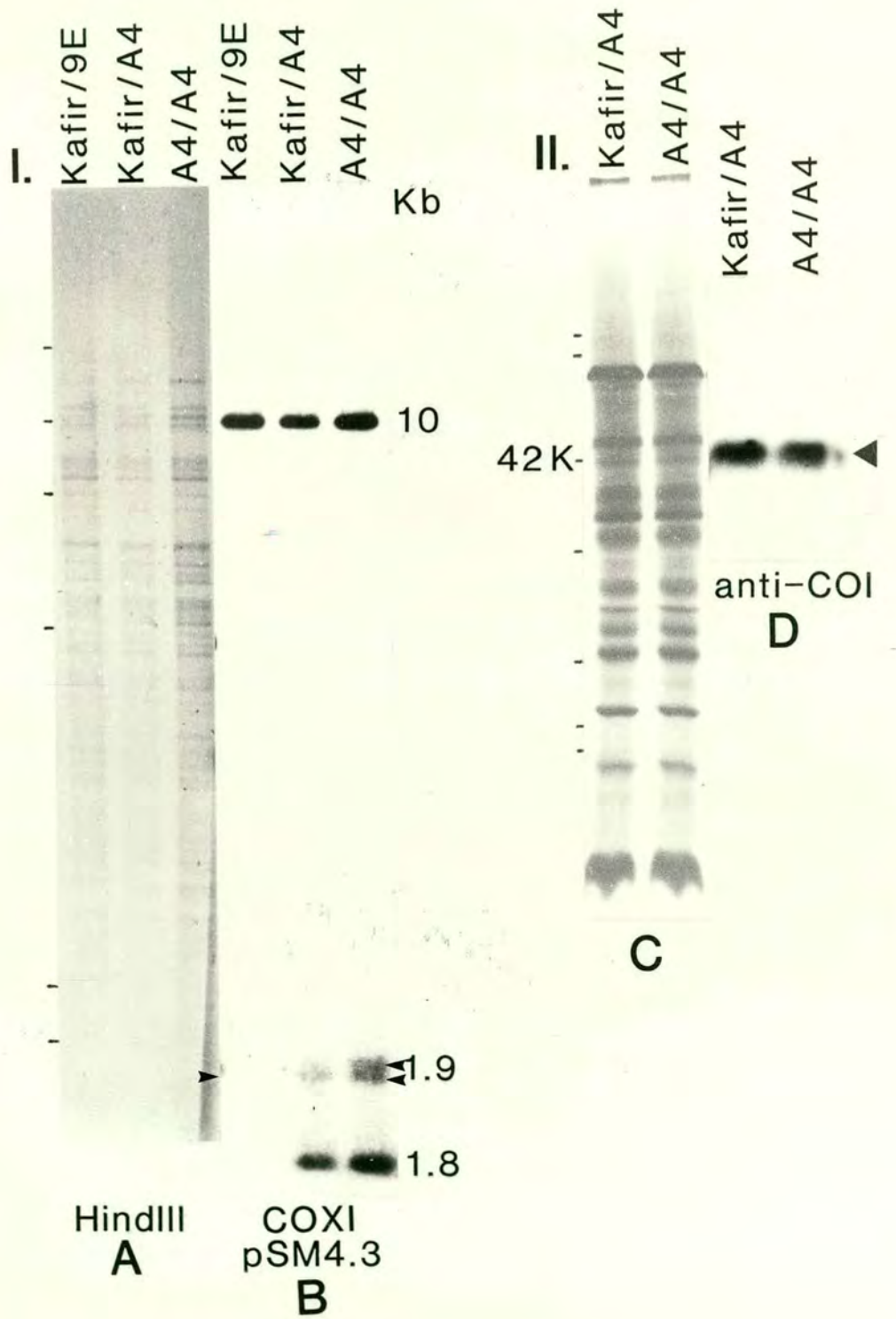

I.
Kafir/9E
Kafir/A4
A4/A4
Kafir/9E
Kafir/A4
A4/A4
Kb
10
1.9
1.8
HindIII
A
COXI
pSM4.3
B
II.
Kafir/A4
A4/A4
Kafir/A4
A4/A4
42K-
C
anti-COI
D

Table 5.1 Sorghum Lines Examined

NUCLEUS	CYTOPLASM	MALE FERTILITY STATUS	% OF EACH NUCLEAR GENOTYPE
Kafir	Kafir	MF	100% Kafir
9ED	9ED	MF	100% 9ED
(referred to as 9ED here, but has IS2483C cytoplasm (Section 4.6))			
M35-1	M35-1	MF	100% M35-1
Kafir	M35-1	CMS	100% Kafir
Kafir + 9ED	M35-1	CMS	50% Kafir + 50%9ED
Kafir + 9ED^{2}	M35-1	CMS	25% Kafir + 75%9ED
Kafir + Milo	M35-1	CMS	50% Kafir + 50% Milo
Kafir + A2	M35-1	CMS	50% Kafir + 50% A2
A3	A3	MF	100% A3
Kafir	A3	CMS	100% Kafir
Kafir + 9ED	A3	CMS	50% Kafir + 50% 9ED
Kafir + 9ED^{2}	A3	CMS	25% Kafir + 75% 9ED
Milo	Milo	MF	100% Milo
Kafir	Milo	CMS	100% Kafir
Kafir + 9ED	Milo	CMS	50% Kafir + 50% 9ED
Kafir + 9ED^{2}	Milo	CMS	25% Kafir + 75% 9ED
Kafir + 9ED^{3}	Milo	CMS	12.5% Kafir + 87.5%9ED
Kafir + A2	Milo	CMS	50% Kafir + 50% A2
Kafir + A3	Milo	CMS	50% Kafir + 50% A3
Kafir + M35-1	Milo	CMS	50% Kafir + 50% A3
A2	A2	MF	100% A2
Kafir	A2	CMS	100% Kafir
Kafir + 9ED	A2	CMS	50% Kafir+50% 9ED
Kafir + 9ED^{2}	A2	CMS	25% Kafir+75% 9ED
Kafir + 9ED^{3}	A2	CMS	12.5% Kafir + 87.5%9ED
A4	A4	MF	100% A4
Kafir	A4	CMS	100% Kafir
Kafir + 9ED	A4	CMS	50% Kafir+50% 9ED
Kafir + 9ED^{2}	A4	CMS	25% Kafir+75% 9ED

Table 5.2 A

MtDNA Restriction Pattern and EcoRI Fragments Identified by Specific MtDNA Probes

LINE NUCLEUS/CYTOPLASM	RESTRICTION PATTERN	FRAGMENTS IDENTIFIED (KB): pSK4.3 COXI	pS9E10.4 COXI	pS9E3.9 ATPA
Kafir/Kafir	Kafir	4.3	9.5, 4.3	5.6, 3,9
9E/9ED	IS2483C	4.3	9.5, 4.3	5.6, 3.9
M35-1/M35-1	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir/M35-1	M35-1	4.3	11, 4.3	3.9
Kafir + 9ED/M35-1	M35-1	4.3	11, 4.3	3.9
Kafir + 9ED^{2}/M35-1	M35-1	4.3	11, 4.3	3.9
Kafir + Milo/M35-1	M35-1	4.3	11, 4.3	3.9
Kafir + A2/M35-1	M35-1	4.3	11, 4.3	3.9
A3/A3	A3	4.3	11, 4.3	3.9
Kafir/A3	A3	4.3	11, 4.3	3.9
Kafir + 9ED/A3	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir + 9ED^{2}/A3	Milo	4.3	9.5, 4.3	3.6, 2.3
Milo/Milo	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir/Milo	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir + 9ED/Milo	9ES	10.4	10.4	3.9, 2.6
Kafir + 9ED^{2}/Milo	9ES	10.4	10.4	3.9, 2.6
Kafir + 9ED^{3}/Milo	9ES	10.4	10.4	3.9, 2.6
Kafir + A2/Milo	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir + A3/Milo	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir + M35-1/Milo	Milo	4.3	9.5, 4.3	3.6, 2.3
A2/A2	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir/A2	Milo	4.3	9.5, 4.3	3.6, 2.3
Kafir + 9ED/A2	A3	4.3	11, 4.3	3.9
Kafir + 9ED^{2}/A2	A3	4.3	11, 4.3	3.9
Kafir + 9ED^{3}/A2	A3	4.3	11, 4.3	3.9
A4/A4	A4	10.4,4.1	10.4	3.9, 2.6
Kafir/A4	A4	10.4,4.1	10.4	3.9, 2.6
Kafir + 9ED/A4	A4	10.4,4.1	10.4	3.9, 2.6
Kafir + 9ED^{2}/A4	A4	10.4,4.1	10.4	3.9, 2.6

Table 5.2 B

Plasmid MtDNAs and Variant Polypeptides

LINE NUCLEUS/CYTOPLASM	MALE FERTILITY STATUS	PLASMID MTDNAs	VARIANT POLYPEPTIDE(S) (K Dalton)
Kafir/Kafir	MF	-	-
9E/9ED	MF	-	-
M35-1/M35-1	MF	-	82 +
Kafir/M35-1	CMS	+++++	82 +++
Kafir + 9ED/M35-1	CMS	+++++	N.D.
Kafir + 9ED^{a}/M35-1	CMS	+++++	N.D.
A3/A3	MF	++	82 +++;12 +
Kafir/A3	CMS	+++++	82 +++,12 +++
Kafir + 9ED/A3	CMS	-	65 ++++
Kafir + 9ED^{a}/A3	CMS	-	65 ++++
Milo/Milo	MF	-	65 +
Kafir/Milo	CMS	-	65 ++++
Kafir + 9ED/Milo	CMS	-	42
Kafir + 9ED^{a}/Milo	CMS	-	42
Kafir + 9ED^{b}/Milo	CMS	-	42
Kafir + A2/Milo	CMS	-	65 ++++
Kafir + A3/Milo	CMS	-	65 ++++
Kafir + M35-1/Milo	CMS	-	65 ++++
A2/A2	MF	-	N.D.
Kafir/A2	CMS	-	65 ++++
Kafir + 9ED/A2	CMS	+++++	N.D.
Kafir + 9ED^{a}/A2	CMS	+++++	N.D.
Kafir + 9ED^{b}/A2	CMS	+++++	N.D.
A4/A4	MF	-	42
Kafir/A4	CMS	-	42
Kafir + 9ED A4	CMS	-	42
Kafir + 9ED^{a} A4	CMS	-	42

Abbreviations: MF - male fertile; N.D. - not determined.
(-) not detected; (+) detection intensity.
Table adapted from Dixon and Leaver, (1982).

Table 5.3

Classification of Sorghum Cytoplasms by Molecular Analyses

CYTOPLASM	VARIANT POLYPEPTIDE [a,d]	MtDNA GROUP [b,d]	CpDNA GROUP[b]	PLASMID LIKE MTDNA[a,c]
Milo	65K	1	1	none
IS6705C	65K			
IS2801C	65K			
IS3063C	65K			
KS34	65K			
KS38	65K			
KS39	65K			
KS35	65K	9		
KS36	65K			
KS37	65K			
A2 (IS12662C)	65K	2	-	
IS1056C	65K	5	2	
A3 (IS1112C)	82K, 12K	3		5.7 Kb 5.3
M35-1	82K	4		5.7 5.3
Millo Blanco*	None[c]	6		none
9E (IS17218)	42K	7	3	
A4 (IS7920C)	42K[c]	8		
Kafir*	None	10	2	
Martin*			-	
IS2483C (9ED)*	None	11	-	

* Martin and Kafir are male fertile lines used to maintain the CMS lines listed in this table.

Millo Blanco and IS2483C lines examined were male fertile.

References: a - Dixon and Leaver, 1982; b - Pring et al., 1982a; c - Pring et al., 1982b; d - Bailey-Serres, unpublished.

5.3 Nuclear-Cytoplasmic Interactions and the CMS Phenotype: A Discussion

The CMS phenotype is expressed in sorghum when a cytoplasm is backcrossed into a non-fertility restoring nucleus (ie. Kafir), and therefore appears to result from incompatibility between a foreign nucleus and cytoplasm. It has been proposed that the CMS phenotype is caused by mitochondrial mutations which are visible as differences in mtDNA restriction endonuclease pattern and variant mitochondrial translation products (Leaver et al., 1985). The results presented here support the hypothesis that the genetic determinants responsible for CMS are carried by mtDNA. In addition, these results demonstrate that nuclear genotype influences mitochondrial genome expression and possibly organization in sorghum. If specific nuclear genes are required for mitochondrial DNA transcription, RNA maturation, or post-translational processing as in fungi (Myers et al., 1985; Rödel et al., 1985; Hill et al., 1985), then correct mitochondrial function may require complementary nuclear and cytoplasmic genomes. Due to the extensive interspecific variation in mitochondrial genome organization, it is not surprising that varying nuclear background can result in altered mitochondrial gene expression and a mutant phenotype. In conclusion, it can be hypothesized that the CMS phenotype results from mitochondrial mutations which are silent in the presence of natural or fertility restoring nuclear gene(s) that:

a) properly regulate mitochondrial gene expression, or
b) compensate for the expression of variant mitochondrial polypeptides.

Nuclear regulation of mitochondrial gene expression is evident from the synthesis of variant polypeptides at high levels in CMS lines and reduced levels in male fertile lines (ie. Sorghum: Milo and A2, 65K polypeptide; M35-1, 82K polypeptide; A3, 12K polypeptide; Maize: CMS-T 13K polypeptide). Nuclear control of mitochondrial

expression is evidently altered by changes in mitochondrial genome organization. In yeast, mtDNA rearrangements can suppress nuclear pet⁻ mutations and restore mitochondrial respiration (Costanza and Fox, 1985; Rödel et al., 1985). These events may parallel the mtDNA rearrangements in maize which are coincident with cytoplasmic reversion to male fertility (Levings et al., 1980; Kemble et al., 1982; Schardl et al., 1985).

Evidence of mitochondrial mutations which are suppressed by nuclear mutations has been reported in fungi (Dujardin et al., 1980; Groudinsky et al., 1981; Labouesse et al., 1985; Kruszewka and Szczesniak, 1985). Nuclear genes may suppress or compensate for mitochondrial mutations which arose perhaps from aberrant genome rearrangements. For example, nuclear genes in 9E (and IS7920C) may have co-evolved with the rearrangements which resulted in the relocation of COXI and the expression of a mutant COI. Perhaps a specific nuclear gene encoded by the 9E nucleus is required during pollen development for efficient transcription and maturation of the 9E COXI precursor mRNA, or assembly and activity of cytochrome c oxidase. It has been proposed that a nuclear mutation in a gene for a nuclear encoded subunit of cytochrome c oxidase suppresses a mitochondrial mutation in COXIII in yeast (Krusazewka and Szczesniak, 1985). Perhaps a similar mutation exists in 9E and A4 (IS7920C) nucleus.

A genetic experiment could be carried out to determine if a single nuclear gene present in 9E nucleus, but absent from the non-fertility restoring nucleus Kafir, is responsible for male fertility in 9E cytoplasm. The following crosses could be made:

1) Kafir/9E ♀ X 9E/9ES ♂

2) F_1 Kafir + 9E/9ES ⊗

↓

F_2

The F_1 progeny could be allowed to self-fertilize, and the F_2 progeny grown and scored for fertility. If a single dominant allele of 9E nucleus is required for male

fertility then the F_2 progeny would be 75% fertile and 25% male sterile. A second genetic experiment would be to determine if A4 nuclear genes suppress the CMS phenotype in 9E cytoplasm and visa versa. These genotypes 9E/A4 and A4/9E could be bred and their fertility status determined.

To date, only circumstantial evidence links the CMS phenotype with a mitochondrial mutation. To unveil the causes of CMS a molecular and cellular analysis of mitochondrial development during microsporogenesis must be undertaken. It has already been demonstrated that during pollen formation there is normally an increase in respiration of anther mitochondria (Ohmasa et al., 1976), number of tapetal cell mitochondria (Warmke and Lee, 1978) and relative amount of mtDNA (Abbott et al., 1985). These increases are less dramatic in CMS lines of maize. Evidence for altered mitochondrial protein synthesis during pollen development has not been examined, however it has been shown that mitochondrial gene expression is developmentally regulated in maize (Newton and Walbot, 1985). It is possible that fine-tuned coordination between nuclear gene expression and mitochondrial function and/or replication is required for sexual reproduction. The following experiments could be carried out to examine this hypothesis:

1) Quantitative analysis of the relative levels of mtDNA and nuclear DNA in seedlings, leaves and anthers. This could be carried out by isolating total cellular DNA from CMS and fertile lines and using specific mitochondrial and nuclear gene probes (ie the COXI, ATPA and maize nuclear rDNA) to quantify the amount of DNA.

2) Isolation of total cellular RNA and determination of the level of transcription of mitochondrial genes relative to a nuclear gene.

3) Quantitative analysis of mitochondrial protein using the western technique and antiserum raised against mitochondrial proteins (ie. COI and α-subunit of F_1ATPase).

Ultimately it may be possible to characterize the nuclear-cytoplasmic interactions necessary for normal mitochondrial function and plant development. From the evidence presented here, it is apparent that variability in mitochondrial genome organization is responsible for altered gene expression and may have distinct phenotypic consequences when a cytoplasm is transferred to a foreign nucleus. In the following chapter the molecular mechanisms which underlie variablility in mitochondrial genome organization are examined.

Chapter VI

GENOME REARRANGEMENTS IN HIGHER PLANT MITOCHONDRIA

6.1 Summary of Results

The mitochondrial genomes of higher plants are large and variable in size and sequence complexity. This variability is so extensive that differences in mitochondrial genome organization can be used to classify cytoplasmic genotypes of a single species. Interspecific variation in male fertile and CMS lines of Sorghum bicolor has been examined by analysis of restriction endonuclease digestion of mtDNA and products of in organello protein synthesis. From these analyses 11 unique cytoplasmic genotypes of sorghum were identified. These cytoplasms were further characterized by hybridization of specific gene probes to Southern blots of EcoRI digested mtDNA, and identification of the fragment location of the genes encoding COI, COII, COB and the α-subunit of F_1ATPase. No differences in the EcoRI fragments which hybridized to the COXII or COB gene probes were detected amongst the lines examined. However, differences in the number and size of EcoRI fragments hybridizing to the ATPA probe were observed. The ATPA gene was detected on two EcoRI fragments in all cytoplasms except two related Indian cytoplasms (A3 (IS1112C) and M35-1), in which only one EcoRI fragment was detected. It was determined that the EcoRI fragment location of the COXI gene correlated with the size of the COI polypeptide synthesized in vivo and in organello. The mitochondrial gene encoding COI was isolated from a line (Milo) that synthesizes the normal COI (apparent molecular weight 38,000) and a line (9E) which synthesizes the variant COI (apparent molecular weight 42,000). Sequence analysis revealed that the normal COI is encoded by a 530 codon gene which shares 98% homology with the corresponding maize gene. In contrast, the variant COI is encoded by a 631 codon gene that diverges completely from the normal sorghum COXI gene

100 bp 5' to the presumed initiator methionine and within the terminal 3' coding sequence. The 3' divergence resulted in a 101 codon continuation of the 9E COXI gene, and thus a 12 K dalton extension to the putative gene product. The carboxy extension of 9E COI is not homologous to any known polypeptide, but DNA with homology to the unique portion of the 9E COXI gene is present in another genomic location in 9E and Milo mtDNA. The novel COXI gene appears to have arisen from at least two genome rearrangements, with consequent effects on transcription and the size of the mature polypeptide. Although no difference in the cytochrome c oxidase activity of seedling mitochondria with the variant COI was observed, it is possible that the genome rearrangements associated with the 9E COXI gene could somehow affect normal plant development. The mutant COI is observed in both male fertile and CMS lines containing 9E and A4 (IS7920C) cytoplasm. These male fertile lines may possess nuclear genes which have co-evolved with the mitochondrial genome rearrangements and compensate for the variant COI polypeptide or any other changes in gene expression. These genes may include the fertility 'restoring' genes which are absent in nuclear-cytoplasmic combinations which express the CMS phenotype. Direct evidence that nuclear genotype can influence mitochondrial gene expression was obtained. When certain cytoplasms are transferred to a foreign nuclear background, variant mitochondrial polypeptides are synthesized at increased levels and the CMS phenotype is observed. In conclusion, these results demonstrate that mitochondrial genome rearrangements can lead to altered gene expression and emphasize the importance of nuclear-cytoplasmic interactions in mitochondrial function and normal pollen development. The molecular mechanisms which underlie the observed variation in mitochondrial genome organization may resemble those which gave rise the mutant COXI gene in 9E cytoplasm. The molecular mechanisms and biological significance of genome rearrangements in higher plants will be discussed in this chapter.

DISCUSSION

6.2 Plant Mitochondrial Genome Structure

The mitochondrial genomes of higher plants are larger than those described in other organisms, yet plant mitochondria appear to synthesize only a few additional proteins. The size and conformation of the mitochondrial genome of higher plants have been estimated by reassociation kinetics, restriction endonuclease digestion, genome mapping and electron microscopy. Size estimates of plant mitochondrial genomes vary from about 215 Kb in Brassica to 2400 Kb in muskmelon (Palmer and Shields, 1984; Ward et al., 1981). Genome size is not correlated with nuclear genome size, mitochondrial volume or taxanomic classification (Ward et al., 1981). Indeed, as shown in this thesis, mitochondrial genome organization can vary within a single species (Levings and Pring, 1977; Chapter V). In general, the restriction endonuclease digestion patterns of plant mtDNA are complex and include fragments present in non-stoichiometric amounts (Quetier and Vedel, 1977; Bonen and Gray, 1980; Spruill et al., 1980; Ward et al., 1981; Borck and Walbot, 1982). This led Quetier and Vedel, (1977) to predict that plant mitochondria possess a heterogeneous population of mtDNA molecules. This prediction has been supported by electron microscope studies which suggest that plant mtDNA is composed of a heterogeneous population of linear and circular molecules of varying sizes (for review, Bendich, 1985). Circular molecules of distinct sizes have been observed in a few species, but these molecules can differ in abundance even within an individual specie (Dale et al., 1981). Circular molecules as large as the predicted genome size have not been observed, however the mitochondrial genome of a monocot (maize) and a dicot (turnip) has been mapped as a single circular molecule (Lonsdale et al., 1984; Palmer and Shields, 1984). In both plants, it has been predicted that "recombination" across repeated DNA sequences on the

"master circle" gives rise to smaller sub-genomic circular molecules (Ibid). Evidence that mtDNA rearrangements can involve specific gene sequences has been provided by studies of wheat, Oenothera, maize and now sorghum (Falconet et al., 1984; Falconet et al., 1985; Bonen and Gray, 1980; Manna and Brennicke, 1986; Leaver et al., 1985; Isaac et al., 1985b). To predict the molecular basis of genome rearrangements in higher plants, such as those associated with the COXI gene of 9E cytoplasm sorghum, the data published to date on genome rearrangements in fungi are reviewed here.

6.3 Molecular Mechanisms of Genome Rearrangements

Genome rearrangement occurs frequently in fungal mtDNA. Three mechanisms have been proposed as a basis for these rearrangements: general recombination, site-specific recombination and transposition.

General recombination describes the genetic exchange between two homologous DNA sequences which is promoted by a general recombinase. For example, when haploid yeast of opposite mating type fuse and form a zygote, the mtDNA of the two parents mix and undergo general recombination and 'sorting-out'. These events are revealed by the symmetrical segregation of parental markers (Dujon and Slonimski, 1976).

Site-specific recombination is the genetic exchange between two homologous sequences which is promoted by an enzyme that recognizes a specific DNA sequence. Examples of site-specific recombination are 1) inversion (flipping) of the yeast "2 micron" plasmid, and 2) integration of mitochondrial intron r1 into the 21S rRNA of yeast lacking that intron (omega⁻ yeast). In the first example, **reciprocal recombination** between two 599 bp inverted repeats results in the inversion of the unique sequences of the yeast plasmid (Vetter et al., 1983). This event requires the expression of a single 2 μm plasmid gene which encodes an endonuclease responsible for cleavage at a specific

site within the large inverted repeats (Andrews et al., 1985). In contrast, **non-reciprocal recombination** occurs when yeast containing the 21S rRNA intron r1 are crossed with yeast lacking r1 (omega⁻): r1 integrates into the 21S rRNA gene and omega⁻ allele is consequently lost (Zinn and Butow, 1985; Butow, 1985). A protein encoded by the r1 ORF is thought to be responsible for site-specific cleavage within the 21S rRNA gene (Macreadie etal.,1985). Non-reciprocal recombination between two alleles of the yeast mitochondrial Var1 gene results in the loss of the shorter allele (Strausburg and Butow, 1981; Butow, 1985).

Recombination events are reportedly responsible for the formation of sub-genomic circular mtDNAs in fungal mutants (yeast petites (de Zamaroczy et al., 1983); N.crassa stopper (Gross et al., 1984)). The excision and selective amplification of mtDNA segments in petite strains of yeast are promoted by either a site-specific or a general recombination mechanism. de Zamaroczy et al., (1983) compared the excision sequences of a large number of S.cerevisiae petite mutants and found perfect direct repeats at each excision site. They interpreted this as evidence for a site-specific recombination mechanism. Recent evidence indicates that sequence topology may have a role in recombination. The site where a class of petite mutants (PIF-dependent) and wild-type mtDNA recombine is capable of forming a hairpin structure (Foury and Van Dyck, 1985). This mtDNA recombination is stimulated by the nuclear gene product of the PIF locus (Ibid).

Transposition is a general term used to describe the movement of distinct genetic elements. Transposition of mobile genetic elements can be conservative (movement to a new genetic location) or replicative (replication and insertion into an additional genetic location). Insertion of a non-mitochondrial 9.0 Kb plasmid into N.intermedia mtDNA is correlated with mtDNA degradation and culture senescence (Bertrand et al., 1985). Transposition may be responsible for the integration of mtDNA into nuclear DNA during senescence in Podospora (Wright and Cummings,1983).

The molecular mechanisms responsible for mitochondrial genome rearrangements are not limited to those described above. A reverse transcription-ligation mechanism has been proposed for the formation of senDNA in Podospora (Kück et al., 1985). Failure in replication or unequal 'sorting-out' of sub-genomic molecules could also result in changes in mtDNA organization and content.

6.4 Evidence for Genome Rearrangement in Higher Plant Mitochondria

Variability in the restriction endonuclease digestion patterns of plant mtDNA is molecular evidence of genome reorganization. Quantitative differences in restriction fragments could result from changes in the copy number of individual sub-genomic molecules, and qualitative changes could result from genome rearrangements. Alterations in mtDNA restriction pattern have been observed in spontaneous revertants of CMS-S maize (Levings et al., 1980), tissue cultures originating from the same plant (Sparks and Dale, 1980), plants transferred to and regenerated from callus culture (Gengenbach et al., 1981; Kemble et al., 1982; McNay et al., 1984), and somatic hybrids (progeny of fusion of two different parental somatic cells) (Petunia: Boeshore et al., 1985, 1983; Nicotiana: Belliard et al., 1979; Nagy et al., 1981; Galun et al., 1982; Brassica: Chetrit et al., 1984; Potato: Kemble and Shepherd, 1984). In these reports progeny with non-parental mtDNA restriction fragments patterns were observed. If mtDNA of angiosperms is indeed maternally inherited (for discussion see Grun, 1976), in the case of the CMS-S revertants and the regenerants from tissue culture, the novel mtDNA fragments may result from **intragenomic** rearrangement of the maternal genome. There is evidence that the novel mtDNA fragments of somatic hybrids may be generated by intragenomic rearrangements (Kemble and Shepherd, 1984) or **intergenomic** rearrangements between the mtDNA of the two parents (Hanson, 1984;

Rothenberg et al., 1985). Intergenomic recombination would require the fusion of the parental mitochondria, and is thought to occur between the parental mtDNAs of human and rodent somatic cell hybrids (Horck et al., 1979).

Genome rearrangements in higher plant mtDNA could result from any of the mechanisms implicated in rearrangements in fungal mtDNA. Perhaps the most valuable data on rearrangements in higher plants comes from genomic mapping studies. In addition to the 'master circle' proposed for turnip and maize, a number of sub-genomic circular molecules have been mapped. Both Palmer and Shields (1984) and Lonsdale et al. (1984) found that mtDNA fragments containing repeated mtDNA sequences of varying size are present in sub-stoichiometric amounts and are flanked by four unique sequences (Figure 6.1 A). This observation has led to the prediction that **homologous recombination** across repeated mtDNA sequences results in a heterogeneous population of mtDNA molecules. Site-specific or general recombination across direct repeats would result in the formation of sub-genomic circular molecules (Figure 6.1 B), as in the formation of yeast petite mutants. Whereas, recombination across indirect repeats would result in inversion of a circular molecule (Figure 6.1 C) as in the yeast "2 micron" circle and proposed for the isomerization of cpDNA (Palmer, 1983). The frequency of homologous recombination across DNA repeats or differences in replication could cause changes in restriction fragment stoichiometry. Novel recombination events could result in formation of unique sub-genomic molecules.

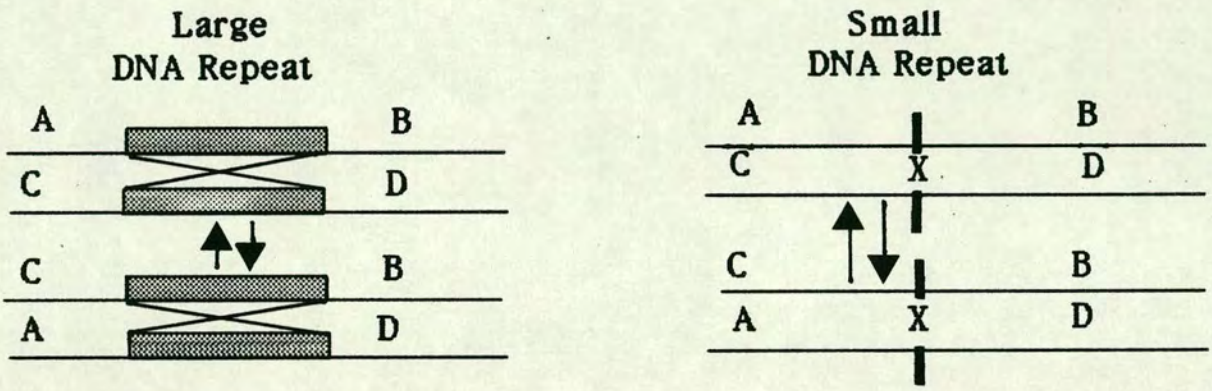
Evidence that repeated mtDNA sequences contain specific genes has been presented for a number of plants and fungi (Table 6.1). These repeated mtDNA sequences may contain all or part of the coding sequence. In maize, for example, the ATPA gene is located within the "12Kb" repeat (Figure 6.1 D) (Isaac et al., 1985b; P.G. Isaac, personal communication). Recombination across the 12Kb repeat gives rise to two sub-genomic circles of 67 Kb and 503 Kb with the "12Kb" repeat situated in two distinct genome

environments (Lonsdale *et al.*, 1984). Genome rearrangements which involve the 26S rRNA gene of *Oenothera* differ from those predicted for the maize *ATPA* gene since only a portion of the gene is repeated (Manna and Brennicke, 1986). The entire 26S rRNA gene is found in one genomic location and a 3' portion is located on a 7.5 Kb sub-genomic circle. Manna and Brennicke (1986) propose that rearrangement between a 10bp DNA sequence located within the 26S rRNA gene and repeated 7.5 Kb downstream of the gene gives rise to the formation of the 7.5 Kb circular molecule (Figure 6.2 B). Since the 5' portion of the 26S rRNA gene is not duplicated within *Oenothera* mtDNA the rearrangement may have generated a sub-genomic circular molecule which was lost, perhaps because of failure to replicate. In CMS-S maize, recombination between the terminal inverted repeats of the linear S1 and S2 plasmids and homologous sequences within the mitochondrial chromosome is thought to result in the linearization of the mtDNA molecules (Schardl *et al.*, 1984) (Figure 6.2 C).

General or site-specific recombination could underlie the genome rearrangement events described above. If the mechanism is site-specific then sequence homology at the various recombination sites is expected. Examination of the various recombination sites (points of divergence) reported to date does not reveal a consensus sequence, however mtDNA rearrangements in a single plant have not been studied in detail. The recombination site within the *Oenothera* 26SrRNA shares homology with the yeast omega locus insertion sequence and YZ endonuclease site in the MAT locus (Manna and Brennicke, 1986; Kostriken *et al.*, 1983). The 5'-ends of the maize S-plasmids share sequence homology with the lambda-phage *attB* sequence (Lonsdale *et al.*, 1984). Short direct or indirect repeated sequences are found at a number of the reported rearrangement sites, the largest of which is the 26bp direct repeat located at the 3' divergence point of the 9E *COX*I gene. Further studies are necessary to determine whether DNA sequence and/or topology is recognized by a 'recombinase'.

Figure 6.1 Recombination Across Repeated DNA Sequences

A. DNA Repeats and Theoretical Linkage Groups



B. Recombination Across Direct Repeats

C. Recombination Across Indirect Repeats

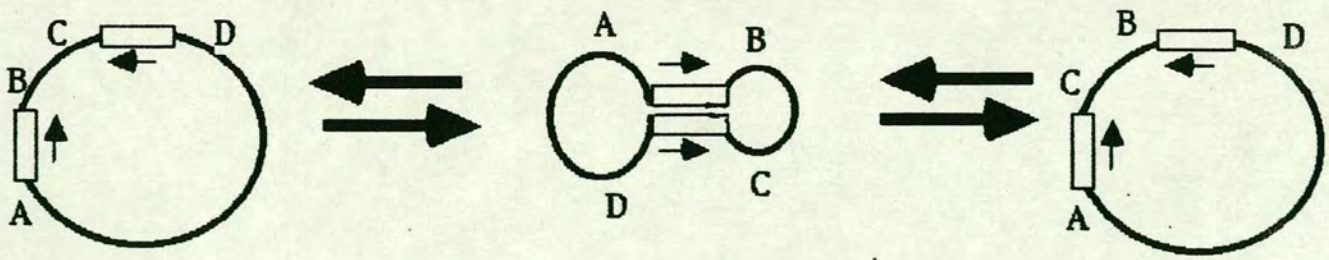
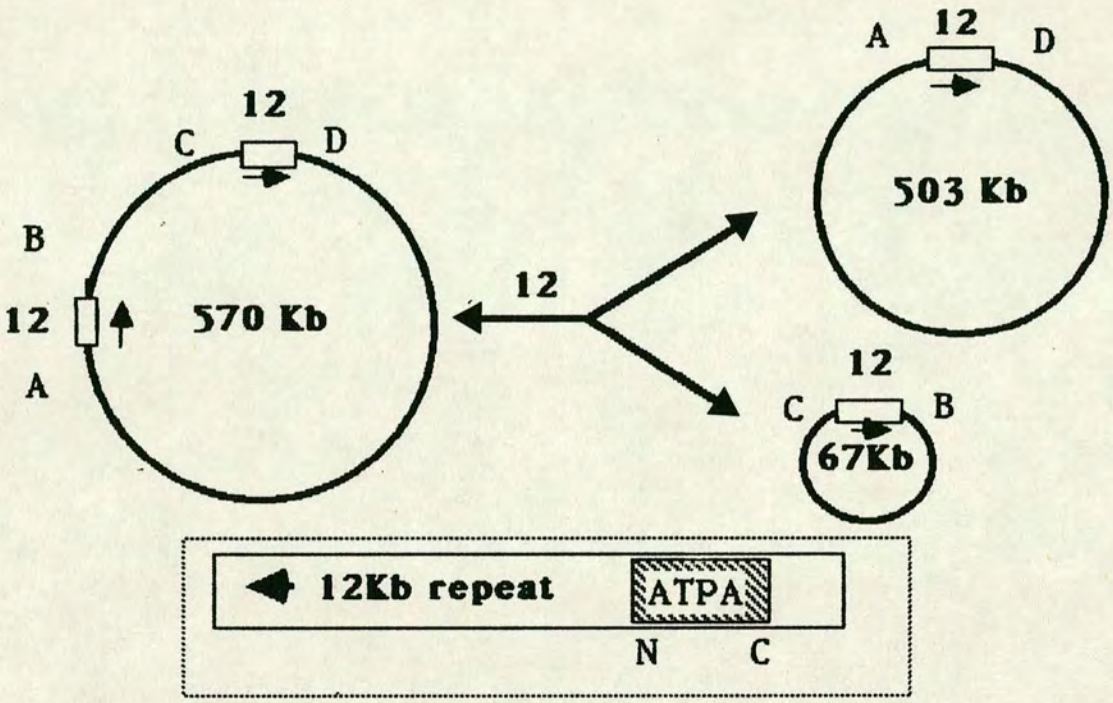
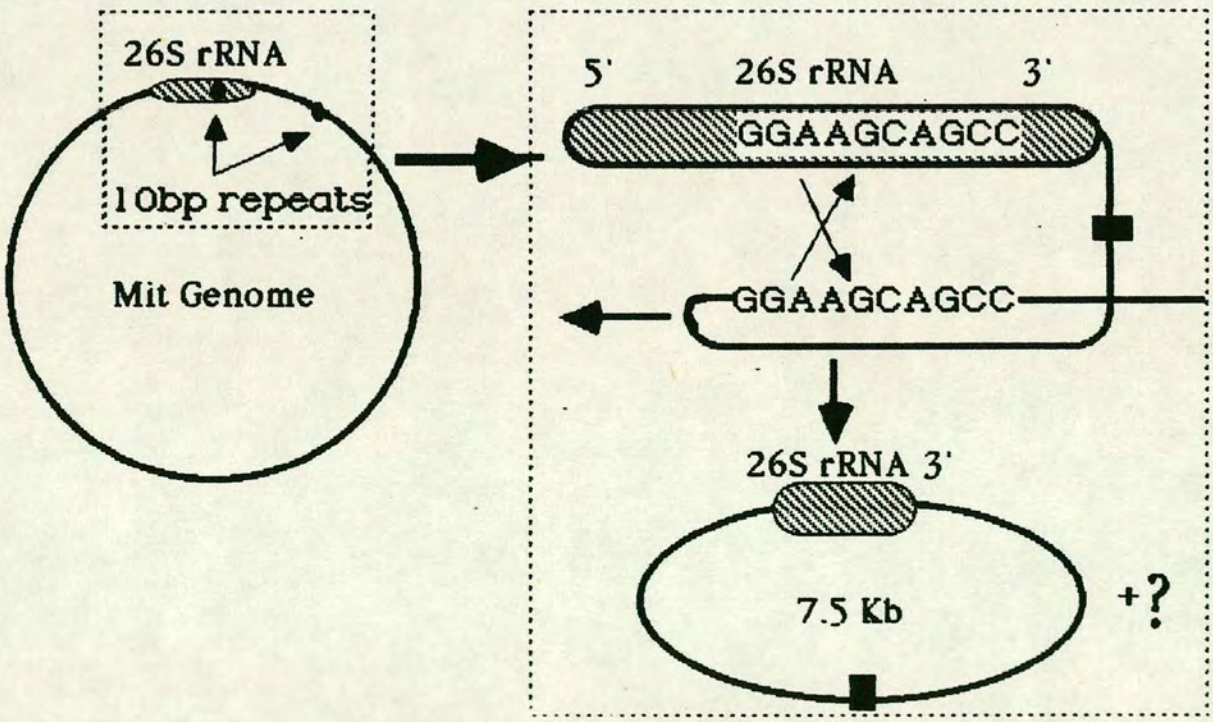


Figure 6.2

A. Maize:Recombination Across the 12Kb Repeat



B. Oenothera:Recombination Across a 10bp repeat



C. CMS-S Maize: Recombination across the Terminal Inverted Repeats (TIRs) of the S Plasmids and Integrated TIR Sequences

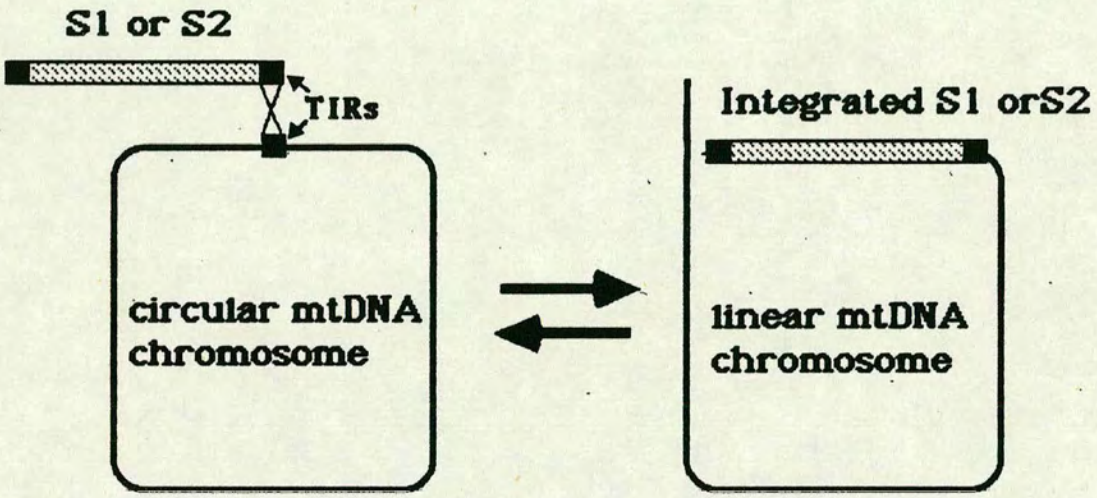


Table 6.1
Repeated Mitochondrial DNA Sequences

ORGANISM	GENE OR GENETIC ELEMENT	PORTION OF GENE OR GENETIC ELEMENT	REPEAT SIZE	REFERENCE(S)
Maize				
N	ATPA	complete	"12Kb"	Isaac etal.,1985b
	S1	1400bp		McNay et al., 1983
	S2	missing complete		
	ATP6	122 bp	"2Kb"	Dewey et al., 1986
	?	-	"14Kb"	Dawson et al.,1986b
	?	-	"10Kb"	
	?	-	"1Kb"	
S and N	S-TIR	portion	"3Kb"	Leaver et al., 1985
S	S1 and S2	complete		Schardl et al., 1984
Sorghum	ATPA	complete (?)		Presented here
	COXI	portion		"
Wheat	COXII	portion Exon I (118 -310 bp)	192bp	Bonen et al., 1984
	26S rRNA	portion		Bonen and Gray,1980 Falconet etal.,1985
	18S + 5S rRNA	complete		Falconet etal.,1984
Oenothera	26S rRNA	portion	10bp	Manna and Brennicke, 1986
	ATPA	portion		A. Brennicke, pers.comm.
	3' COXII & 3' ATPA		50bp "	"
Phytolacca	26S rRNA	?		Stern and Palmer,1984b
Spinach	26S rRNA	?	"5Kb"	"

Table 6.1 (continued)
Repeated Mitochondrial DNA Sequences

ORGANISM	GENE OR GENETIC ELEMENT	PORTION OF GENE OR GENETIC ELEMENT	REPEAT SIZE	REFERENCE(S)
Brassica campestris	*COX*II		"2Kb"	Stern and Palmer, 1984b
Soybean culture	5S rRNA	complete		Morgens *etal.*, 1984
Kloecker africana	Large rRNA	partial		Clark-Walker *et al.*, 1981b
Tetrahymena pyriformis	Large rRNA	complete		Goldbach *et al.*, 1978b
Achlya ambisexualis	Large + Small rRNA	complete		Hudspeth *et al.*, 1983
Saccharomyces petites	various	various		de Zamoroczy *et al.*, 1983
Podospora sen mutants e.g.	*COX*I	Intron I		Kück *et al.*, 1985
N.crassa	$tRNA^{met}$	complete		Gross *et al.*, 1984

MtDNA recombination in higher plants has yet to be directly demonstrated *in vitro* and *in vivo*. An *in vitro* demonstration of mtDNA recombination could be carried out using a cloned mtDNA fragment containing a sequence thought to be involved in recombination. For example, the Milo cytoplasm 4.3 Kb fragment clone containing the *COX*I gene and a 9E mtDNA clone containing the novel 3' portion of the 9E *COX*I gene (note, this clone has not yet been identified) could be incubated in a mitochondrial protein extract from 9E cytoplasm. *In vitro* formation of the novel *COX*I gene would be direct evidence for recombination. If recombination is detected the 'recombinase' could be further purified by FPLC. Unfortunately, a negative result would raise the possibility that the 'recombinase' is only present at a specific developmental stage or that this particular rearrangement occurred once during the evolution of a sorghum.

Reassociation kinetic analysis suggests that 5 - 10% of the mtDNA of cucurbits, pea and maize is present as short repeated sequences (Ward *et al.*, 1981). Do all repeated sequences act as sites for general recombination? Genome mapping studies of maize suggests that the "12Kb" and "3Kb" repeats are highly recombinogenic, whereas the "2Kb" repeat rarely undergoes recombination and the "10Kb" repeat may be completely inactive (Lonsdale *et al.*,1984). This may be interpreted as support of a site-specific recombination mechanism. However, in the case of the "2Kb" repeat, recombination is probably limited because this repeat is an integral portion of the *ATP*6 gene and is located just 5' to the *COX*II gene (Dewey *et al.*, 1986).

If recombination is indeed the source of molecular heterogeneity in higher plant mtDNA, then when does it occur, how is it regulated and how is a new allele fixed in a population? The mtDNA restriction pattern and circular molecule populations of whole plant tissues and plant cell cultures often appear to be stable (Levings and Pring, 1976; Dale *et al.*, 1983; Nikiforova and Negruk, 1983; Boeshore *et al.*, 1985). Alterations in mtDNA

organization are stimulated by cell culture or changes in nuclear background (P.G. Isaac and I. Small, personal communication). Nuclear genotype appears to affect the relative stoichiometries of mtDNA fragments containing COXI in CMS-S maize. These differences might result from the amount or activity of a nuclear encoded 'recombinase' responsible for recombination between S-plasmids and a homologous 'target sequence' 5' to the COXI gene. Alternatively, nuclear genotype may affect the replication rate of the sub-genomic molecules containing the various COXI copies. This observation suggests that mitochondrial genome organization may be under some form of nuclear control. Further examination of rearrangement events which result from the introduction of a foreign nucleus into a cytoplasm may help to define the molecular basis and phenotypic consequences of genome rearrangements in higher plant mitochondria.

6.4 The Consequences of Genome Rearrangements in Higher Plants

The mitochondrial genomes of higher plants may consist of various circular and linear molecules, each with a different sequence arrangement of the necessary genetic information. The overall organization of a plants mtDNA may reflect the genome rearrangement events which have occurred during evolution. If genome rearrangements are a steadily occurring source of molecular heterogeneity in higher plants then how is genome stability maintained? Perhaps only rearrangements which do not alter mitochondrial respiration are tolerated. Silent rearrangements may be responsible for the observed variation in mtDNA restriction patterns in related lines of sorghum or maize and in somatic hybrids. It seems likely that silent rearrangements could ultimately lead to construction of new mitochondrial genes (Figure 6.3). The event which led to the extension of the COXI ORF in 9E cytoplasm is an example of how a rearrangement might lead to a mutant gene

and altered gene expression. Genome rearrangements are circumstantially linked to the expression of the CMS phenotype in numerous plants. CMS cytoplasms of maize differ from N cytoplasm because of specific mtDNA rearrangements and deletions which correlate with the synthesis of variant mitochondrial polypeptides. Variant polypepetides are also synthesized by CMS lines of tobacco, wheat and V.faba (Boutry et al., 1984). A mtDNA rearrangement in a Petunia somatic cell hybrid correlates with the expression of the CMS phenotype (M. Hanson, personal communication). Within this novel mtDNA fragment a transcribed 1062 bp ORF which is unique to this CMS line has been identified.

A tentative hypothesis is that mtDNA rearrangements result in the formation of novel polypeptides which could be associated with specific enzyme complexes (ie. 9E cytoplasm COI). These variant polypeptides may have no evident effect during normal vegetative growth but during microsporogenesis, when there is an increased demand for mitochondrial biogenesis and respiration, they may alter normal mitochondrial function and result in the failure to produce and release functional pollen. Nuclear fertility restorer gene products may suppress the synthesis of a variant polypeptide, or compensate for its presence.

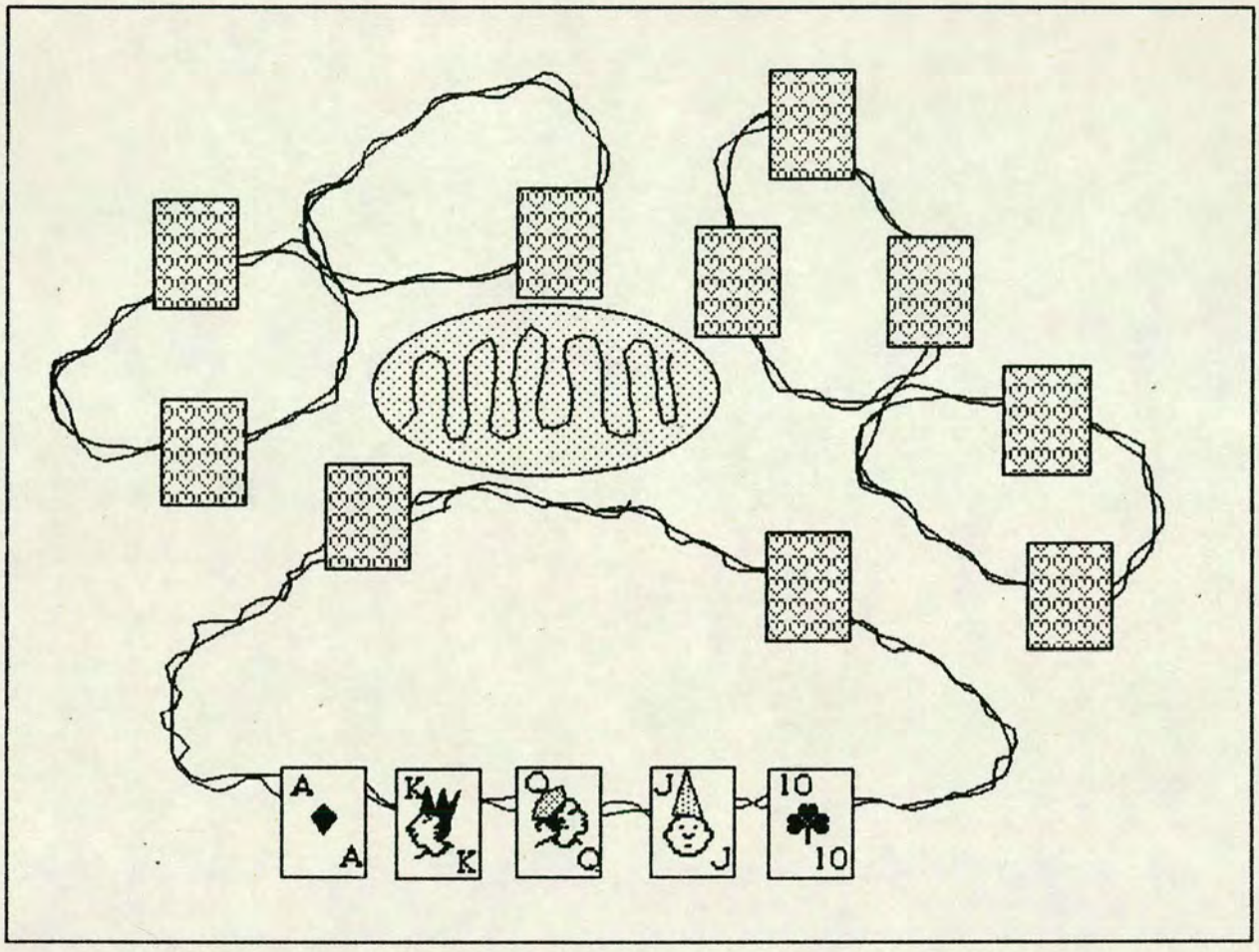


Figure 6.3 The Molecular Card Game

Genome rearrangements in higher plant mtDNA could result in the construction of novel mitochondrial genes. This possibility can be likened to playing a molecular poker game. If mtDNA which undergoes rearrangement is represented by a deck of playing cards, in a game where a player is dealt five cards, the possibility of a hand with an Ace, King, Queen, Jack and '10' may approximate the possibility that mtDNA rearrangements would place a promoter, ribosome binding site, start codon, ORF and stop codon into order. Thus, the acquisition of what is called a 'Royal Flush' may be likened to the formation of a novel mitochondrial gene! Perhaps an example of such a construction is the novel COXI gene of 9E cytoplasm sorghum .

6.6 Conclusion and Prospectus

Specific nuclear-mitochondrial interactions are required for normal plant development. These interactions include nuclear regulation of mitochondrial gene expression, biogenesis and function. Due to the interspecific diversity in mitochondrial genome organization, specific nuclear genes may have evolved to complement a particular mitochondrial genome. When a cytoplasm is transferred into a foreign nuclear background, the nuclear genes capable of regulating mitochondrial gene expression and function may be absent. Their absence may result in altered mitochondrial replication or function and therefore expression of cytoplasmic mutations such as toxin sensitivity or cytoplasmic male sterility. A model mutation may be the mtDNA rearrangement which resulted in the formation of novel COXI gene and the expression of a variant COI polypeptide in 9E cytoplasm sorghum. This mutation may be masked by 9E nuclear genes but could underlie the CMS phenotype when those genes are absent. To further the understanding of the CMS phenotype in 9E cytoplasm the following strategies might be pursued:

1) characterization of the mtDNA rearrangement event which led to the formation of the variant COXI,
2) examination of mitochondrial gene expression and cytochrome c oxidase activity in the developing microspores and anther tapetum.
3) examination of the role of 9E nuclear fertility gene(s) in expression of COXI, or function of cytochrome c oxidase.

These strategies could be applied to the study of other mtDNA rearrangements and variant mitochondrial polypeptides associated with the CMS phenotype. The further examination of the CMS phenotype is not only likely to unravel the mysteries of the large and variable mitochondrial genome of higher plants, but may reveal the specific nuclear-mitochondrial interactions necessary for plant development.

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APPENDIX I

SORGHUM LINES EXAMINED

Nuclear-Cytoplasmic Combinations, International Sorghum Number, Male Fertility Status and Apparent Molecular Weight of COI.

Nucleus	Cytoplasm	IS Number	Phenotype	COI (K daltons)
9ES	9ES		MF	42
9ED	9ED	IS2483C ?	MF	38
Martin	9E		CMS	42
Kafir	9E		CMS	42
Kafir + Kaolingi	9E		PF	42
Kafir + Korigi	9E		PF	42
Restorer of Milo	9E		PF	42
Restorer of Feterita #1	9E		PF	42
Restorer of Feterita #2	9E		PF	42
Kafir + Milo	9E		PF	42
Martin + IS12662	9E		PF	42
Martin + IS2801	9E		PF	42
Martin + TX430	9E		PF	42
Martin + SA7078	9E		PF	42
Martin + IS1112	9E		PF	42
IS7920	IS7920	IS7920C	MF	42
Martin	IS7920		CMS	42
Martin + IS2483	IS7920		CMS	42
Kafir	Kafir		MF	38
Martin	Martin		MF	38
Yellow Feterita	Yellow Feterita		MF	38
IS2483C	IS2483C		MF	38
IS12662	IS12662	IS12662C	MF	38
Milo	Milo		MF	38
Kafir	Milo		CMS	38
Martin + IS12662	Milo		CMS	38
Martin + IS1112	Milo		CMS	38
Martin + SA7078	Milo		CMS	38
Martin	Milo		CMS	38
Martin + IS2801	Milo		CMS	38
Martin + TX430	Milo		CMS	38
Dwarf Kafir	IS3063	IS3036C	CMS	38
Dwarf Kafir	IS2801	IS2801C	CMS	38
Dwarf Kafir	IS1056	IS1056C	CMS	38
Dwarf Kafir	IS3379	IS3379C	CMS	38
Martin	IS6705	IS6705C	CMS	38

Nucleus	Cytoplasm	IS Number	Phenotype	COI (K daltons)
Kafir	IS12662	IS12662C	CMS	38
Kafir	IS1056	IS1056C	CMS	38
M35-1	M35-1		MF	38
Golden Kafir	M35-1		CMS	38
Yellow Feterita	M35-1		CMS	38
IS1112	IS1112	IS1112	MF	38
Kafir	IS1112		CMS	38
Milo	IS1112		CMS	38
Yellow Feterita	IS1112		CMS	38
Zera-Zera	IS1112		CMS	38
Feterita	IS1112		CMS	38
Milo + Kafir	IS1112		CMS	38

The sorghum lines examined are classified by nuclear-cytoplasmic genotype, International Sorghum (if any), and male fertility status. The apparent molecular weight of COI in these lines was determined by *in organello* mitochondrial protein synthesis and/or by the western immunolabelling technique. Additional lines examined are listed in Table 5.1.