VARIATION IN THE ORGANIZATION OF THE MAIZE MITOCHONDRIAL GENOME

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I declare that this thesis was written and composed by myself, and that the data contained within is my own, unless otherwise stated.

Ian Small

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Abstract

Maize cytoplasms can be divided into four classes on the basis of their mitochondrial DNA restriction endonuclease patterns; N, C, T and S. Mitochondria from the S cytoplasm of maize contain two linear DNA plasmids, which by integrating into homologous regions of the main mitochondrial genome, can convert the genome into a partially linear form. Specific DNA probes for S plasmids, and for sequences flanking their integration sites, were used to investigate the organization of the mitochondrial genome in S cytoplasm. An effect of nuclear genotype was discovered on the ratios of substrates to products of this integration reaction.

Male sterile S cytoplasms can revert to fertility, and these revertants show alterations in their mitochondrial DNA when compared to their sterile parents. A number of cytoplasmic revertants from different sources were compared. Using DNA probes for S plasmids, and sequences flanking their integration sites, a number of mitochondrial DNA configurations were shown to be connected with reversion to fertility, which were almost entirely defined by the nuclear genotype of the revertant plant. No one mitochondrial DNA alteration in the revertant cytoplasms could be identified that correlated with the newly acquired fertile phenotype.

The mitochondrial DNA organization around the atpA gene was investigated with specific hybridization probes in a number of different maize cytoplasms. This area of the maize mitochondrial genome was shown to be extremely variable between cytoplasms. Differences in atpA copy number and sequence organization were described even within the cytoplasmic groups of N and S. In most cytoplasms, sub-stoichiometric restriction endonuclease fragments (termed 'sublimons') could be detected that corresponded to arrangements that were characteristic of other cytoplasms.

The mitochondrial genome organization of two closely related fertile cytoplasms, OY and LF, were investigated. These cytoplasms contained one and two copies of the atpA gene respectively. The second arrangement of the atpA gene, specific to LF cytoplasm, was detected at low levels in the mitochondrial genome of OY cytoplasm. These low copy number arrangements were shown to be probably produced by homologous recombination between a pair of 181bp repeats. In another case, it was shown that the low level arrangements could not be easily explained by recombination. The possible implications of these observations on various modes of mitochondrial genome evolution are discussed, and speculations are made on the possibilities for sudden genome rearrangements in plant mitochondria based on the amplification of rare recombinant molecules now shown to be present in the genome.

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Abbreviations

′	minute
ATP	Adenosine 5'triphosphate
atpA, atp6, atp9	Mitochondrial genes encoding subunits α, 6 and 9 of
the	ATP synthase complex
bisacrylamide	N, N'-methylene bisacrylamide
bp	Base pairs
BSA	Bovine serum albumin
CMS	Cytoplasmic male sterility, cytoplasmic male sterile
cob	Mitochondrial gene encoding apocytochrome b
COI	Cytochrome c oxidase subunit I polypeptide
coxI, coxII, coxIII	Mitochondrial genes encoding subunits 1, 2 and 3 of
the	cytochrome c oxidase complex
cpDNA	Chloroplast deoxyribonucleic acid
Da	Dalton
dCTP	2' deoxycytidine 5' triphosphate
DEAE	Diethyl aminoethyl
DNA	Deoxyribonucleic acid
ds	Double stranded
EDTA	Ethylene diamine tetra acetic acid (disodium salt)
EGTA	Ethylene glycol bis-(β-aminoethyl ether)
	N,N,N',N'-tetraacetic acid
X g	average maximum relative gravitational force
hrs	hours
IPTG	Isopropyl thio-β-D-galactoside
kb	kilobase (pair(s))
kDa	kilodalton
LS	Mitochondrial sequence showing homology to the chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase
MOPS	Morpholinopropane sulphonic acid
mtDNA	Mitochondrial deoxyribonucleic acid
mtRNA	Mitochondrial ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NCS	Non-chromosomal stripe
ND1	Mitochondrial gene for subunit 1 of the
NADH:ubiquinone	oxidoreductase complex
N-terminal	Amino-terminal
ORF	Open reading frame
PEG	Polyethylene glycol

Rf	Nuclear fertility restorer gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S13	Mitochondrial gene encoding a polypeptide homologous to subunit 13 of the small ribosomal subunit of *E. coli*
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate, defined on p. 31
STIR	S plasmid terminal inverted repeat
TAE	Tris-actetate electrophoresis buffer, defined on p.31
TBE	Tris-borate electrophoresis buffer, defined on p.31
TE	Tris-EDTA buffer, defined on p. 31
TEMED	N,N,N',N'-tetramethylethylene-diamine
Tricine	N-tris (hydroxy methyl) methyl glycine
Tris	Tris (hydroxymethyl) amino methane
tRNA	Transfer ribonucleic acid
w/v	Weight per volume (given as a percentage)
X-gal	5-bromo 4-chloro 3-indoyl β-galactoside

Chapter 1 - Plant mitochondrial genome organization and evolution

1.1 Introduction

Each of the nuclear, chloroplast and mitochondrial compartments in higher plant cells contains its own genetic information. The chloroplast and mitochondrial genomes are small relative to the nuclear genome and contain only a part of the genetic information necessary for their biogenesis and function. Higher plant mitochondria synthesize only about 20 polypeptides (5-10% of their total polypeptide complement) as shown by analysis of their in organello labelled translation products (Leaver and Gray 1982). The remaining gene products are encoded by the nuclear genome. These observations imply a coding capacity for higher plant mitochondrial DNA (mtDNA) similar to that of other eukaryotic mtDNAs. All the animal mitochondrial genomes so far examined (including Homo sapiens, Xenopus and Drosophila) encode the same thirteen proteins (Anderson et al. 1981, 1982, Bibb et al. 1981, Clary and Wolstenholme 1985, Roe et al. 1985). However, higher plant mitochondrial genomes are invariably at least 10 fold larger than animal mtDNAs, and several times bigger than the known fungal mitochondrial genomes (for reviews see Leaver and Gray 1982, Pring and Lonsdale 1985, Lonsdale 1987). In addition, higher plant mitochondrial genomes vary greatly in size (from 200-2400kb), even between species within a single family (Ward et al. 1981). On the evidence of DNA reassociation kinetics, this size variation is not thought to be due to the presence of repetitive sequence duplications.

The large and variable size of the mitochondrial genome of higher

plants makes it an interesting subject for investigation, as does the apparent rapidity and scale of its structural variation on an evolutionary timescale. This thesis attempts to explore some of these areas by concentrating on variation in the mitochondrial genome of maize, as typified by a few specific mitochondrial genome rearrangements, and then discussing these results in relation to the general field of plant mitochondrial genome evolution. A novel hypothesis is discussed that could help explain some forms of plant mitochondrial genome reorganization.

1.2 Higher plant mitochondrial genome size and organization

The mitochondrial genomes of several plant species have been mapped by ordering cloned overlapping mtDNA fragments (table 1.1). These maps share the common feature that they can be arranged to form a 'master circle' accounting for all the sequence complexity, which generally includes one or more pairs of large direct repeats. Homologous recombination between these repeats would be expected to give rise to families of smaller subgenomic circles (fig. 1.1). Clones have been obtained for the recombinant rearrangements as well as for the master circle configuration, so it seems likely that higher plant mtDNA does exist as a multipartite genome (Palmer and Shields 1984, Lonsdale et al. 1984). Differences in replication and/or recombination rates of these subgenomic molecules allow the possibility of complex stoichiometries of genome fragments, detectable by restriction endonuclease analysis (Quetier and Vedel 1977, Pring and Levings 1978, Borck and Walbot 1982). From an evolutionary standpoint, it is interesting to note that this complex mix of molecules is stable, at least for a few sexual

Table 1.1 Plant mitochondrial genomes with complete restriction maps

Species: Common name	Latin name	Genome size (kb)	No. of repeats	References
	Brassica hirta	208	0	1
Turnip	Brassica campestris	218	1	2
Cabbage	Brassica oleracea	219	1	3,4
Oilseed rape	Brassica napus	221	1	4
Black mustard	Brassica nigra	231	1	4
Radish	Raphanus sativa	242	1	4
Spinach	Spinacea oleracea	327	1	5
Wheat	Triticum aestivum	440	10	6
Maize	Zea mays	570	6	7

References:

1 Palmer and Herbon 1987a

2 Palmer and Shields 1984

3 Chetrit et al. 1984

4 Palmer and Herbon 1987b

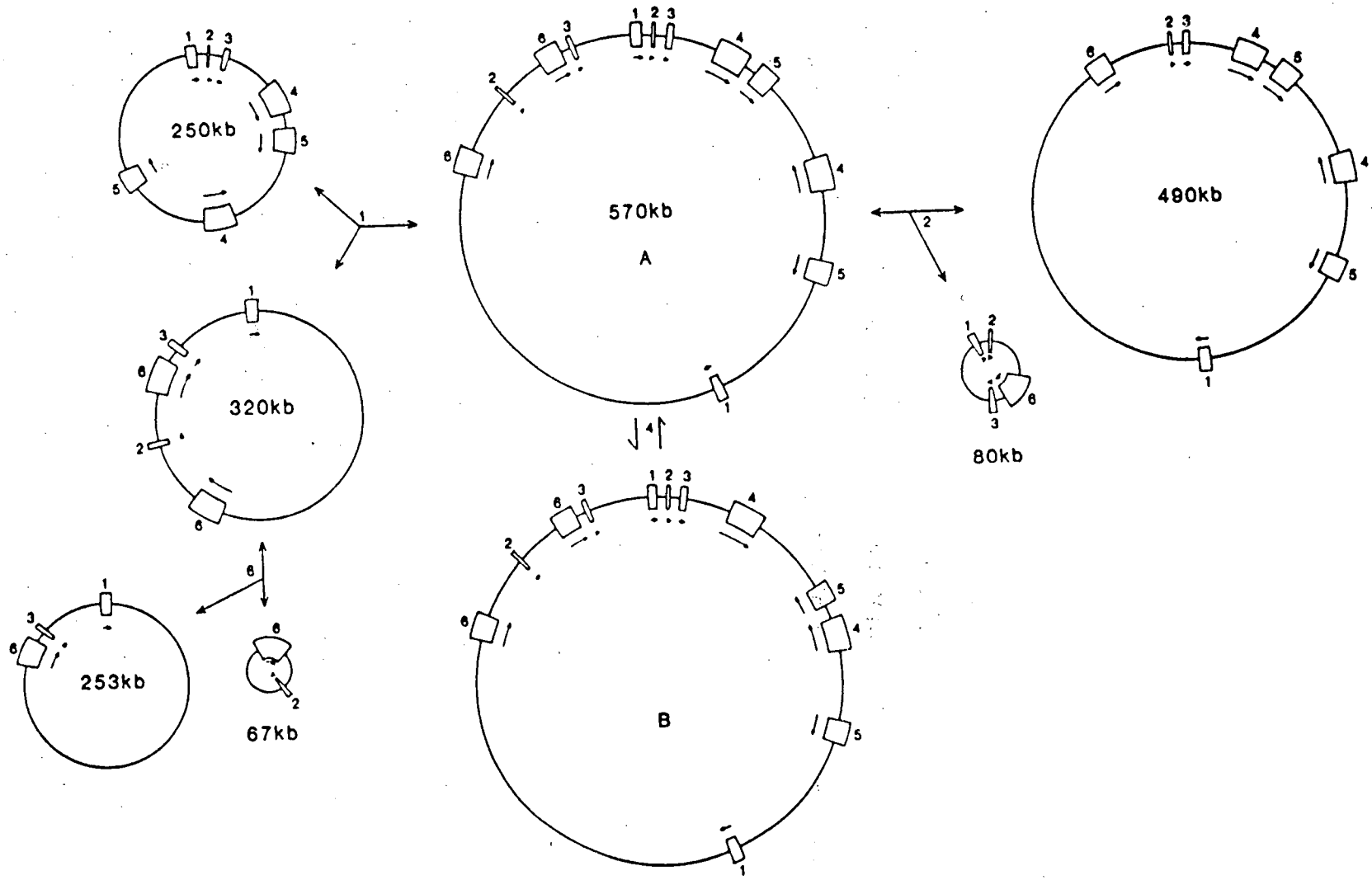
5 Stern and Palmer 1986

6 Quetier et al. 1985

7 Lonsdale et al. 1984

Fig. 1.1. The multipartite mitochondrial genome of maize mitochondria (from Lonsdale 1987).

Six repeats of between c. 1kb and 14kb have been identified on the 570kb master circle (Lonsdale et al. 1984). Recombination between the single pair of indirect repeats (repeat-4) interconverts two isomers of the master circle (**A** and **B**). Recombination between the five pairs of direct repeats gives rise to a multitude of subgenomic circles, some of which are shown. Many other circular forms arising from both inter- and intramolecular recombination events between these repeats can be predicted but are not illustrated.


250kb
320kb
253kb
67kb
570kb
A
B
490kb
80kb
1
2
3
4
5
6

generations (Oro et al. 1985), implying an equilibrium has been reached which is not perturbed by segregation during seed formation, germination or subsequent differentiation of tissues (Quetier and Vedel 1977). However, quantitative variations in amounts of specific restriction endonuclease fragments has been observed in tissue culture cells (McNay et al. 1984, Kemble and Shepard 1984, Morgens et al. 1984, Negruk et al. 1986, Rode et al. 1987).

It should be noted that purified higher plant mtDNA, when examined by electron microscopy, usually appears as an assortment of linear molecules of varying sizes (Wolstenholme and Gross 1968, Mikulska et al. 1970, Wong and Wildman 1972, Kim et al. 1982a, Manna et al. 1985). Circular molecules are rare, (less than 5% of the population) and when isolated are generally heterogenous in size and much smaller than the master circle, as predicted from summing the sizes of restriction endonuclease fragments (Synenki et al. 1978, Levings et al. 1979, Sparks and Dale 1980, Fontarnau and Hernandez-Yago 1982, Negruk et al. 1982, Bailey-Serres et al. 1987). It is unclear whether this accurately reflects the in vivo situation, or whether the high preponderance of linear molecules is due to shearing of the mtDNA during isolation (Wolstenholme and Gross 1968). Some mitochondrial genomes have been convincingly demonstrated to be wholly or partially linear in vivo (e.g. Tetrahymena (Morin and Cech 1986); Chlamydomonas (Gray and Boer 1988); Candida (Kovac et al. 1984)) including the maize mitochondrial genomes from S (Schardl et al. 1984) and RU cytoplasms (Lonsdale et al. 1988). It is possible that all higher plant mtDNAs consist largely of circularly permuted linear molecules rather than as a collection of closed circles.

1.2.1 The maize mitochondrial genome

The mitochondrial genome of fertile (N) maize can be represented as a 'master circle' of 570 kb, containing six pairs of large repeats (Lonsdale et al. 1984). Five of these pairs of repeats are in direct orientation, and recombination between them is predicted to give rise to a mixed population of subgenomic circular molecules of varying sizes (fig 1.1).

A number of protein coding genes have been isolated and sequenced from maize mtDNA (table 1.2). In all but one case, these genes code for polypeptide constituents of the protein complexes of the inner mitochondrial membrane, which are involved in electron transport and the coupled synthesis of ATP. These include the genes for subunits I, II, and III of the cytochrome c oxidase complex, apocytochrome b of the cytochrome bc_1 complex, and the genes coding for three constituents of the ATP synthase complex; subunits 6 and 9 of the F_0 membrane fraction, and the α-subunit of the F_1 catalytic fraction (table 1.2). In addition, part of the gene coding for subunit 1 of the NADH:ubiquinone oxidoreductase complex has been identified (Bland et al. 1986).

Plant mitochondria encode their own protein synthesizing machinery, some of which is encoded in the mitochondrial genome. Maize mtDNA encodes the 26S, 18S and 5S ribosomal RNAs and at least one ribosomal protein (table 1.2), and also a number of tRNA genes. It is not currently clear whether maize mtDNA lacks some of the tRNAs required for protein synthesis, as is the case in some algal mitochondria (Suyama 1986, Gray and Boer 1988). All these genes have been located on the 570kb master circle map (fig. 1.2) (Dawson et al. 1986, Lonsdale 1987).

As well as the N mitochondrial genome, three other major classes of mitochondrial genome organization are known in maize (designated C, T and S).

Fig. 1.2. Genetic map of the maize mitochondrial genome (after Dawson et al. 1986, Lonsdale 1987).

The approximate size, orientation (inner arrows) and location of the six large repeats in the genome (numbered as in fig. 1.1) are shown (open boxes). The solid boxes represent known genes, including those presumed to be derived from plastid DNA (LS, 16S, ct5S). The direction of transcription of these genes is indicated by the outer arrows. Only part of the ND1 gene has been sequenced. The locations of transfer RNAs are marked by thin bars. For a fuller description of the known maize mitochondrial genes, see table 1.2. The hatched box represents sequence of chloroplast DNA origin. The repeat-1 sequences have been labelled α-R1 and R2-β according to notation used by Houchins et al. (1986). -R1 and R2- are integrated forms of the linear R1 and R2 plasmids (see **3.2**). The atpA gene lies on a 12kb direct repeat (repeat-6). The two copies of the gene are labelled 1 and 2 according to the designation used in chapter 5 (Small et al. 1987).

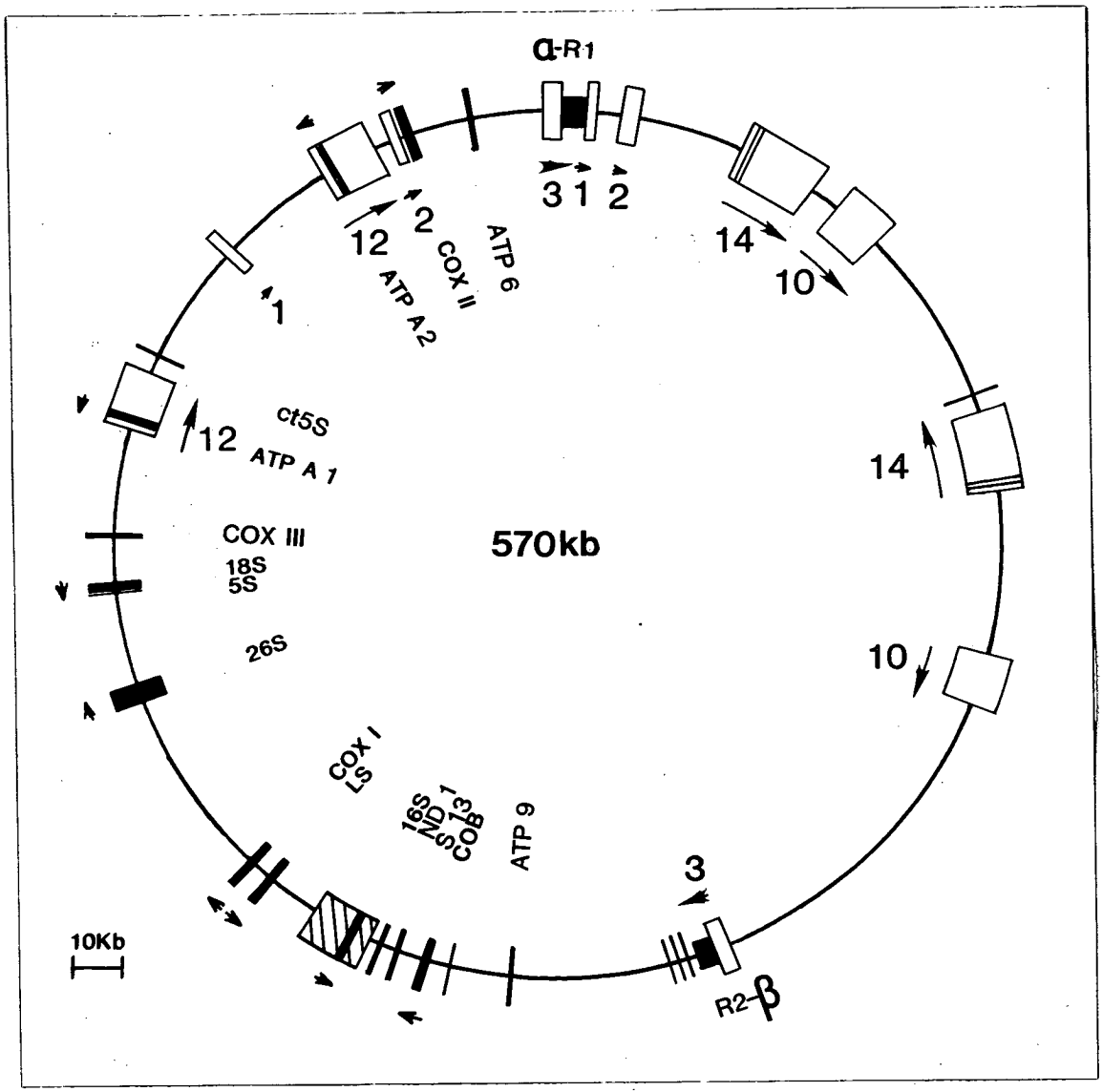

α-R1
3 1 2
12
2
COX II
ATP 6
1
ATP A2
14
10
12
ct5S
ATP A 1
14
COX III
18S
5S
570kb
26S
10
COX I
LS
16S
ND1
SS13
COB
ATP 9
3
10Kb
R2-β

Table 1.2 Genes isolated and sequenced from maize mtDNA

Gene		Length (bp)	Reference
Cytochrome c oxidase complex			
Subunit I	coxI	1584	Isaac et al. 1985b
Subunit II	coxII	780	Fox and Leaver 1981
Subunit III	coxIII	795	Hauswirth et al. pers. comm.
Cytochrome bc_1 complex			
Apocytochrome b	cob	1164	Dawson et al. 1984
ATP synthase complex			
α- subunit (F_1)	atpA	1524	Braun and Levings 1985, Isaac et al. 1985a
subunit 6 (F_0)	atp6	873	Dewey et al. 1985a
subunit 9 (F_0)	atp9	222	Dewey et al. 1985b
Ribosomal proteins			
small subunit	S13	387	Bland et al. 1986
Ribosomal RNAs			
26S rRNA		3546	Dale et al. 1984
18S rRNA		1968	Chao et al. 1984
5S rRNA		126	Chao et al. 1983
Transfer RNAs (tRNAs)			
f-Met		74	Parks et al. 1984
Met		74	Parks et al. 1984
Asp		74	Parks et al. 1985
His		76	Iams et al. 1985
Cys		71	Grienenberger*
Ser		88	Grienenberger*

* cited in Lonsdale 1987

These four cytoplasmic groups can be differentiated by the restriction endonuclease digestion patterns of their mtDNA (Levings and Pring 1976, Pring and Levings 1978, Thompson et al. 1980, Kemble et al. 1980). These variant mtDNA organizations are linked with the trait of cytoplasmic male sterility (CMS) (see **1.3**).

1.2.2 Extrachromosomal DNAs

Many plant mitochondria contain in addition to the main genome (or mitochondrial chromosome) small extrachromosomal species, often referred to as plasmids. These plasmids can be differentiated into circular and linear classes.

Circular plasmids are present in high copy number relative to the main genome, and are apparently unrelated to any sequences in the main genome (for reviews see Sederoff and Levings 1985, Pring and Lonsdale 1985, Lonsdale 1987). For these reasons they are presumed to contain their own replication origins, and thus are of interest. Sequence analysis has identified potential 'hairpin' structures (Wahleithner and Wolstenholme 1987) similar to those at replication origins in mammalian (Chang et al. 1985) and yeast mtDNA (de Zamaroczy and Bernardi 1985) or sequences resembling yeast ARS (autonomously replicating sequence) elements (Ludwig et al. 1985). Different cytoplasms even within one species often show their own characteristic pattern of extrachromosomal plasmids (Kemble and Bedbrook 1980, Powling 1981, Kemble et al. 1983, Smith et al. 1984, Carlson and Kemble 1985). This implies the plasmids are not essential for mitochondrial function, an idea which is supported by the fact that although some of these plasmids are transcribed, they seem to contain no uninterrupted open reading frames (ORFs) and therefore presumably lack coding

functions (Hansen and Marcker 1984, Wahleithner and Wolstenholme 1987), although there are possible exceptions (Ludwig et al. 1985, Bedinger et al. 1987).

Some plant mitochondria contain linear extrachromosomal plasmids or episomes. Linear plasmids have been identified from Zea (Pring et al. 1977, Thompson et al. 1980, Weissinger et al. 1982, Levings and Sederoff 1983, Timothy et al. 1983, Paillard et al. 1985, Bedinger et al. 1987), Sorghum (Dixon and Leaver 1982, Pring et al. 1982b, Chase and Pring 1986) and Brassica (Palmer et al. 1983, Erickson et al. 1986b, Turpen et al. 1987). The best studied of these linear plasmids are the S1 and S2 DNAs of mitochondria from the S cytoplasm of maize, which will be discussed in detail in Chapter 3. Autonomously replicating dsRNA plasmids have also been described in mitochondria from the S cytoplasm of maize (Finnegan and Brown 1986), from male sterile Vicia faba (Grill and Garger 1981), and from fertile and male-sterile sunflower (Brown et al. 1986).

1.2.3 Promiscuous DNA in the mitochondrial genomes of plants

Part of the explanation for the large size of plant mitochondrial genomes is the large amounts of DNA sequence included within them which appears to be of non-mitochondrial origin (reviewed in Lonsdale 1987, Schuster and Brennicke 1987a). The bulk of the 'foreign' or 'promiscuous' DNA identified to date is of plastid origin. The original finding was of 12kb of contiguous sequence in maize mitochondria showing homology to chloroplast DNA (cpDNA) (Stern and Lonsdale 1982). Other plastid homologies in maize include a large part of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (Lonsdale et al. 1983). These are shown on fig. 1.2. Observations of cpDNA homology

in mtDNA were soon extended to many other plant species (Stern and Palmer 1984a, Lonsdale 1987, Schuster and Brennicke 1987a).

Protein-coding sequences derived from plastids found in plant mitochondria are probably non-functional, due to the differences in transcription/translation signals and machinery in the two organelles, but there is a possibility that some tRNA genes in plant mitochondria which show considerable homology to their plastid counterparts are functional in the mitochondrion (Bedinger et al. 1987).

Recently mtDNA sequences have been identified in Oenothera which appear to have nuclear origin, including part of the nuclear 18S rRNA gene (Schuster and Brennicke 1987b). Thus it appears that throughout the evolution of plant mtDNA it has acquired sequences from both the other cellular compartments containing genetic information.

1.3 Cytoplasmic male sterility

Cytoplasmic male-sterility (CMS) is a maternally inherited trait characterized by a failure of the affected plants to produce functional pollen. It is a common trait, and has been described in over 140 different species of flowering plant (Laser and Lersten 1972). The CMS phenotype is used extensively in the commercial production of F_1 hybrids of several crop plants, including maize and sorghum, as it prevents self-fertilization of the female parent. To produce male-fertile F_1 hybrids (required for crop production), the male parent of the cross must contain a dominant nuclear gene which supresses the CMS phenotype. Such nuclear genes are termed fertility-restorer (Rf) genes.

A range of evidence associates CMS with changes in the mitochondrial genome rather than the chloroplast genome (Leaver and Gray 1982), most

convincingly demonstrated by the segregation of the CMS phenotype with mitochondrial and not plastid genotype following protoplast fusion experiments (reviewed in Hanson and Conde 1985). Although first shown in maize (Levings and Pring 1976, Pring and Levings 1978), mtDNA alterations linked with CMS have been demonstrated in several other species, including Sorghum (Conde et al. 1982, Pring et al. 1982a), Vicia faba (Boutry and Briquet 1982), sugarbeet (Powling 1982), tobacco (Belliard et al. 1979, Boutry et al. 1984), Petunia (Boeshore et al. 1985), sunflower (Leroy et al. 1985), Brassica sp. (Erickson et al. 1986a), and rice (Mignouna et al. 1987). Maize cytoplasms that can confer the CMS phenotype are classified as either T (Texas), C (Charrua) or S (USDA), on the basis of the effects of different nuclear fertility-restorer genes (Duvick 1965). N (normal) cytoplasms allow fertile pollen development in all nuclear backgrounds.

CMS is important because it is one of the very few phenotypic mutations known to have its origins in plant mitochondria. The study of yeast mitochondrial biogenesis is so much further advanced than that of plants because of the ability of yeast to survive in anaerobic conditions without requiring mitochondrial function. This ability allows experimenters to create and investigate mutations in yeast mtDNA and in nuclear genes coding for mitochondrial proteins that would invariably be lethal in other organisms. Plants cannot survive without functional mitochondria, and thus most mutations in mtDNA or in nuclear genes encoding mitochondrial proteins are either lethal or undetectable because they have no phenotypic effect. CMS is not a lethal mutation, and it only affects a few specific cells during pollen formation. The interest in the molecular basis for CMS also stems partly from the fact that the definition covers a multitude of subtly different phenotypes (depending

on the stage at which anther differentiation is affected), due probably to an even greater number of initial mutations. In maize, mitochondria from the three types of CMS cytoplasms can be distinguished by restriction endonuclease digestion patterns of their mtDNA, variations in their mtRNA transcription patterns, and by differences in their translation products (Leaver and Gray 1982, Lonsdale 1987), and thus different alterations in mtDNA organization and related gene expression appear to give rise to CMS even within the same species.

Another phenotype, nonchromosomal stripe (NCS), has recently been linked with mtDNA rearrangements in maize (Newton and Coe 1986). Plants carrying the mutation have poor growth, abnormal morphology and exhibit leaf striping. All the mutants reported have arisen from the closely related lines WF9-T and H49-T, but the phenotype is not affected by the nuclear genes that restore pollen fertility to CMS-T plants. The two mutants that have been characterized so far are distinguishable on the basis of mtDNA restriction endonuclease patterns, and as the WF9 nuclear background appears to induce these mutations at a frequency of around 1%, novel plant mitochondrial mutants may soon be available for study (Newton and Coe 1986).

1.3.1 Chimaeric genes and variant polypeptides in mitochondria from male-sterile plants.

A common feature of mitochondria from CMS lines is the synthesis of variant polypeptides absent from fertile control lines (Forde et al. 1978, 1980, Forde and Leaver 1980, Dixon and Leaver 1982, Boutry et al. 1984, Bailey-Serres et al. 1986a, 1986b). In several of these cases the variant polypeptides have been shown to be coded for by a novel open reading

frame (ORF) generated by fusion of several unrelated sequences creating a 'chimaeric' gene (Dewey et al. 1986, 1987, Bailey-Serres et al. 1986b, Young and Hanson 1987, Levings and Dewey 1988).

In the Sorghum 9E cytoplasm a variant 42kDa polypeptide was shown to be coded for by an extended coxI gene. In this cytoplasm DNA recombination has added 101 codons to the 3' end of the normal coxI ORF, increasing the apparent molecular weight of the COI protein from 38kDa to 42kDa (Bailey-Serres et al. 1986b). The origin of the extra coding sequence is not known. It has not yet been possible to demonstrate any significant phenotypic effect of the extended COI protein on the function of 9E mitochondria.

Similar chimaeric genes have been described from the male-sterile maize C cytoplasm. In CMS-C mtDNA a novel gene has been created by 13 codons from the 5' end of the atp9 gene becoming coupled to 147 codons from an unidentified gene (showing homology to the chloroplast genome) which is in turn linked to 268 codons from the 3' end of the atp6 gene. Elsewhere in the genome the 5' end of the atp6 gene is fused to the majority of the coxII gene (Levings and Dewey 1988). In this case it has not been demonstrated that the proteins predicted by these rearranged reading frames are expressed (although the novel genes are transcribed) or that they are responsible for the male-sterile phenotype.

Conclusive links between chimaeric genes and male-sterility have been shown in two cases. Boeshore et al. (1985), by screening Petunia plants with recombinant mitochondrial genomes produced by fusing protoplasts derived from sterile and fertile lines, identified a mtDNA restriction endonuclease fragment that consistently segregated with the CMS phenotype. Sequence analysis of this fragment has revealed that it contains a novel chimaeric gene, designated Pcf. The Pcf gene contains

the first 35 codons of the atp9 gene, 158 codons from coxII and 157 codons from an unidentified gene, urfS (Young and Hanson 1987). Transcripts of this gene are elevated in anther tissue relative to leaves, and although a protein product from this gene has yet to be demonstrated, there is strong evidence that this gene is in some way responsible for the CMS phenotype in Petunia.

Probably the most intensively studied CMS system is CMS-T in maize. This is due in part to the fact that it was the predominant cytoplasm used in commercial breeding of F_1 maize hybrids. In 1970, this cytoplasmic uniformity in the maize crop (over 90% of commercially grown maize hybrids carried this cytoplasm) led to a disastrous epidemic of Southern Corn Leaf Blight, a disease caused by the pathogenic fungus Helminthosporium maydis Race T, which preferentially attacked CMS-T plants (Hooker et al. 1970). Mitochondria isolated from T cytoplasm, but not from N, C or S cytoplasm are sensitive to low concentrations of the T-toxin produced by the fungus (Miller and Koeppe 1971). The T-toxin (a linear polyketole) promotes leakage of NAD^+ and Ca^{++} (Holden and Sze 1984), uncouples oxidative phosphorylation (Bednarski et al. 1977) and dissipates the potential across the inner mitochondrial membrane (Holden and Sze 1987). These results suggest that the T-toxin, in association with a factor specific to CMS-T mitochondria, probably increases the permeability of the inner mitochondrial membrane to protons, and possibly other ions (Holden and Sze 1987).

Mitochondria isolated from CMS-T plants synthesize a 13kDa polypeptide not present in other maize cytoplasms (Forde et al. 1978). Synthesis of this polypeptide is reduced in lines containing the nuclear genes Rf1 and Rf2, both of which are necessary for male-fertility

restoration of CMS-T plants (Forde and Leaver 1980). The 13kDa polypeptide is also lacking in plants which have permanently reverted to fertility (Dixon et al. 1982). The presence of this 13kDa polypeptide correlates with both the CMS-T phenotype and T-toxin sensitivity.

Using differential hybridization of mtRNA from N and T mitochondria to cloned T mtDNA, Dewey et al. (1986) identified an abundantly transcribed restriction endonuclease fragment unique to CMS-T mtDNA. This fragment included a novel chimaeric open reading frame (designated urf13-T) predicted to code for a polypeptide of 12,961Da. The chimaeric fragment containing this ORF appears to have been produced by at least seven separate recombination events, involving the 5' flank of atp6, flanking and coding sequences of the 26S rRNA gene, an unidentified ORF coding for a predicted 25kDa protein, and a sequence showing homology to a chloroplast tRNA -Arg gene (fig. 1.3) (Dewey et al. 1986). An antibody raised against a synthetic oligopeptide based on a portion of the coding sequence of urf13-T selectively immunoprecipitates the CMS-T specific 13kDa polypeptide (Dewey et al. 1987, Wise et al. 1987a, Leaver et al. 1988). It is interesting to note that urf13-T is entirely composed of sequence from the 3' flanking region of the 26S rRNA gene and a short sequence from within the coding region of the 26S RNA gene, sequences which are not normally protein-coding. This contrasts with the rearrangements described in Sorghum, Petunia and the maize C cytoplasm which involve modifications of pre-existing protein-coding genes.

Four transcripts of the urf13-T region have been identified (Dewey et al. 1986, 1987). The smallest of these is present only in lines containing the Rf1 fertility-restorer gene, and the identification of its 5' terminus indicates it cannot encode the entire 13kDa polypeptide. CMS-T lines with Rf1 also synthesize reduced levels of the 13kDa polypeptide,

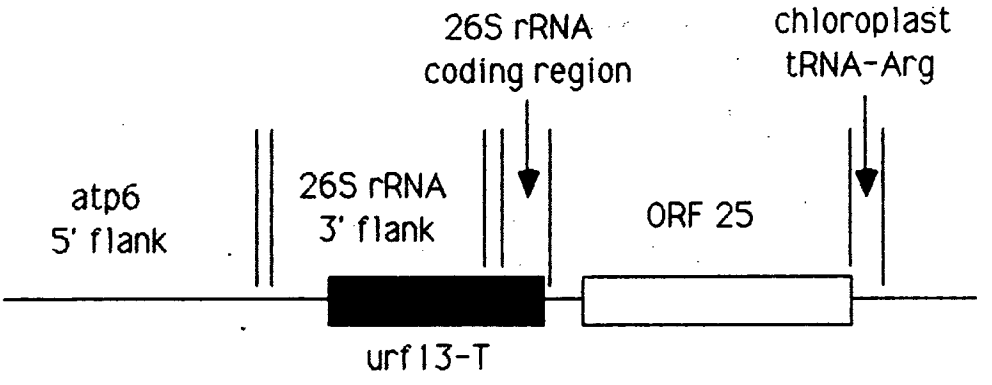


Fig. 1.3. The urf13-T region of CMS-T mtDNA (based on the data of Dewey et al. 1986)

Vertical lines indicate the sites of the recombination points involved in the formation of this region. Other sequences homologous to this region are indicated where known. The urf13-T gene is shown by a solid box, the open reading frame encoding a putative 25kDa polypeptide (ORF 25) is shown by an open box. Nine codons of the urf13-T gene, between the two regions of 26S rRNA homology, are of unknown origin.

suggesting that the Rf1 gene product may suppress the CMS-T phenotype by processing the mRNA encoded by the urf13-T gene (Dewey et al. 1987). However, the nuclear gene Rf2 is also obligatory for suppression of the CMS-T phenotype, and this gene does not appear to influence the expression of urf13-T (Dewey et al. 1987).

As mentioned earlier, the presence of the 13kDa polypeptide also correlates with T-toxin sensitivity. The best direct evidence of this is that if the urf13-T gene is expressed in E. coli, then T-toxin sensitivity is conferred on the bacterial respiration. This T-toxin effect is not apparent in E. coli expressing an N-terminal truncated version of the 13kDa polypeptide (Levings and Dewey 1988).

Some other variant polypeptides synthesized by plant mitochondria are probably coded for by the linear plasmids described in **1.2.2.** In some Sorghum lines the presence of certain high molecular weight polypeptides correlates with the presence of linear plasmids (Dixon and Leaver 1982, Bailey-Serres et al. 1986a), whilst in maize the linear plasmids characteristic of the S cytoplasm have been shown to encode high molecular weight polypeptides by using portions of these plasmids to direct protein synthesis in vitro, and then using antibodies to these proteins to detect similar polypeptides in CMS-S mitochondria (Manson et al. 1986, Zabala and Walbot 1987).

1.3.2 Reversion to fertility

An interesting feature of S-type CMS is the spontaneous heritable reversion to male fertility that sometimes occurs in such plants (Laughnan et al. 1981, Laughnan and Gabay-Laughnan 1983). Such reversion events can be due either to mutations located in the nucleus (i.e. show Mendelian inheritance) or in the cytoplasm (in which case they show maternal inheritance and are linked with specific alterations in the mitochondrial genome). A full discussion of cytoplasmic reversion to fertility in CMS-S cytoplasms is given in Chapter 4.

Reversion from CMS-C or CMS-T has never been found in field-grown plants, but reversion from CMS-T has been shown in plants regenerated from callus cultures, either with (Gengenbach et al. 1977) or without T-toxin selection (Brettell et al. 1980, Umbeck and Gengenbach 1983). These reversion events are associated with either loss or reduction in synthesis of the 13kDa polypeptide characteristic of CMS-T mitochondria (Dixon et al. 1982). These reversion events are also associated with alterations in the mtDNA. In the vast majority of these revertants a 6.7kb XhoI fragment has been replaced by a 6.3kb XhoI fragment (Umbeck and Gengenbach 1983) due to a small deletion following recombination between two 55bp direct repeats (Rottman et al. 1987). The deleted sequence includes the urf13-T gene thought to be closely linked to the CMS-T phenotype and T-toxin sensitivity (see above). In one revertant, a tandem 5bp duplication has resulted in a frameshift within the urf13-T gene leading to the synthesis of a truncated gene product (Wise et al. 1987b).

Reversion from CMS-S has also been demonstrated in plants regenerated from tissue culture (Earle et al. 1987). A study of the mtDNA

from CMS-S revertants obtained in this way is given in chapter 4.

1.4 Evolution of higher plant mitochondrial genomes

The sequence homology between mtDNA from related cytoplasms or species is extremely high. Table 1.3 shows the homology between mitochondrial genes of maize and those of other higher plants. There is in the order of 90% nucleotide sequence homology between the genes of maize and dicotyledonous plants such as Oenothera, soybean, pea and Petunia, which are evolutionarily quite unrelated to maize. This remarkable conservation of sequence extends to non-functional sequences, such as the cpDNA insertions described in **1.2.3** (Marechal et al. 1987, Schuster and Brennicke 1987c), and thus is not solely due to selection pressure. The base substitution rate is very low for higher plant mtDNA when compared to mtDNAs from animals (Chao et al. 1984, Grabau 1985). A study of mitochondrial genome evolution in the genus Brassica has suggested point mutation rates 100-fold slower for Brassica mtDNA than that for animal mtDNA and 4-fold slower than that for Brassica cpDNA (Palmer and Herbon 1987b). Both copies of large repeats in plant mtDNA are generally identical (Houchins et al. 1986, Isaac et al. 1985a, Dewey et al. 1986, Hiesel et al. 1987). These observations suggest that efficient copy correction/mismatch repair systems are present, which is probably not the case for animal mitochondria (Brown 1983). These processes may be integral to the homologous recombination system presumed to be active in higher plant mitochondria.

However, mtDNAs from closely related species and even from different cytoplasms within the same species can be readily

distinguished by their mtDNA restriction endonuclease profiles. Many of the characterized differences between related mtDNAs appear to involve aberrant recombination events between short regions of homology (Leaver et al. 1985, Schardl et al. 1985, Dewey et al. 1986). Others involve insertions and deletions of sequences, particularly the creation of novel repeats or loss of previously existing ones (Palmer and Herbon 1987b, Small et al. 1987, Pring et al. 1987, 1988). Thus the predominant feature in the mtDNA evolution of higher plants appears to be rapid structural reorganization of the genome (Palmer and Herbon 1987b).

One aspect of the plant mitochondrial genome which may help explain this mode of evolution is the prevalence of non-coding sequences (up to 90% and more of the larger genomes (Ward et al. 1981)). Of equal importance is the fact that all but a few genes in plant mtDNA are independently transcribed (Makaroff and Palmer 1987). Therefore the plant mitochondrial genome might be expected to be very tolerant of structural reorganization and changes in gene order, as it indeed seems to be. In contrast, higher plant cpDNAs are densely packed with genes, many of which are cotranscribed (reviewed in Gray 1986), whilst animal mtDNAs lack non-coding sequence almost entirely, and are transcribed from a single promoter (Clayton 1984, Brown 1985). This compact arrangement must severely constrain opportunities for structural alterations in the genome.

1.4.1 Structural evolution of the maize mitochondrial genome

The origins of maize are a matter of some debate. Some workers believe that modern maize (Zea mays) is derived from a wild corn that has since become extinct (Mangelsdorf 1986), but the majority view is that modern maize is a direct descendant of its closest living relative,

Table 1.3 Homologies between maize mitochondrial genes and those of other plant species

Species	DNA sequence homology (%)	Amino acid homology (%)	References
18S ribosomal RNA			Chao et al. 1984
wheat	97	-	Spencer et al. 1984
Oenothera	87.9	-	Brennicke et al. 1985
soybean	85	-	Grabau 1985
5S ribosomal RNA			Chao et al. 1983
wheat	93.2	-	Spencer et al. 1981,1984
soybean	87.2	-	Morgens et al. 1984
Oenothera	86.3	-	Brennicke et al. 1985
Cytochrome c oxidase subunit I			Isaac et al. 1985a
sorghum	98	99.2	Bailey-Serres et al. 1986b
wheat	98.1	97.5	Bonen et al. 1987
soybean	93.9	94.2	Grabau 1986
Oenothera	92.7	92.2	Hiesel et al. 1987
Cytochrome c oxidase subunit II			Fox and Leaver 1981
rice	99.5	100	Kao et al. 1984
wheat	98.9	98.8	Bonen et al. 1984
Oenothera	88.8	88.1	Hiesel and Brennicke 1983
pea	90.4	86.4	Kao et al. 1984, Moon et al. 1985
Apocytochrome b			Dawson et al. 1984
wheat	98.8	98.5	Boer et al. 1985
Oenothera	95.3	94.2	Schuster and Brennicke 1985
ATP synthase (F_1) α-subunit			Braun and Levings 1985, Isaac et al. 1985b
Oenothera	92.2	92.7	Schuster and Brennicke 1986
ATP synthase (F_0) subunit 9			
petunia	93	96.1	Young et al. 1986

the teosinte Z. mexicana, with which it crosses freely, and is the product of 10,000 years of selective breeding (Galinat 1971). The mtDNA of Z. mexicana closely resembles that from modern N maize, and probably can be considered as ancestral to it (Timothy et al. 1979, Sederoff et al. 1981).

The origins of the three male sterile maize cytoplasms are even less clear. Restriction endonuclease fragment analysis of the four classes of maize mtDNA (N, C, T, S) reveals widespread differences between them (Pring and Levings 1978). Pairwise comparisons show that about 30% of the fragments are different between any two lines (Borck and Walbot 1982). Comprehensive studies of the mtDNA from ancestral maize cytoplasms from South America (Weissinger et al. 1983) and Mexico (Kemble et al. 1983) as well as contemporary US inbred cytoplasms (Levings and Pring 1977, Pring et al. 1980, 1987, McNay et al. 1983, Sisco et al. 1985) reveal abundant minor variations within the four maize cytoplasmic classes, but they have not identified any obvious intermediate forms. Two theories have ben suggested to explain this situation:

(i) The maize CMS cytoplasms were derived from teosinte species related to maize. In many species CMS cytoplasms have been obtained by crossing two related species or cultivars with different mitochondrial genomes, and then repeatedly backcrossing the hybrid to the male parent. As mitochondria are not transmitted through the pollen in most higher plants (including maize), the breeder eventually obtains a line with the mitochondrial genome from the original female parent and the nuclear genome from the male parent. Incompatability between the two genomes is thought to be responsible for the CMS phenotype. However, a study of the mtDNAs from all the extant Zea sp. has shown that with the

exception of Z. mexicana, they do not resemble N mtDNA, neither are they at all related to the mtDNAs of the male-sterile maize cytoplasms (Timothy et al. 1979, Sederoff et al. 1981). Only Z. mexicana can be crossed to maize. Thus if the male-sterile cytoplasms are derived from ancestral teosintes, these ancestors are now extinct.

(ii) An alternative hypothesis is that the CMS-linked mitochondrial genomes are derived from the original Z. mexicana genome. In some ways this latter hypothesis seems more likely, as there is evidence that male-sterile cytoplasms post-date some of the fertile cytoplasms, e.g. the fertile RU cytoplasm of some primitive South American races appears to be ancestral to CMS-S cytoplasm because of the relationship between their linear plasmids (see **3.2**). As stated above, no intermediates between the N, C, S, and T genomes are known, implying they have either been lost, or that the mitochondrial genome reorganizations occurred suddenly.The induction of catastrophic genome alteration from N-type to S-type has recently been claimed (Lemke et al. 1985). For a discussion of these experiments see **7.4**.

Two well documented examples exist of rapid changes in mitochondrial genome organization in higher plants. The most drastic occurs following protoplast fusion to produce hybrid or cybrid cells (Belliard et al. 1979, Galun et al. 1982, Boeshore et al. 1983,1985, Fluhr et al. 1983, Nagy et al. 1981,1983, Chetrit et al. 1985, Kemble et al. 1986). Widespread recombination between parental genomes gives rise to new mitochondrial genotypes containing novel recombinant fragments (Rothenberg and Hanson 1987, Morgan and Maliga 1987). Amplification of pre-existing low abundance fragments has also been described following this procedure (Morgens et al. 1984, Morgan and Maliga 1987).

Cytoplasmic reversion to fertility is also associated with sudden

changes in the mtDNA. CMS-S cytoplasmic revertants usually lose the characteristic linear S1 and S2 plasmids and also have specific deletions in the main genome following aberrant recombination events (Levings et al. 1980, Kemble and Mans 1983, Schardl et al. 1985). These alterations occur within a single plant life cycle. For a full discussion see Chapter 4. Culture-derived CMS-T revertants show very specific mtDNA alterations, as described earlier (**1.3.2**).

Chapter 2- Materials and methods

2.1 Materials

2.1.1 Maize seed

The genotypes of the maize lines listed below are given as nuclear genotype-cytoplasmic genotype (where both are known).

Maize seed with the genotypes B37-N, C, T, S, SRf; B73-N, S; WF9-N, C, S, T, TRf; WM13-S; ECU321; ECU398; PUN6; CUN443 were supplied by Pioneer Hi-bred International, Des Moines, Iowa, USA.

Maize seed with the genotypes A632-CA; Ay191-71-CA; B14A-CA; B37Ht-CA; CO109-CA; Mo17-CA; MS74-CA; NyD410-CA; W182BN-CA; W64A-CA, N; WF9-N, S and the WF9-S revertant 85:6838 were supplied by V.E. Gracen, Cornell University, New York, USA.

The seed from the progeny of W182BN plants regenerated from tissue culture (samples 1B to 20B, used for the experiments in Chapter 4) and the control lines W182BN-N, 181, CA, LBN, S, T were supplied by E.D. Earle, Cornell University, New York, USA.

Maize seed with the genotypes M825-N, S; M825/Oh07-VG and revertants thereof; WF9-ML, RD and revertants thereof were supplied by S. Gabay-Laughnan and J.R. Laughnan, University of Illinois, Illinois, USA.

The maize seed for the experiment shown in fig. 7.1 and the control lines W182BN-181 and R181-N were supplied by C.A. Lemke, Cornell University, New York, USA.

Maize seed with the genotypes CO192xWJ-LF, OY, F, Q, N, 234, SG, J, MY, H, PS, G, I, B, SD, L, D, CA, 181 were produced by R. E. Gunn, Plant Breeding Institute, Cambridge using cytoplasmic stocks from V.E. Gracen, Cornell University, New York, USA (Forde et al. 1980).

2.1.2 Maize mitochondrial DNA clones

The following single-stranded M13 clones were kindly supplied by Dr. P.G. Isaac (Department of Botany, University of Edinburgh):

M3A3, M2C1; clones containing respectively the 5' and 3' ends of the maize mitochondrial coxI gene (Isaac et al. 1985b)

ALXR18, BLSC1, AN6, BM4; clones containing portions of the atpA genes and surrounding sequences from N-type maize mtDNA (Isaac et al. 1985a).

The clones IS1E5, S1B5 and RHAB59 were constructed during the course of my research (see **2.2.4**).

The double-stranded plasmid clones S1339 (containing the S1 linear plasmid of CMS-S mtDNA), S2341 and S2342 (containing internal PstI fragments of S2) were gifts from Prof. C.S. Levings III (Department of Genetics, North Carolina State University, USA).

The plasmids pHSB3, pIII and HS3C5 were supplied by Dr. P.G. Isaac. The plasmid pZmE67 was from a clone library of B37-N mtDNA constructed by Dr. A. Dawson (Department of Botany, Edinburgh). The plasmids pAB5 and T3H4 were made during the course of my research (chapter 6).

The cosmid clones G4 and G5, covering the 12kb repeats from WF9-N maize mtDNA were donated by Dr. D.M. Lonsdale, Plant Breeding Institute, Cambridge.

2.1.3 Chemicals

Chemicals were supplied by British Drug Houses, Poole, Dorset or Sigma Chemicals, Poole, Dorset unless indicated otherwise.

Agarose: Miles agarose for minigels

Sigma type II agarose for fractionation of mtDNA and

	restriction endonuclease mapping of plasmids
Agar:	Difco Bacto Agar for minimal media plates
	Oxoid technical Agar for L broth agar plates

Caesium chloride:	Fisons Scientific Apparatus
Deoxy and Dideoxy-N-5' triphosphates:	Boehringer Mannheim
Herring sperm DNA:	Serva Feinbiochemica
Sephadex:	Pharmacia
X-gal:	NBL enzymes
IPTG:	NBL enzymes
oligodeoxynucleotide primers:	Pharmacia

2.1.4 Enzymes

Restriction endonucleases:	Bethesda Research Laboratories, NBL Enzymes, Amersham International, Boehringer Mannheim
Alkaline phosphatase:	Amersham International
DNA polymerase I:	Amersham International
Large fragment of DNA polymerase (Klenow):	Bethesda Research Laboratories, Pharmacia
Lysozyme:	Sigma
Proteinase K:	Boehringer Mannheim
T4 DNA ligase:	Bethesda Research Laboratories, Boehringer Mannheim
T4 polynucleotide kinase:	Amersham International

Reactions were carried out according to the suppliers instructions.

2.1.5 Radioisotopes

Supplied by Amersham International:

Deoxycytosine 5'[α-^{32}P]triphosphate (dCTP) as triethylammonium salt, specific activity 15.17TBq mmol^{-1}

Adenosine 5'[γ-^{32}P]triphosphate (ATP) as triethylammonium salt, specific activity 111TBq mmol^{-1}

2.1.6 Buffers and solutions

TE:	0.1mM EDTA, 10mM Tris-HCl pH8.0
10X TBE:	0.89M Boric acid, 0.02M EDTA, 0.89M Tris pH8.0
10X TAE:	0.2M sodium acetate, 0.02M EDTA, 0.4M Tris pH8.0
20X SSC:	3.0M sodium chloride, 0.3M sodium citrate pH7.0
Hybridization buffer:	4X SSC, 0.1% (w/v) SDS, 50mM sodium phosphate (pH 5.5), 0.2% (w/v) BSA, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinyl pyrrolidone (mol. wt. 40000) and 200μg/ml denatured herring sperm DNA

2.1.7 Bacterial growth media

All percentages are w/v.

Minimal agar:	1.5% agar, 0.2% $(NH_4)_2SO_4$, 1.4% KH_2PO_4, 0.6% K_2HPO_4, 0.1% sodium citrate, 0.02% $MgSO_4$, 0.2% glucose, 2.5 X 10^{-4}% thiamine hydrochloride
BBL top agar:	1% agar, 0.65% trypticase, 0.5% NaCl
L broth:	1% Difco Bacto Tryptone, 0.5% Difco Bacto yeast extract, 0.5% NaCl; pH7.2

L agar: L broth including 1.5% agar

2.1.8 Centrifugation equipment

Sorvall RC-5B centrifuges were used in conjunction with Sorvall GSA rotors (250ml bottles, 100ml tubes) or Sorvall SS-34 rotors (50ml tubes, 30ml or 15ml Corex tubes) for isolation of mitochondria and initial pelleting of mtDNA.

Sorvall OTD65B ultracentrifuges were used in conjuction with a Sorvall AH-627 rotor (17ml or 36ml tubes for sucrose gradients) or a Beckman 70.1 Ti rotor (Beckman heat-sealed tubes for CsCl gradients).

Eppendorf or MSE Micro Centaur microcentrifuges were used in conjunction with Treff 1.5ml microcentrifuge tubes for all manipulations involving purified DNA, including enzyme reactions, and for the isolation of plasmid, cosmid and 'phage DNA.

2.2 Methods

2.2.1 Purification of maize mitochondria

Maize seeds were imbibed for 4-16 hours in running water before sowing on cellulose wadding wetted with 1mM calcium chloride. After 4 days growth in enclosed seed trays in the dark at 29^{0}C, the coleoptiles were removed and cooled to 4^{0}C prior to mitochondrial extraction. Immature cobs (5-10cm in length) were obtained from plants grown in soil in greenhouses.

The following procedures were carried out at 4^{0}C, with pre-cooled centrifuges and equipment, and are a modification of those described by Leaver et al. (1983). When more than one sample was treated at one time, common equipment was washed thoroughly in cold distilled water. The volumes of buffers, the type of rotor and the size of centrifuge tubes

used varied with the amount of starting tissue.

The tissue (either coleoptiles or immature cobs) was added to approximately twice the tissue weight of grinding buffer (0.4M mannitol, 25mM MOPS buffer, 1mM EGTA, 0.1% (w/v) BSA, 8mM cysteine; pH7.5) and homogenised in a pestle and mortar before being filtered through 4 layers of muslin. The filtrate was centrifuged for 5' at 1000g to remove starch, cell wall debris, nuclei and most plastids. The supernatant was then centrifuged for 15' at 10000g to pellet the mitochondria. This pellet was resuspended in 1-2ml of wash medium (0.4M mannitol, 5mM MOPS, 1mM EGTA, 0.1% BSA; pH7.5) and layered on a 13ml or 31ml sucrose step gradient (0.6M-0.9M-1.2M-1.45M-2.0M; 1mM EGTA, 10mM Tricine; pH7.2) which had been left for 1-2 hours to form a smoother gradient. When dealing with small amounts of tissue, as for the fertility revertant samples in chapter 4, a simpler 14ml step gradient of 1.2-1.6M sucrose (containing 10mM potassium phosphate buffer, pH7.5) was used (Boutry and Briquet 1982). This type of gradient gave a higher yield of mitochondria, but the resultant mtDNA showed signs of degradation and contamination with degraded nuclear or plastid DNA. In retrospect this procedure is not recommended for mtDNA isolation. Both types of sucrose gradient were centrifuged at 50000g for 60'. The purified mitochondria were removed from the gradient (at the 1.45M and 1.6M interfaces respectively) and diluted with 4 volumes of wash buffer before pelleting at 10000g for 15'. This pellet can be kept frozen at -80^{0}C for over one year without signs of mtDNA degradation.

2.2.2 Mitochondrial DNA isolation

MtDNA was isolated by solubilization of the mitochondria in 0.5% N-lauroyl sarcosine, 100mM EDTA, 100mM Tris-HCl (pH 8.0), 0.1mg/ml Proteinase K for 1 hour at 60^{0}C, followed by CsCl density gradient

centrifugation in the presence of 75µg/ml ethidium bromide as described by Fox (1979). After centrifugation, the ethidium bromide was removed by extraction with butan-1-ol (pre-saturated with NaCl and TE). CsCl was removed by diluting the sample with two volumes of TE, adding ethanol to 70% and precipitating the mtDNA overnight at -20^{0}C. The pellet (usually containing some CsCl) was collected by centrifugation at 10000g for 30' at 0^{0}C, dissolved in 400µl TE, transferred to 1.5ml tubes and reprecipitated with 50µl of 3M sodium acetate (pH6.0) and 1ml of ethanol at -20^{0}C. The mtDNA was pelleted by centrifugation at 12000g for 10-15' in a microcentrifuge, and repeatedly washed with 70% ethanol until free of salt. This purified mtDNA was stored at 4^{0}C dissolved in TE buffer.

2.2.3 Gel electrophoresis, blotting and probing of DNA

Approximately 2µg of mtDNA from each maize line was digested to completion with the chosen restriction endonuclease, and fractionated on a 1X TAE 0.8% agarose gel (20 X 20 X 0.5 cm) at 1.5-2.0 V/cm overnight. The gels were stained with 0.0001% ethidium bromide for 30' and photographed (Ilford HB5 film). HindIII digested λ DNA was used as size markers on all gels. The mtDNA in the gels was then depurinated, denatured and capillary blotted (modified from Southern 1975) onto nylon filters (Hybond-N, Amersham) according to the manufacturers instructions. These filters were pre-hybridized for 30-60 minutes in hybridization buffer at 65^{0}C. ^{32}P labelled DNA probes (see **2.2.9**) were hybridized to the nylon filters under stringent conditions (65^{0}C in hybridization buffer) overnight. The filters were then washed in one or two changes of fresh hybridization buffer (lacking herring sperm DNA) at 65^{0}C, followed by washing in 2X SSC or distilled water for 30 minutes at

X-ray film (Curix RP1)

room temperature. The filters were then exposed to pre-flashed for 1-7 days at -80^{0}C with the aid of intensifying screens. In some cases, the hybridized probe was removed by treating the filter in 0.4M NaOH for 1-2 hours at 45^{0}C followed by neutralization at 45^{0}C for 30' in 0.1X SSC, 0.1%(w/v) SDS, 0.2M Tris-HCl pH7.5.

The above procedures were modified slightly for the detection of sublimons (Chapters 5 and 6). Approximately 5µg of mtDNA was used per gel lane (or the entire mtDNA content of an immature cob) and blotting was extended to 48 hours or longer. After probing, the filters were fluorographed with Amersham Hyperfilm.

When probing with oligonucleotide probes, the hybridization buffer was modified to 6X SSC and 250mg/ml herring sperm DNA, and the hybridization/washing temperature was lowered to 45^{0}C.

2.2.4 Cloning and screening in M13 vectors

M13 is a single-stranded coliphage that has been modified to make screenable cloning vectors (M13mp vectors) by the introduction of part of the β-galactosidase gene from E. coli (Messing et al. 1977). This lac region can complement the lesion in certain lac$^-$ hosts (e.g. JM101, Messing 1983). The M13 derived vectors mp18 and mp19 contain an array of unique restriction sites (polylinker) within this lac region (Norrander et al. 1983). Insertion of DNA into these sites abolishes lac function. In the presence of X-gal and the inducer IPTG, host cells transformed with vector DNA form blue plaques when plated on a lawn of untransformed log-phase cells, whilst cells transformed with recombinant DNA form white plaques (Messing 1983).

The construction and identification of the clone S1B5 will be given as an example of the use of this system. Relevant details of the clones IS1E5 and RHAB59 are given in chapters 3 and 6 respectively.

The requirement was for a clone containing sequence specific to S1 and lacking sequence common to S2. 2µg of S1339 and 1µg of M13mp19 DNA were digested with HindIII, and the enzyme removed by phenol extraction and ethanol precipitation. The linear DNAs were mixed and incubated with T4 DNA ligase for 3.5 hours at room temperature. One-tenth of this mixture was used to transform competent JM101 cells (prepared according to Dagert and Ehrlich 1979). The ligation mixture was incubated with the competent cells for 40' on ice, heat-shocked at 37^{0}C for 5-10' and mixed with 200µl of log-phase JM101 cells. 10µl of 50mg/ml IPTG, 10µl of 50mg/ml X-gal (both dissolved in dimethylformamide) and 3.3ml of BBL top agar were added, shaken and the resultant mixture poured evenly on a minimal plate. After overnight incubation at 37^{0}C, recombinant clones (white plaques) were picked onto three replica sets of gridded minimal plates covered by a fresh lawn of JM101 cells (200µl log-phase cells plus 3.3ml BBL top). After further overnight incubation the 'phage were transferred directly to nitrocellulose filters (Schleicher and Schuell) and the DNA denatured (5' on an absorbent pad soaked in 1.5M NaCl, 0.5M NaOH), neutralized (5' on 1.5M NaCl, 10mM EDTA, 0.5M Tris-HCl pH7.2), rinsed in 2X SSC and fixed by baking at 80^{0}C in a vacuum oven. One filter was probed with S2342 (to show clones containing pUC fragments or S2 homology), and the other with gel-purified fragments (see **2.2.8**) of S1339 known to be from entirely within the S1-specific sequence. Clones hybridizing to the second but not the first probe were presumed to be S1-specific, and included S1B5. Using labelled S1B5 as a probe on BamHI and HindIII digested S mtDNA gave the expected hybridization pattern.

2.2.5 Preparation of single-stranded DNA from M13 clones

Single-stranded M13 DNA was prepared from a single 'phage plaque as follows:

A 10^{-2} dilution of an overnight culture of JM101 was grown for 90' at 37^{0}C in L-broth with vigorous shaking and then inoculated with 'phage using a toothpick. After four hours growth 1ml of this culture was removed into an Eppendorf tube, and the cells pelleted by 3' centrifugation at 12000g in a microcentrifuge. 'Phage particles were precipitated from the supernatant by the addition of 200µl of 20% PEG-6000, 2.5M NaCl for 30', followed by 5' microcentrifugation. After thorough removal of the PEG, the DNA was purified from the 'phage particles by repeated phenol extraction and ethanol precipitation.

2.2.6 Cloning and screening in pUC vectors

pUC plasmid vectors contain the ampicillin resistance gene and the origin of replication from pBR322 and the lac region from M13mp vectors (Ruther 1980, Vieira and Messing 1982). pUC18 and 19 contain the same polylinker as mp18 and 19 (Norrander et al. 1983). Recombinant clones can be identified by the same blue-white screening procedure as is used for M13mp vectors. Transformed cells can be selected against untransformed cells by growing cells in the presence of ampicillin.

The OY mtDNA library used in chapter 6 was made as follows:

OY mtDNA was digested with XhoI and ligated to SalI cut pUC18 which had been treated with alkaline phosphatase to inhibit religation (enzymes were removed by phenol extraction prior to ligation). The ligation mixture was used to transform competent JM101 cells as described by Maniatis et al. (1982). The transformation mixture (with added IPTG and X-gal, see **2.2.4**) was plated onto L-broth plates containing 100µg/ml ampicillin. After overnight growth, recombinant colonies (white) were picked onto gridded replica plates (L-broth,

100µg/ml ampicillin). After further overnight growth the colonies from one set of plates were transferred to Whatman 541 filter paper, and laid on L-broth plates for 16 hours containing 250µg/ml chloramphenicol to amplify the plasmids. DNA from the colonies was isolated and fixed to the filters by the denaturing and neutralization steps described in **2.2.5**.

2.2.7 Preparation of plasmid and cosmid DNA

Plasmid and cosmid DNA was purified by a modification of the alkaline lysis procedure of Birnboim and Doly (1979).

Cells picked from a single colony were grown overnight at 37^{0}C in 5ml of L-broth containing 100µg/ml ampicillin (200µg/ml for cosmid isolation). Cells from 1ml of overnight culture were pelleted by 1' microcentrifugation and resuspended in 100µl of lysis buffer (10mM EDTA, 1% (w/v) glucose, 2mg/ml lysozyme, 25mM Tris-HCl pH8.0) at room temperature. After 5', 200µl of ice-cold 0.2M NaOH/1% (w/v) SDS was added and gently mixed. After a further 5' on ice, 150ml of ice-cold 3M sodium acetate (pH4.9) was added and gently mixed. The precipitated bacterial chromosomal DNA was pelleted by 10' microcentrifugation, and the supernatant deproteinated by phenol extraction. The plasmid DNA was subsequently precipitated by the addition of 0.9ml of ethanol.

2.2.8 Gel-purification of restriction endonuclease fragments

Restriction endonuclease digests of the donor plasmid were carried out such that the chosen fragment was easily distinguishable in size from other fragments, and was preferably the largest fragment present. The products of the digest were separated by electrophoresis in 1X TBE agarose mini-gels (10 X 7 X 0.3 cm) containing 1.25µg/ml ethidium bromide. The percentage agarose used (typically 1% w/v) and the

electrophoresis conditions varied according to the size of the fragment to be isolated. These mini-gels were also used routinely to check the progress of restriction endonuclease digestion of plasmids and mtDNA. Small strips of DEAE membrane (Schleicher and Schuell) were inserted into the gel on either side of the chosen fragment. On the resumption of electrophoresis the stained fragment collected on the DEAE membrane in 'front' of the fragment and could be eluted by 15' incubation at 65^{0}C in 200µl of 1.5M NaCl, 1mM EDTA, 10mM Tris-HCl pH8.0 before purification by ethanol precipitation.

2.2.9 DNA hybridization probes

Single-stranded M13 clones were labelled with [α-^{32}P]dCTP by a modification of the method of Hu and Messing (1982). A 15- or 17-base oligodeoxynucleotide universal sequencing primer was annealed to the single stranded M13 template DNA and used to prime second strand synthesis by Klenow. Unincorporated nucleotides were removed by passing the reaction mixture through a G-50 Sephadex column (Maniatis et al. 1982). The labelled and template strands were separated by boiling and rapid quenching on ice before being added to the hybridization buffer.

Double-stranded plasmids and cosmids were labelled with ^{32}P by nick-translation (Rigby et al. 1977). Double-stranded gel-purified DNA fragments were boiled to separate the strands and then labelled with ^{32}P by using annealed random hexanucleotide primers to prime second strand synthesis by Klenow.

Oligonucleotides were labelled by incubating 10pmol of oligonucleotide, 10pmol [γ-^{32}P]ATP and 10 units of polynucleotide kinase at 37^{0}C for 1-2 hours in kinase buffer (0.5M Tris-HCl (pH7.6), 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA). The entire reaction

mixture was added to the hybridization buffer.

2.2.10 DNA sequencing

The sequencing of the M13mp19 clone RHAB59 was performed by the chain-termination method (Sanger et al. 1977, 1980), using both 15 and 17-base synthetic oligonucleotide sequencing primers (Messing 1983), and [α-^{32}P]dCTP as a radioactive label. Analysis of the reaction products was by electrophoresis in 8% acrylamide gels (8M urea, 8% (w/v) acrylamide, 0.25% (w/v) bisacrylamide, 1X TBE, 0.1% (w/v) ammonium persulphate, 0.005% TEMED), followed by autoradiography of the dried gel (Curix RP1 X-ray film, Agfa-Gevaert).

Chapter 3- Mitochondrial genome organization in CMS-S lines of maize

3.1 CMS-S maize

The United States Department of Agriculture (USDA) source of cytoplasmic male-sterility in maize, now known as CMS-S, was identified as being separate from the CMS-T source many years ago on the basis of differential nuclear effects on fertility (Jones et al. 1957, Duvick et al. 1965). A single dominant restorer gene, designated Rf3 is required for fertility restoration (or perhaps more accurately sterility suppression) in CMS-S (for reviews see Duvick 1965, Laughnan and Gaby-Laughnan 1983). This gene is gametophytic in action, i.e. an Rf3rf3 plant produces half normal and half aborted sterile pollen grains, whereas restorer genes for the other maize male-sterile cytoplasms completely suppress sterility for all pollen grains in a heterozygote and are therefore sporophytic. The gametophytic action of Rf3 implies that pollen abortion in CMS-S maize is due to developmental abnormalities late in microsporogenesis. Ultrastructural investigations of pollen abortion in CMS-S maize add weight to this view, revealing that microsporogenesis in N and S cytoplasms is identical until the developing pollen is almost mature (Lee et al. 1980). The tapetum is unaffected, whereas in CMS-T or C plants, tapetal cells show signs of mitochondrial degeneration before separate pollen grains have formed (Warmke and Lee 1977, Lee and Warmke 1979, Lee et al. 1979).

Several different sources of CMS-S have been discovered, and these can often be distinguished by minor differences in the restoration efficiency of different nuclear genotypes and by minor variations in restriction endonuclease profiles of their mtDNA (Sisco et al. 1985). The

major groups of S cytoplasms are given in Table 3.1.

Table 3.1 CMS-S sub-groups as classified by Sisco et al. (1985)

1	B, D
2	CA, E, F, G, H, IA, J, K, L, M, ML, MY, PS, R, SD, TA, VG, W
3	LBN
4	ME
5	S, TC, I

Most of my investigations have been with the two commonest cytoplasms, S (the original USDA cytoplasm after which the whole group is named) and CA, which is the type cytoplasm for the largest sub-group of S cytoplasms.

3.2 S1 and S2 linear plasmids

The mitochondrial genomes of S-type cytoplasms of maize are easily distinguished by the presence of two linear DNA episomes S1 (6397bp) and S2 (5453bp) in high copy number relative to the main mitochondrial genome (Pring et al. 1977). These linear DNAs have identical 208bp terminal inverted repeats (STIRs) (Kim et al. 1982b, Levings and Sederoff 1983, Paillard et al. 1985). Other linear plasmids from maize cytoplasms share significant homology with S1 and S2. The R1 (7.5kb) and R2 (5.4kb) plasmids from Latin American RU cytoplasms (Weissinger et al. 1982, 1983) and also the D1 (7.5kb) and D2 (5.4kb) plasmids from Zea diploperennis (Timothy et al. 1983) have been compared to S1 and S2 by heteroduplex analysis (Levings et al. 1983, Timothy et al. 1983). The results suggest that S2, R2 and D2 are virtually identical to each other,

and that R1 and D1 are also nearly indistinguishable. However, S1 differs from R1/D1 by containing significant homology (1.5kb including the STIR) to S2 at one end (Kim et al. 1982b, Levings et al. 1983). This S2 homologous sequence replaces about 2.6kb of sequence found only in R1/D1, explaining the length difference between S1 and R1/D1 (fig. 3.1). It has been suggested that S1 was formed by recombination between R1 and R2 (Levings et al. 1983, Elmore-Stamper and Levings 1986). It should be noted that cytoplasms containing R or D plasmids are male fertile.

Sequence analysis of S1 (Paillard et al. 1985) and S2 (Levings and Sederoff 1983) has revealed four open reading frames (fig 3.1). S1 contains an open reading frame (ORF 4) which is lacking from the R1/R2 system. These open reading frames are transcribed (Traynor and Levings 1986), and the two largest probably encode the large (more than 100kd) polypeptides characteristic of mitochondria from S (Forde and Leaver 1980) and RU cytoplasms (Liddell and Leaver unpublished), on the evidence of antibodies raised to protein synthesized in E.coli from constructs containing parts of ORF 1 (Manson et al. 1986) and ORF 3 (Zabala and Walbot 1987).

Small linear DNA replicons have since been identified in numerous other unrelated organisms, including the filamentous bacterium Streptomyces rochei (Hirochika et al. 1984) and the fungi Kluyveromyces lactis (Fujimura et al. 1987), Gaeumannomyces graminis (Honeyman and Currier 1986), Fusarium oxysporum (Kistler and Leong 1986), Ascobolus immersus (Meinhardt et al. 1986) and Ceratocystis fimbriata (Gianon and Lalonde 1987) as well as in the higher plants Brassica (Palmer et al. 1983) and Sorghum bicolor (Pring et al. 1982b). The replication mechanism of these linear DNAs is of interest because it is likely to differ from that of supercoiled circular plasmids and that of the

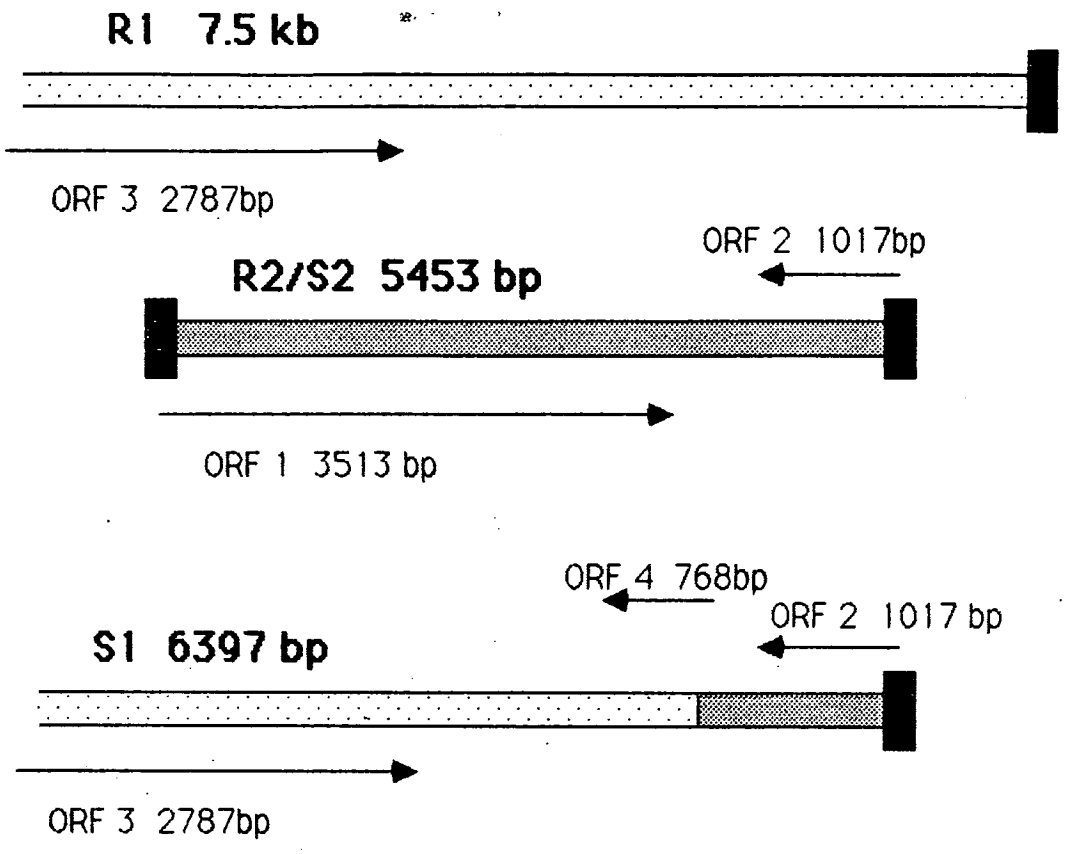


Figure 3.1 The R and S linear plasmids of maize mitochondria.

Terminal inverted repeat (STIR or TIR)

R1 sequence

R2/S2 sequence

The four open reading frames contained within these plasmids are shown. ORF 3 of S1 probably encodes a DNA polymerase (Kuzmin and Levchenko 1987). The suggested derivation of S1 by a recombination event between R1 and R2 (Levings et al. 1983) is indicated by the shading.

mitochondrial chromosome.

Many viruses have linear replicons, of which the best studied are the adenoviruses and Bacillus phages such as ϕ29. In these cases priming of DNA replication is aided by a protein which is covalently linked to the 5' terminal phosphate of the DNA chain, replication proceeding bidirectionally by strand displacement (for a review see Salas 1983). A protein has been shown to be covalently linked to the 5' ends of S1 and S2 (Kemble and Thompson 1982), which is involved in holding the plasmids in a circular configuration, and probably primes bidirectional replication (Sederoff and Levings 1985). It thus seems likely that these linear plasmids are replicated in an analogous manner to adenoviruses and Bacillus phage DNAs. Corroboration comes from the finding of homology between ORF 3 of S1 and several viral DNA polymerases, particularly that of ϕ29 (Kuzmin and Levchenko, 1987). The evidence points to a viral origin for these plasmids; certainly they are not a functional requirement, as, for example, the T and C genomes lack homology to them nearly entirely (Thompson et al. 1980, Spruill et al. 1980, Koncz et al. 1981, Pring and Lonsdale 1985). The interest in these plasmids stems from their effect on the mitochondrial chromosome with which they are associated.

3.3 Integration of linear plasmids into mitochondrial chromosomes

Sequences homologous to S1 and S2 have been found in N mtDNA (Thompson et al. 1980) and subsequently cloned and mapped (Lonsdale et al. 1981, Koncz et al. 1981). This mapping data, together with heteroduplex (Levings et al. 1983) and sequence analysis (Houchins et al. 1986) has shown that N mtDNA contains nearly complete copies of R1

(91%) and R2 (94%) (rather than S1 and S2) integrated next to a 5.27kb repeat (Repeat-1, see fig. 1.2). Each of these integrated plasmid sequences lacks one of the STIRs found on the free plasmid. The integrated R2 sequence has point mutations within it that introduce stop codons into ORF1, and thus it presumably has no coding function (Houchins et al. 1986).

Plasmid sequences are also found integrated into the main genome in S mitochondrial DNA. A high proportion of the mitochondrial genome in S cytoplasms is present as linear molecules with STIR sequences at their termini (Schardl et al. 1984). These linear molecules probably arose by recombination between S1 or S2 and sequences homologous to their STIRs found in the main mitochondrial genome (Schardl et al. 1984, Isaac et al. 1985, Braun et al. 1986) (fig. 3.2). These regions of STIR homology have been termed σ-σ', σ-φ', φ-σ' and φ-φ' (Schardl et al. 1984). I shall refer to them as 'targets' for STIR recombination.

3.4 Rearrangement of coxI containing sequences in S mtDNA

The maize gene for subunit 1 of the cytochrome c oxidase complex (coxI) has been sequenced along with its 5' and 3' flanking regions (Isaac et al. 1985b). When probes including the gene are hybridized to Southern blots of HindIII or BamHI digested mtDNA, striking differences are seen between the N and S genomes (fig 3.3; Isaac et al. 1985b). In S mtDNA many fragments contain coxI homology. Using probes overlapping both ends of the gene, I was able to show that each of the hybridizing fragments contains a whole copy of the gene (fig 3.3B).

Isaac et al. cloned the largest HindIII fragment, and subsequent analysis showed that the gene was contained within a 4.75kb BamHI sub-fragment. The sequence of this fragment revealed that the N and S

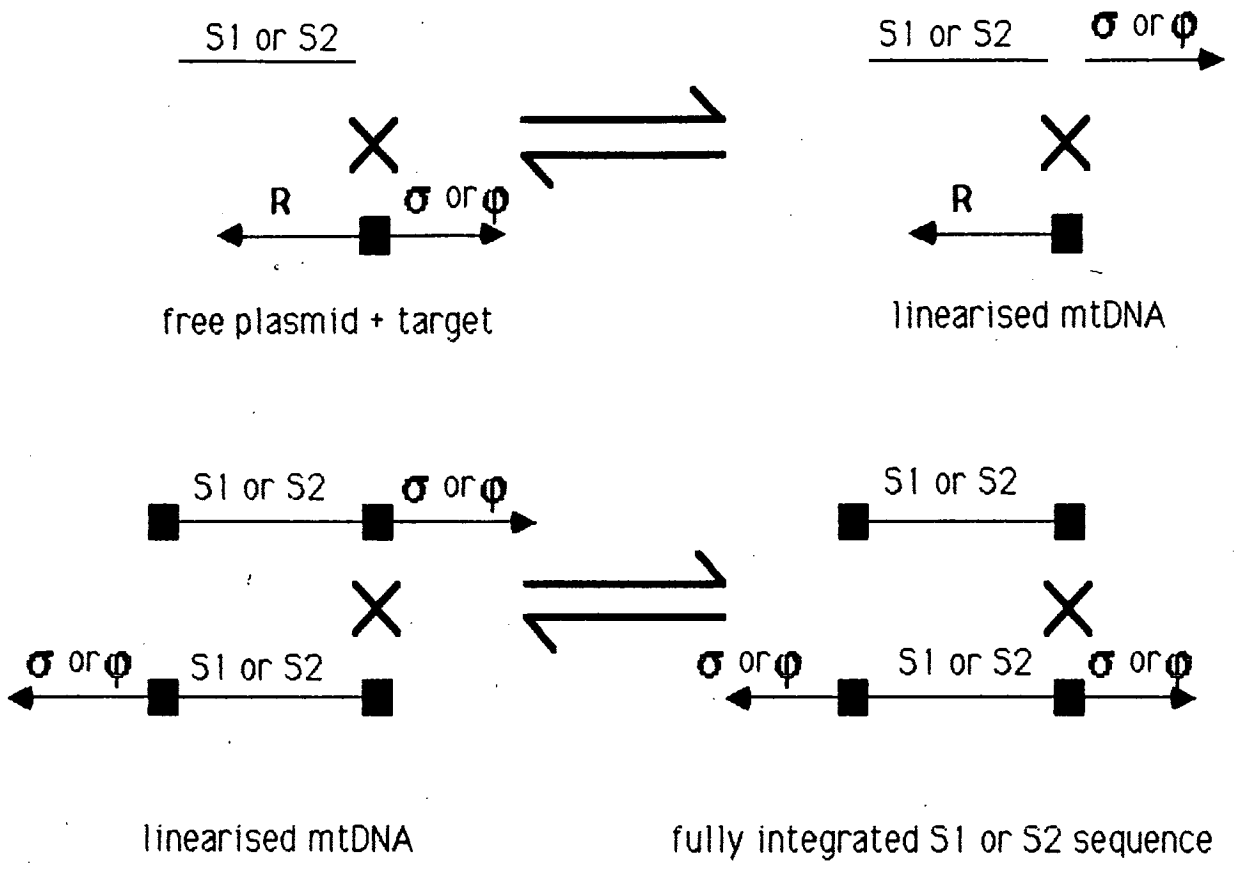


Figure 3.2 Linearisation of S-type maize mitochondrial DNA.
Recombination between the STIRs of the free plasmids and homologous sequences within the main genome can give rise to linear mitochondrial chromosomes with STIR sequences at their termini. Further recombination between two linear chromosomes can reform a free plasmid and create a circular molecule including a fully integrated plasmid sequence. σ and φ are the designations given to the sequences flanking STIRs in the genome by Schardl et al. (1984). **R** defines a repeat in the S genome (including a STIR) part of which is identical to the unique end of the R1 plasmid.

Figure 3.3 Comparison of coxI arrangements in N and S mtDNA

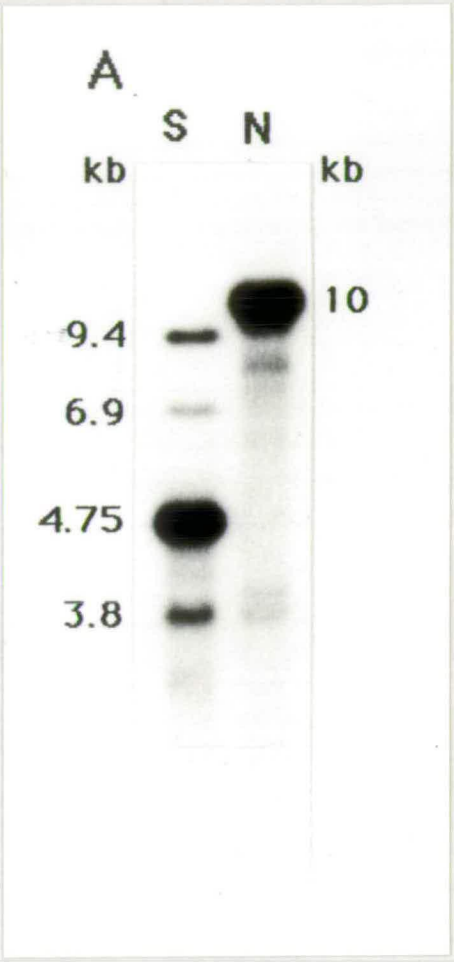
A: Southern blot of BamHI digested B73-S and B73-N mtDNA probed with a coxI probe (M2C1). Four fragments are clearly identified in S mtDNA (the most prominent is 4.75kb) compared with a single major 10kb fragment in N mtDNA.

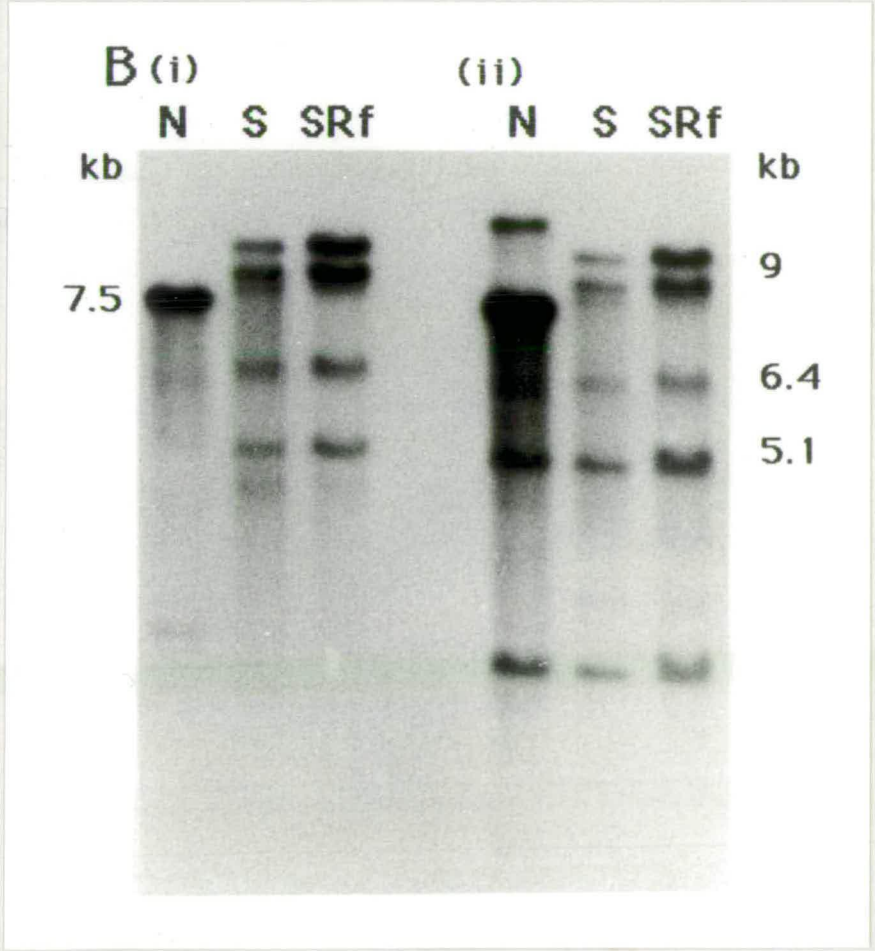
B: Southern blots of HindIII digested mtDNA from B37-N, B73-S and B37-SRf (S restored to fertility) probed with:

(i) a probe specific to the 3' end of coxI, and including 3' flanking sequences (M2C1)

(ii) a probe specific to the 5' end of coxI, and including 5' flanking sequences (M3A3)

All the fragments detected in (i) are detected in (ii) and thus probably represent complete copies of the coxI gene. The extra fragments hybridizing in (ii) (in both N and S mtDNA) probably indicate some sequence at the 5' of coxI is repeated elsewhere in the genome. There is no difference between the restored and non-restored S lines.


A
S
N
kb
kb
10
9.4
6.9
4.75
3.8


B (i)
(ii)
N
S
SRf
N
S
SRf
kb
kb
7.5
9
6.4
5.1

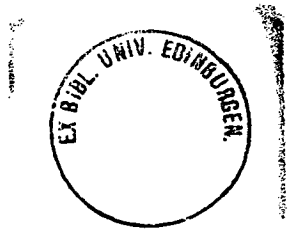
mtDNA sequences were identical from the 3' BamHI site to a point 174bp 5' of the start codon. At this point in the S genome there is a 185bp sequence showing exact homology with the free end of the STIR sequence of the R and S plasmids (preceded by an A instead of the terminal T residue of a STIR) (Isaac et al. 1985b). This sequence resembled the STIR targets described by Schardl et al. (1984). Comparison of our hybridization and sequence data with that of Schardl et al. allowed the identification of the coxI-containing sequence as σ. On the other side of the STIR was a stretch of sequence of unknown origin. To discover the identity of this sequence, I constructed M13 clones of Sau3AI digested pHSB3 (a clone of the 4.75kb coxI containing BamHI S mtDNA fragment) and selected those clones which hybridized to pHSB3 but not to a clone of coxI from N mtDNA. One such clone, IS1E5, hybridizes strongly to R1 but not to R2, S2 or S1 and to three fragments in S mtDNA (fig. 3.4). This sequence forms a repeat in S mtDNA. Both σ' and φ' contain about 2kb of repeated sequence which includes sites for the restriction enzymes BamHI and SalI (Lonsdale personal communication and data not shown), and thus σ–σ' and σ–φ' give the same 4.75kb fragment in a BamHI digest. In this thesis I shall refer to this repeat as **R** (figs 3.2, 3.4).

Using the STIR recombination scheme of Schardl et al. (fig. 3.2), together with restriction enzyme maps and sequence data from S1 (Paillard et al. 1985), S2 (Levings and Sederoff 1983) and σ-**R** (Isaac et al. 1985b), it is possible to calculate all the possible coxI hybridizing fragments in BamHI and HindIII digests (fig. 3.5). The predicted patterns closely resemble those actually observed (fig. 3.3).

Fig. 3.4. Identification of the sequence 5' of the STIR linked to coxI in S mitochondrial DNA as being homologous to the unique end of R1.

A IS1E5 was used to probe BamHI digested mtDNA from B73-N, B73-S and several South American accessions. ECU398 and CUN443 contain R1 and R2 but ECU321 and PUN6 lack R plasmids. The probe hybridizes strongly to the 2.5kb fragment derived from the unique end of R1, but shows no hybridization to S1, S2 or R2. The probe also detects the 4.75kb coxI (σ) containing target from which it was derived, and two other fragments in S mtDNA. The largest fragment is the φ-containing target, proving that the two target fragments share R1 homology in addition to their common STIR sequences. I have designated this repeated sequence **R**. The smallest fragment is the **R**-containing linear chromosome end generated by recombination between a target sequence and a free S plasmid (fig. 3.2). The probe also hybridizes to B73-N mtDNA as R1 homologous sequence is found next to a large repeat in N mtDNA (Houchins et al. 1986).

B Diagrammatic representation of the 4.75kb coxI-containing BamHI fragment (σ-**R**) from S mtDNA showing the positons of the STIR and R1 homologies relative to the coxI gene. The area covered by the probe IS1E5 and the extent of the **R** repeat are also shown.



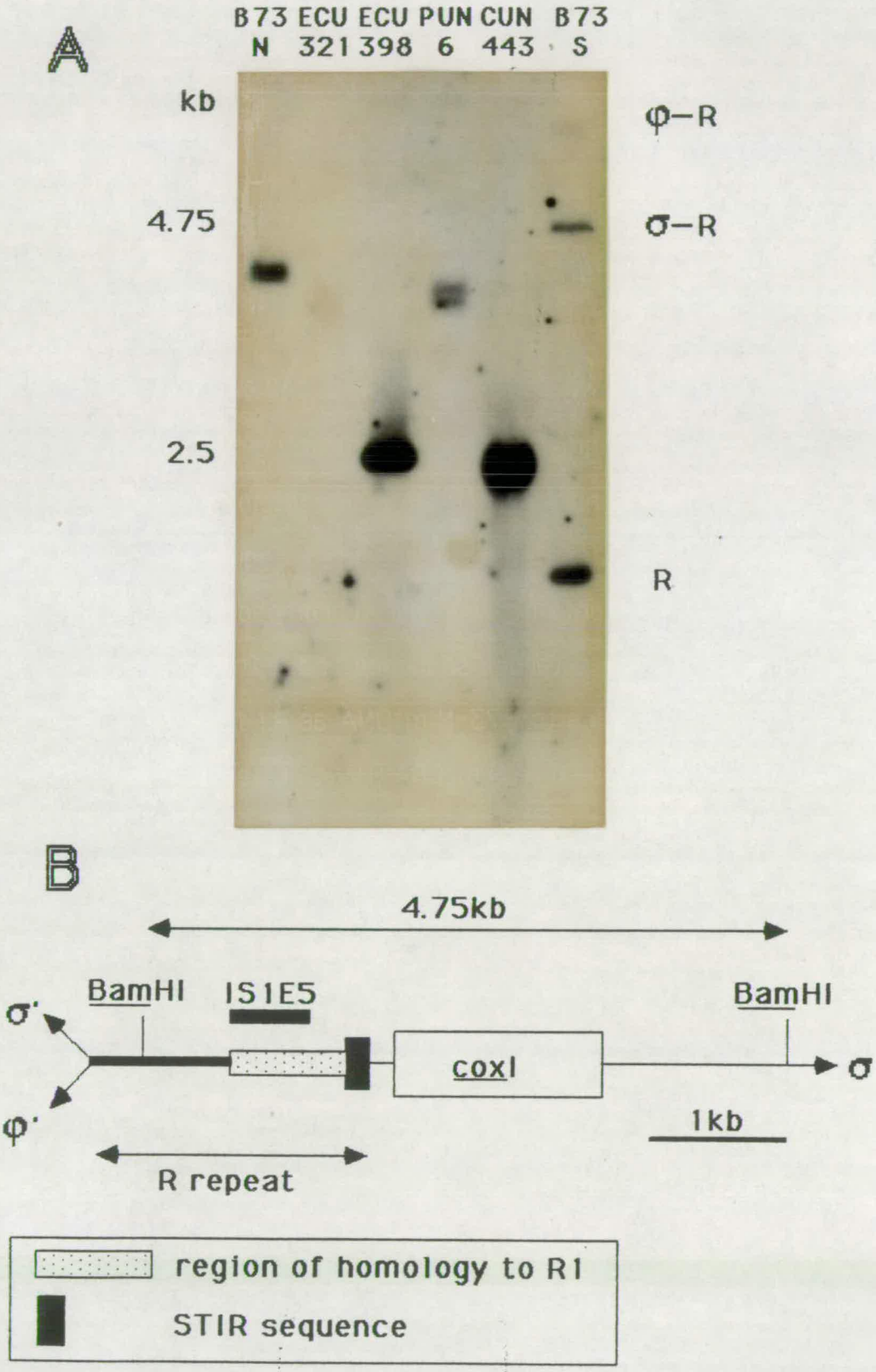
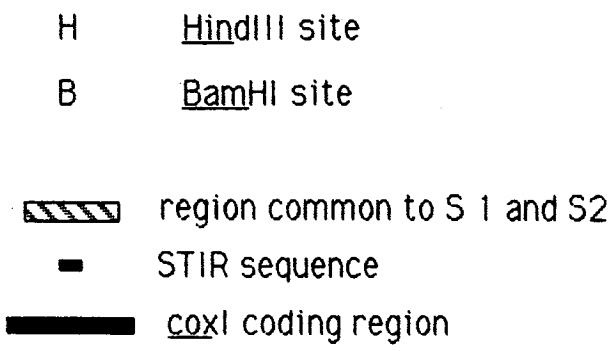
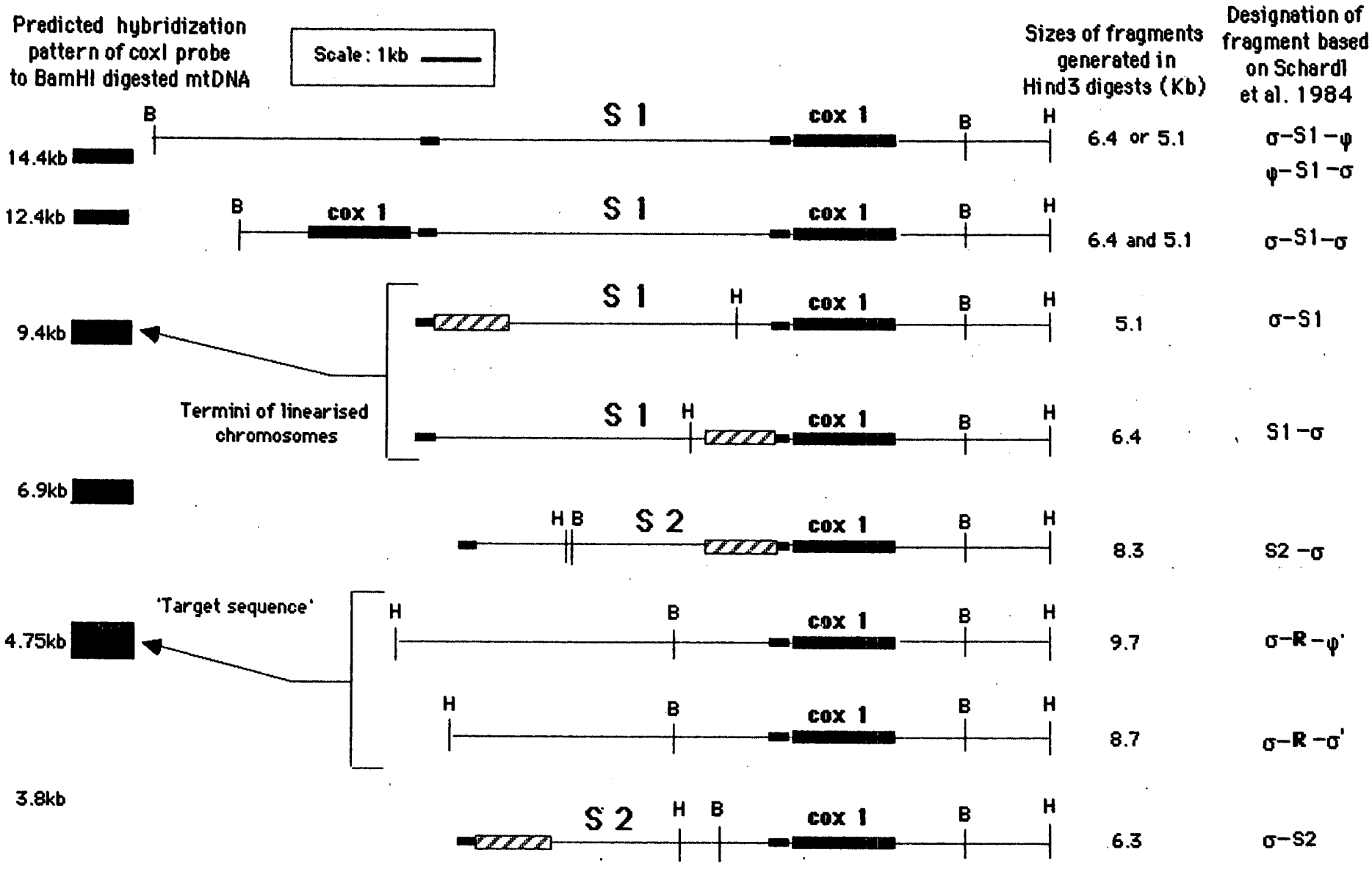

A
B73 N
ECU 321
ECU 398
PUN 6
CUN 443
B73 S
kb
4.75
2.5
φ–R
σ–R
R
B
4.75kb
BamHI
IS1E5
BamHI
σ'
φ'
coxI
σ
1kb
R repeat
region of homology to R1
STIR sequence

Figure 3.5 Calculated sizes of coxI containing fragments in BamHI and HindIII digests of CMS-S maize mtDNA



BamHI and HindIII sites are given for the coxI target sequences and the six possible S1 or S2-linked products of STIR recombination. The predicted BamHI hybridization pattern is shown. This compares well with the observed pattern (fig. 3.3A). The fragments have been designated according to the nomenclature of Schardl et al. (1984).


Predicted hybridization pattern of coxI probe to BamHI digested mtDNA
Scale: 1kb
Sizes of fragments generated in Hind3 digests (Kb)
Designation of fragment based on Schardl et al. 1984
14.4kb
12.4kb
9.4kb
6.9kb
4.75kb
3.8kb
Termini of linearised chromosomes
'Target sequence'
S 1
S 2
cox 1
B
H
6.4 or 5.1
σ-S1-ψ
ψ-S1-σ
6.4 and 5.1
σ-S1-σ
5.1
σ-S1
6.4
S1-σ
8.3
S2-σ
9.7
σ-R-ψ'
8.7
σ-R-σ'
6.3
σ-S2

3.5 Formation of the σ–R arrangement found in S mtDNA

The immediate progenitor of S cytoplasm is not known with any certainty, but due to the relationship of the S plasmids with the R plasmids of the primitive South American RU cytoplasms, RU is usually considered as the progenitor of S (Lonsdale 1987). The coxI arrangement in mtDNA from several primitive South American cytoplasms, including the RU cytoplasms ECU398 and CUN443, are the same as that of N mtDNA (fig 3.6). Given the presence of the abundant R1 homology adjacent to the STIR in the **R** repeat (fig 3.4), it seems tempting to suggest that the rearrangement of the coxI gene in S occurred initially via a rare aberrant recombination event between the terminus of R1 and the slight STIR homology present upstream of coxI in N mtDNA described by Isaac et al. (1985b) (fig 3.7). As only part of R1 is present adjacent to coxI in S, at least one further recombination event must have occurred before fixation of the S-type arrangement. If S cytoplasm derived not from RU, but from N cytoplasm, as claimed by Lemke et al. (1985), then a similar sequence of events can be envisaged, as N mtDNA contains the same R1 sequence at the flank of a large repeat (Houchins et al. 1986). This alternative hypothesis has been described in Leaver et al. (1985).

3.6 Nuclear effects on mtDNA in S cytoplasms

The dominant restorer gene Rf3 has no effect on the mtDNA organisation of S cytoplasm. The restriction endonuclease profiles of restored and non-restored lines are identical (not shown), and the coxI hybridization pattern, a more sensitive test, is also identical (fig 3.3B). However, nuclear genotype effects on S mtDNA are known. In the presence of most nuclear genotypes, the ratio of free S1 to S2 is 1:1, but in cytoplasms with the M825 nuclear background the ratio is 5:1, whilst

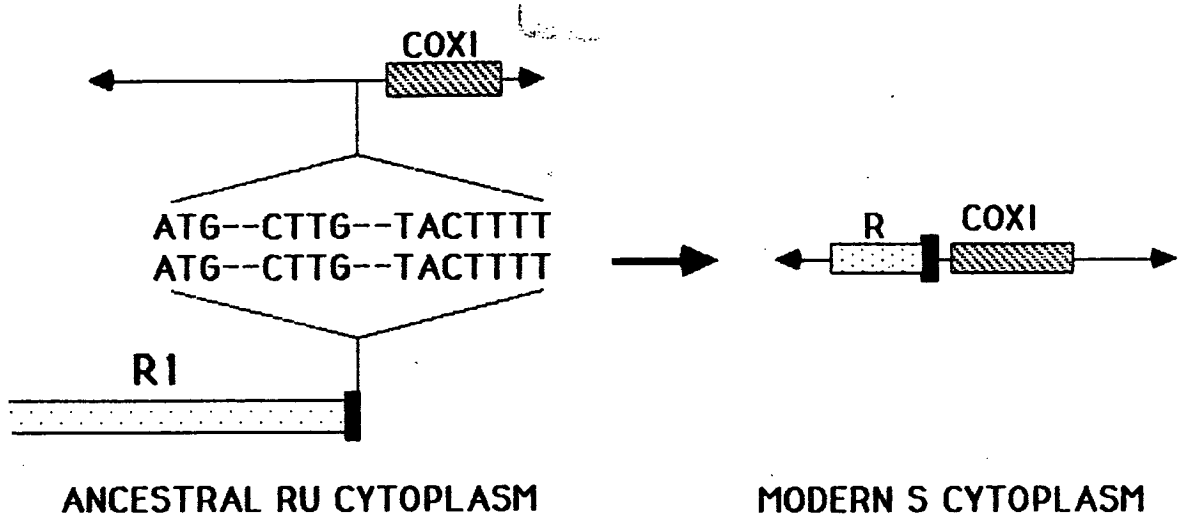


Figure 3.7 Possible pathway for the generation of the STIR 'target' sequence 5' to the coxI gene in CMS-S maize.

The sequence in N mtDNA at the point of insertion of the STIR in S mtDNA shows 14/18 homology to the terminal 18bp of the maize linear plasmids (Isaac et al. 1985). A rare recombination event between the sequences shown may have been the primary event in forming the arrangement characteristic of modern S cytoplasms, in which part of the R1 plasmid is found integrated 176bp 5' of the coxI gene.

with the nuclear background 38-11 the ratio is approximately 1:3 (Laughnan et al. 1981). The involvement of nuclear genes in these cases has been proved conclusively by backcrossing M825 or 38-11 lines (as recurrent male parents) to other lines with varied types of S cytoplasm.

I have found that the nucleus appears to influence the ratio of 'targets' to recombination products within the STIR recombination system in S mitochondria. Lines with the nuclear genotypes WF9 and NyD410 show relatively lower levels of the **σ–R** target and increased levels of the S1/S2 linked recombination products when their mtDNA is hybridized to a σ-specific probe than do lines with other nuclear genotypes (fig. 3.8). It seems likely therefore that at least some of the proteins required for STIR recombination and/or replication of the sequences involved must be nuclear encoded (some of these data have been published (Leaver et al. 1985)).

3.7 Discussion

The mitochondrial genome of CMS-S plants differs markedly from that of fertile N plants. The most obvious difference is the presence of the linear S plasmids and the associated chromosome linearization. The evidence for STIR recombination in CMS-S mitochondria is strong, and supports the notion of a homologous recombination system in plant mitochondria. However, in the majority of lines (exemplified by B73-S) the recombination substrates (targets and free plasmids) are considerably more abundant than the recombination products, suggesting that the recombination system is apparently not at equilibrium. This in turn would suggest that recombination is infrequent and the stoichiometries of the products are predominantly governed by other influences (e.g. relative rates of replication).

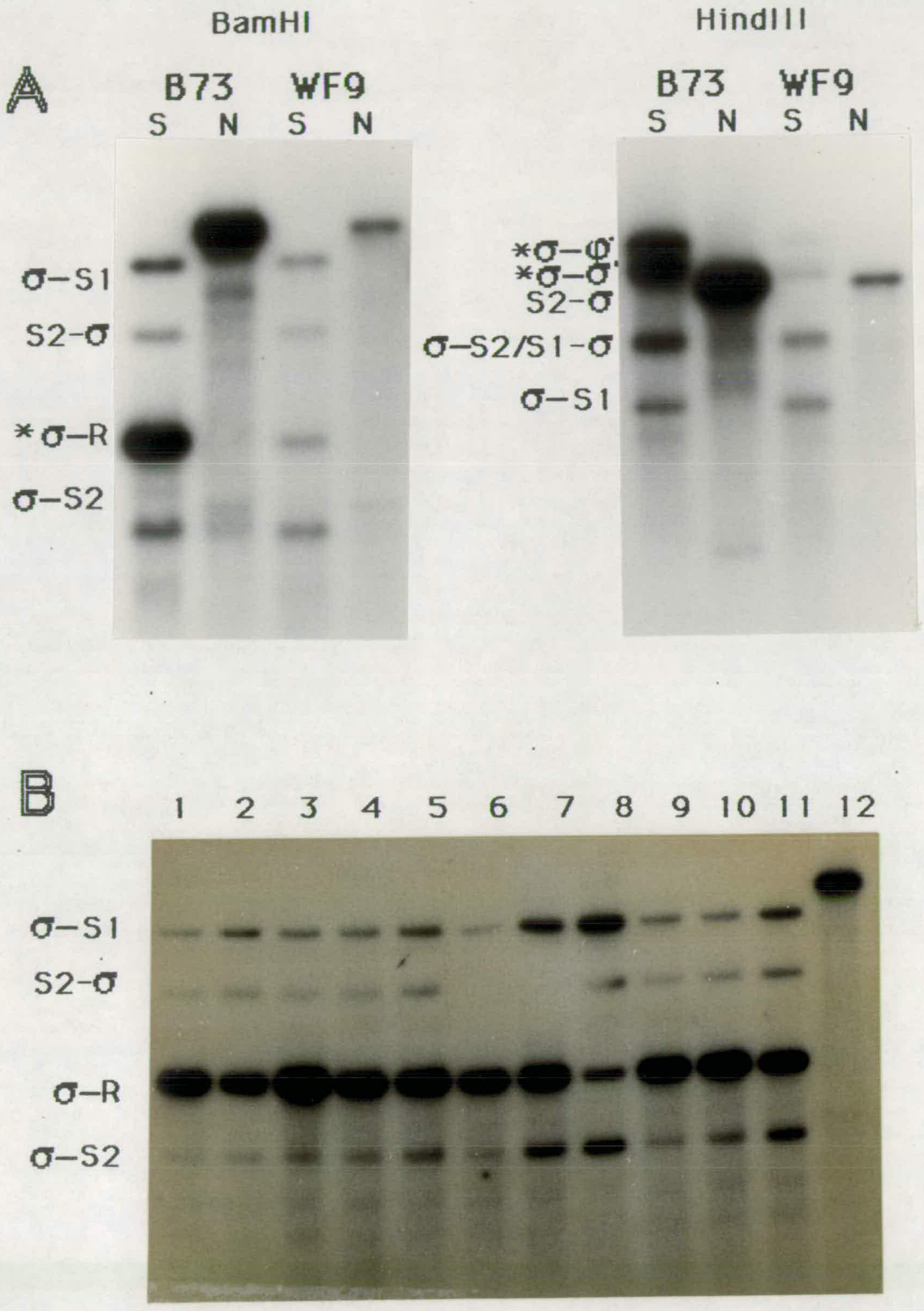
Fig. 3.8. Effect of nuclear genotype on ratio of STIR recombination substrates and products.

A Hybridization of M2C1 to BamHI or HindIII digested mtDNA from B73-N and S, WF9-N and S. In B73-S mtDNA the target fragments (marked *) are considerably more abundant than the products of STIR recombination. In WF9-S mtDNA the four hybridizing fragments are in approximately equal stoichiometry.
N and S lanes of one genotype are loaded at equal levels. Compare the N lanes to estimate the difference in loading between B73 and WF9 mtDNA.

B Hybridization of M2C1 to BamHI digested mtDNA from:

1	A632-CA	7	MS74-CA
2	Ay191-71-CA	8	NyD410-CA
3	B14A-CA	9	Oh43-CA
4	B37Ht-CA	10	W182BN-CA
5	CO109-CA	11	W64A-CA
6	Mo17-CA	12	W64A-N

All the genotypes shown resemble the B73-S pattern of hybridization (**A**) with the exception of NyD410-CA, which closely approaches the WF9-S pattern. All the CMS-S lines shown have the cytoplasmic genotype CA, thus the difference revealed must be due to the difference in nuclear genotype.


BamHI
HindIII
A
B73
WF9
S
N
S
N
σ–S1
S2–σ
*σ–R
σ–S2
*σ–σ
*σ–σ
S2–σ
σ–S2/S1–σ
σ–S1
B
1
2
3
4
5
6
7
8
9
10
11
12
σ–S1
S2–σ
σ–R
σ–S2

In spite of the considerable information now available on the CMS-S mitochondrial genome, no link with the CMS phenotype has been identified. The expression of the coxI gene is apparently unaffected by the close proximity of the STIR sequence (23bp from the start of transcription) and ensuing recombination (Isaac et al. 1985b). In fact the DNA (fig. 3.3) and the CMS-S specific proteins (Forde and Leaver 1980) from fertile restored CMS-S lines show no alterations from those of sterile CMS-S lines. However, it should be borne in mind that all these molecular studies are conducted with material from young etiolated seedlings. CMS-S is only expressed at a very late stage of microsporogenesis, and so it may be unreasonable to expect the molecular basis for the phenotype to be evident in the mitochondria from young shoots. A further complicating factor is the numerous and widespread nature of the differences between N and S mitochondria, which make correlations difficult. An alternative strategy is to examine revertants to fertility, which have been obtained from a number of CMS-S lines. This approach will be explored in the next chapter.

Chapter 4- Mitochondrial DNA Alterations in Cytoplasmic revertants to fertility from S-type male-sterile lines of maize

4.1 Reversion from CMS-S

Some CMS-S sources of maize spontaneously revert to fertility in the field (reviewed in Laughnan et al. 1981). These fertility changes are heritable and can be divided into two classes: those in which the genetic change is cytoplasmic (i.e. shows maternal inheritance); and those in which the genetic change is nuclear (i.e. shows Mendelian inheritance). Nuclear revertants appear, in effect, to have acquired a dominant restorer gene. Genetic analyses of several independent revertants has revealed that these acquired 'restoring elements' are at different sites on different chromosomes, leading to the suggestion that episomal transposition events may be invoved (Laughnan and Gabay 1973, 1975).

The nuclear genotype also affects the frequency with which cytoplasmic revertants arise, ranging from 0% in lines such as N6-S to 10.9% per generation for the line M825-VG (Laughnan et al. 1981). Concomitantly, there is a shift from predominantly nuclear reversion in lines not prone to frequent reversion events, to predominantly cytoplasmic reversion in lines which display very high reversion frequencies (e.g. reversion is 95% cytoplasmic in M825-VG) (Laughnan et al. 1981). Cytoplasmic reversion events are stable (there are no reports of fertile revertants regaining sterility), and in all cases examined so far, linked to alterations in the mtDNA.

4.2 Mitochondrial DNA alterations in cytoplasmic revertants from CMS-S

The first cytoplasmic revertants to be studied were in lines with the M825-VG or M825/Oh07-VG genotype. These revertants had lost free S1 and S2 plasmids (Levings et al. 1980, Laughnan et al. 1981) but showed a novel organization of S2-homologous sequences in their main genome (Levings et al. 1980, Kemble and Mans 1983). Detailed analysis of the main genome in these revertants revealed that they lacked fragments diagnostic of linear chromosome termini, but had increased levels of completely integrated S2 sequences (Schardl et al. 1985). These integrated S2 sequences, however, had undergone a deletion of their left (unique) ends including the TIR, explaining their differing mobility to the homologous fragments from sterile parent plants. It was tentatively suggested that the loss of free plasmids and the apparent recovery of a circular genome accounted for the fertility reversion (Schardl et al. 1985). The difficulties of generalising from a few examples (all of similar genotypes) became apparent when it was reported that cytoplasmic revertants with the nuclear genotype WF9 retain S plasmids at the same levels as their sterile parents (Escote et al. 1986, Ishige et al. 1985). Thus it is important to investigate further the mtDNA alterations in cytoplasmic revertants with a range of nuclear backgrounds, particularly with reference to both free and integrated S plasmid sequences. This chapter contains a comparison of the mtDNA from cytoplasmic revertants with the nuclear backgrounds W182BN, M825/Oh07, WF9 and 38-11. In the light of these observations, the molecular mechanisms underlying reversion, and the possibility of identifying the molecular basis of the CMS-S phenotype are discussed.

4.3 W182BN cytoplasmic revertants

All CMS-S revertants studied to this date have been spontaneous reversions in field-grown plants. This limits the number and variety of revertants that can be investigated, as many CMS-S lines revert at low frequency or not at all (Laughnan et al. 1981). Recently CMS-S revertants have been obtained after regeneration from callus cultures of immature embryos from sterile plants with the nuclear genotype W182BN (Earle et al. 1987). The cytoplasms of the parent plants used in these experiments included CA, S and LBN and were from three different CMS-S subgroups (table 3.1). These genotypes have never been known to revert during normal sexual propagation, and yet many regenerated plants from 16 out of 18 separate cultures were fertile or produced fertile progeny after pollination with the non-restoring line W182BN-N (Earle et al. 1987). Breeding evidence indicated that the apparent reversion events were stable and cytoplasmically inherited. By selfing the fertile plants and pollinating sterile plants with W182BN-N, cytoplasmically continuous lines were established for each regenerant. DNA analysis revealed that all the fertile regenerant lines had lost free S1 and S2 plasmids (Earle et al. 1987).

I have studied the mtDNA from some of these regenerant lines in detail (see table 4.1 for sources of seed used in these experiments). Specific probes for STIR-linked sequences (fig. 4.1) were hybridized to BamHI or SalI digests of the mtDNA from each of these regenerant lines. BamHI does not cut S1, so allowing one to distinguish between free S1, S1 at a linear chromosome terminus and S1 as part a circular mitochondrial chromosome (fig. 3.5, table 4.2). SalI does not cut S2, so allows an analogous separation of S2 recombination products (table 4.2). In addition, neither SalI or BamHI differentiate between σ' and φ' as the **R** repeat includes sites for both these enzymes. These hybridization experiments showed

Table 4.1 Origins of the W182BN seed used in these experiments

Sample	Fertility	Cytoplasm	Culture	Time in culture (months)	S1 integrates
1B	S	CA	398	3	(+)
2A	F	CA	398	3	+
2B	F	CA	398	3	+
3A	F	CA	406	15	*
5A	F	LBN	436	13	(+)
6B	F	S	1981	17	-
7B	F	S	1981	16	-
8A	S	S	460	12	(+)
9A	F	S	460	12	+
10A	F	S	468	3	+
11A	F	S	468	12	-
12A	S	S	484	3	(+)
13A	F	S	484	3	+
17A	F	S	484	4	+
18A	S	S	484	4	(+)
19A	F	CA	398	14	*
20B	F	CA	398	3	+

F male fertile

S male sterile

\+ present

(+) present but at low level

\- absent

* present but rearranged

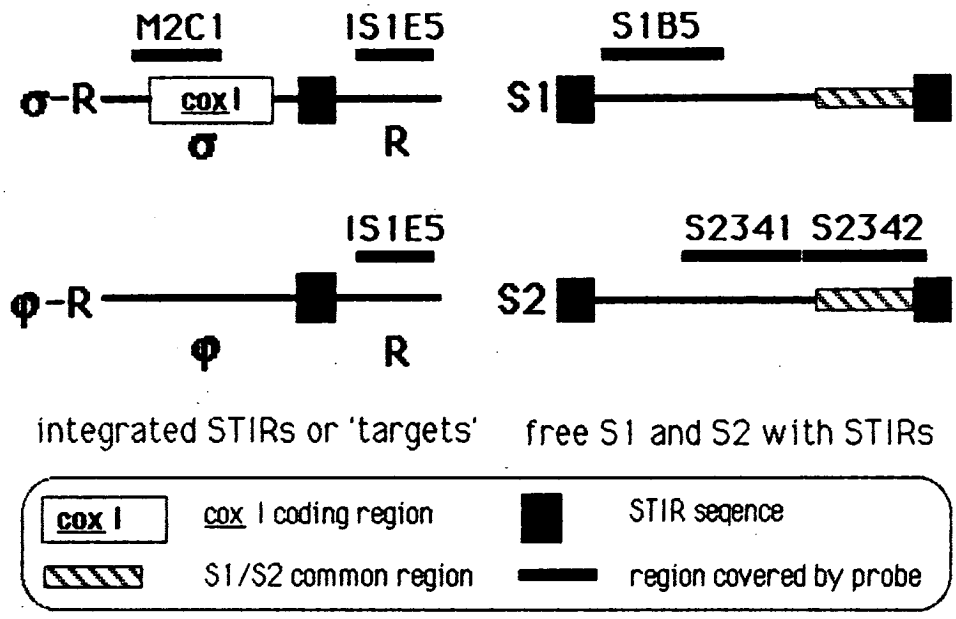


Fig. 4.1. The derivation of the hybridization probes used to investigate mtDNA from cytoplasmic revertants.

M13 clones:-

M2C1- MspI clone specific for the σ sequence, and derived from the 3' end of the coxI gene (Isaac et al. 1985)

IS1E5 - SauIIIA clone specific for the R sequence

S1B5 - HindIII clone specific to S1

pUC 8 clones:-

S2341 - PstI clone of S2 containing 1330bp common to S1 and S2

S2342 - PstI clone specific to S2

many differences between the fertile and sterile regenerants (the sterile regenerants 1B, 8A, 12A and 18A were in all cases identical to their sterile parents) (figs. 4.2, 4.3 and 4.4).

IS1E5, a probe for the **R** sequence, reveals that all the fertile regenerants lack a 1.75kb BamHI fragment characteristic of S mtDNA (fig. 4.2). This fragment is the **R** linear chromosome end generated by STIR recombination (fig. 3.2, table 4.2). In addition, the fertile regenerant 6B lacks the largest hybridizing 5.75kb BamHI fragment (fig. 4.2, lane 16), (the **φ-R** 'target' sequence), indicating that the not all the fertile regenerant plants have the same mitochondrial DNA alterations. Probing the fertile regenerant mitochondrial DNA with M2C1 (specific to σ) (fig. 4.3) confirms the presence of the **σ-R** target sequence and also confirms the lack of linear chromosome end fragments (there is no 9.4kb fragment indicating the σ-S1, S1-σ linear ends). The fertile regenerant mitochondrial DNA also lacks the 3.6kb and 6.9kb σ-S2 and S2-σ fragments. Furthermore, probing these DNAs with S2341 (specific to S2) shows that all these fertile regenerants apparently lack S2 sequences (either free or integrated) entirely (not shown). However, the most interesting finding from the M2C1 probing is the presence in some of the fertile regenerants of two σ-containing BamHI fragments of over 12kb. The size of these fragments suggested that they could include integrated complete copies of S1 (two σ-containing S1 integrates of this size are predicted by the STIR recombination model (fig. 3.5, table 4.2), but are rarely detectable in W182BN-S mitochondrial DNA by hybridization). Probing the same blot with S2342 (which includes sequence common to both S1 and S2) after removing the previous probe produces three bands in the same region (fig. 4.4). The two smaller bands are coincident with the doublet which hybridizes to M2C1. The same pattern is obtained using a

Table 4.2 Predicted hybridization of probes to STIR recombination products in mitochondrial DNA from CMS-S maize

		BamHI				
		kb	**IS1E5**	**M2C1**	**S2342**	**S1B5**
	φ-S1-φ	16.3			+	+
	σ-S1-φ, φ-S1-σ	14.4		+	+	+
	σ-S1-σ	12.5		+	+	+
*	φ-S1, S1-φ	10.4			++	++
*	σ-S1, S1-σ	9.4		++	++	++
	S2-φ	7.9			++	
	S2-σ	6.9		++	++	
	S1	6.4			++++++	++++++
	φ-R	5.75	+++		+	
	σ-R	4.75	+++	+++	+	
	φ-S2	4.7			+	
	S2-	3.9			++++++	
	σ-S2	3.6		++	+	
*	**R**	1.75	+++		+	
	-S2	0.6			+	

		SalI	
		kb	**S2341**
	φ-S2-φ	19.3	+
	φ-S2-σ, σ-S2-φ	14.2	+
*	φ-S2, S2-φ	12.4	++
	σ-S2-σ	10.3	+
*	σ-S2, S2-σ	7.9	++
	S2	5.5	++++++

No. of crosses indicates approximately the predicted hybridization signal expected.

* indicates this fragment is derived from the terminus of a linear chromosome, and is therefore diagnostic of linearisation by STIR recombination

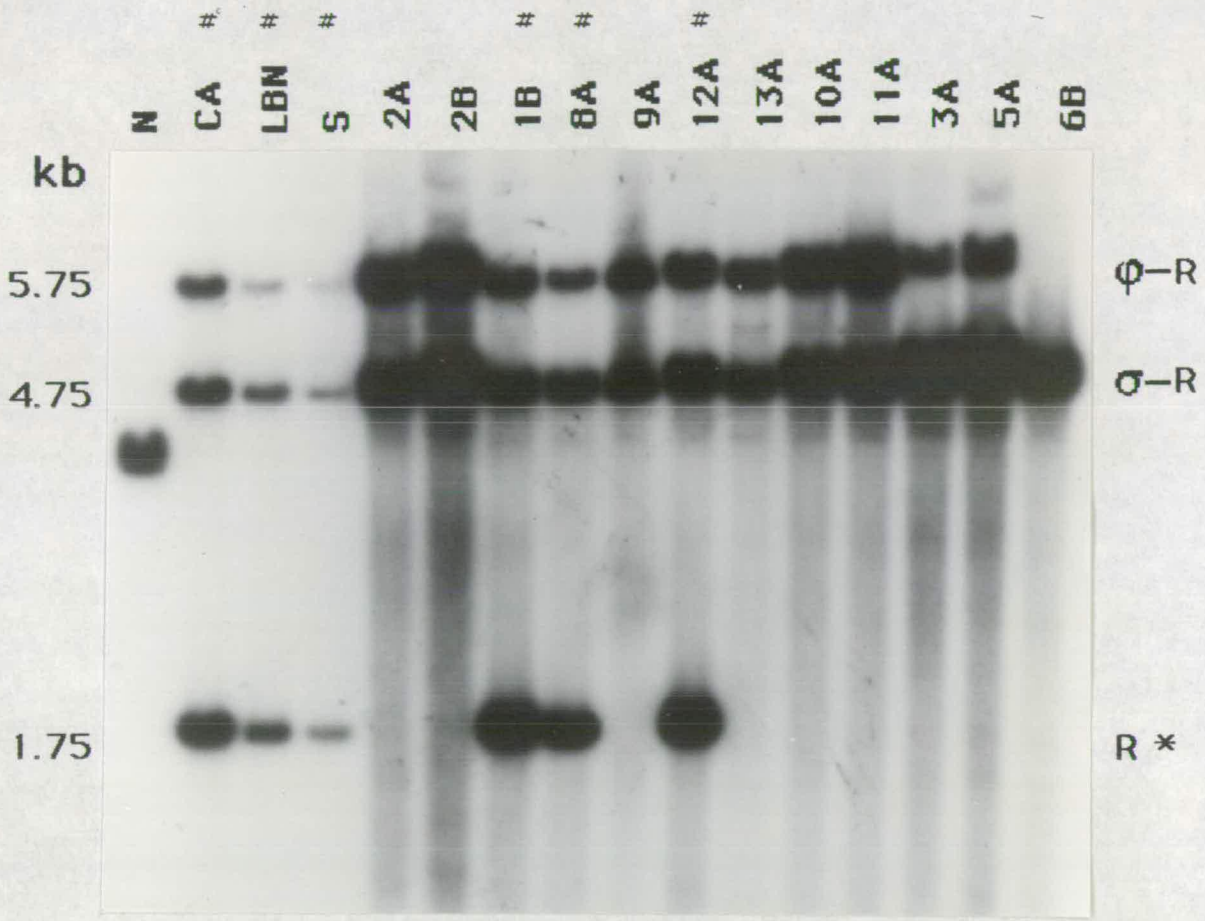
Fig. 4.2 Hybridization of IS1E5 (specific for the **R** repeat) to a Southern blot of BamHI digested mitochondrial DNA from the W182BN regenerant lines compared with fertile (N) and sterile parental (CA, LBN, S) mitochondrial DNA.

1B, 8A and 12A are male-sterile regenerant lines and retain the 1.75kb **R** linear chromosome terminus. All other regenerant lines are fertile (revertant) and lack this fragment. All lines except 6B retain the φ-**R** and σ-**R** targets characteristic of CMS-S. 6B lacks φ-**R**, and other lines, e.g. 3A and 5A, appear to have reduced amounts of this fragment.

No consistent differences are observed between fertile regenerants from CA (2A, 2B, 3A), LBN (5A) or S (6B, 9A, 10A, 11A, 13A).

Differences in hybridization signal intensity are due to inconsistencies in the amount of mitochondrial DNA loaded per lane.

* denotes fragments derived from the termini of linear chromosomes



# denotes male sterile lines

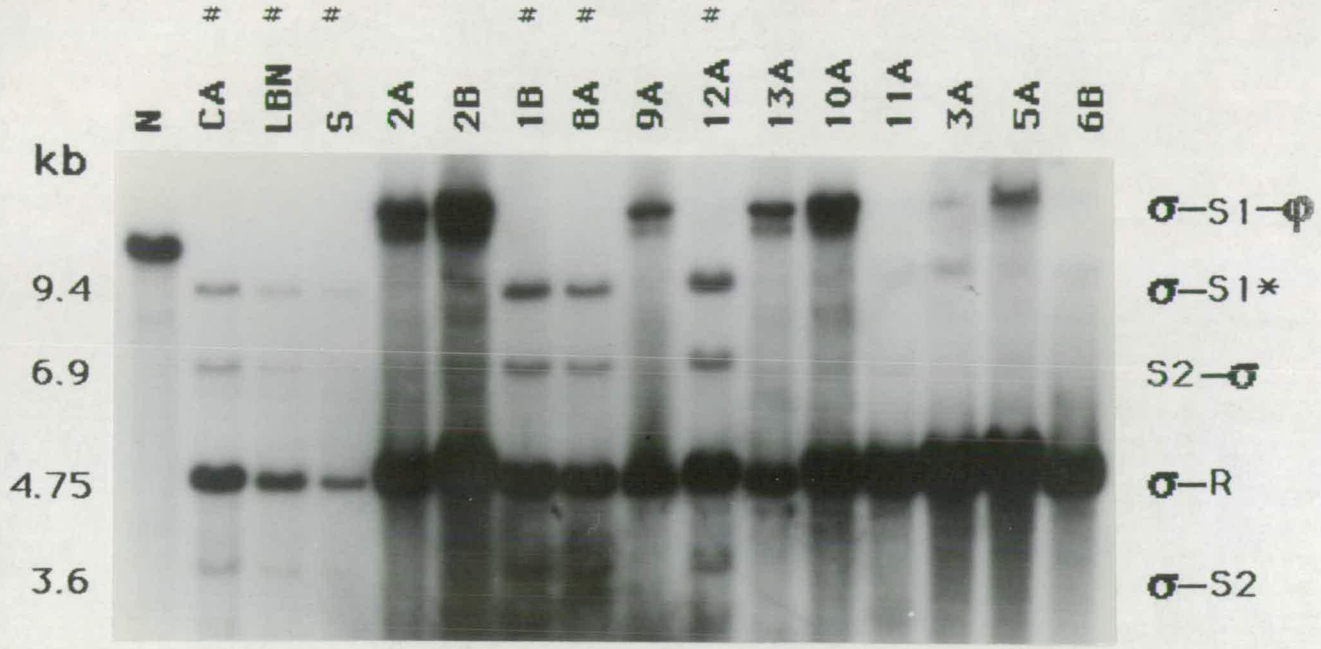
Fig. 4.3 Hybridization of M2C1 (specific for the σ sequence) to a Southern blot of BamHI digested mitochondrial DNA from the W182BN regenerant lines compared with fertile (N) and sterile parental (CA, LBN, S) mitochondrial DNA.

The sterile regenerant lines 1B, 8A and 12A show a pattern indistinguishable from that of the parental CMS-S lines (CA, LBN, S□. All the fertile regenerants retain the σ-**R** target but lose the products of S plasmid recombination seen in the sterile lines. However, many of the fertile regenerants show two hybridizing fragments in the 12-15kb region. These can be interpreted as the σ-S1-φ and σ-S1-σ double integration products (fig. 3.5), which are not visible in the sterile lines.

No consistent differences are observed between fertile regenerants from CA (2A, 2B, 3A), LBN (5A) or S (6B, 9A, 10A, 11A, 13A).

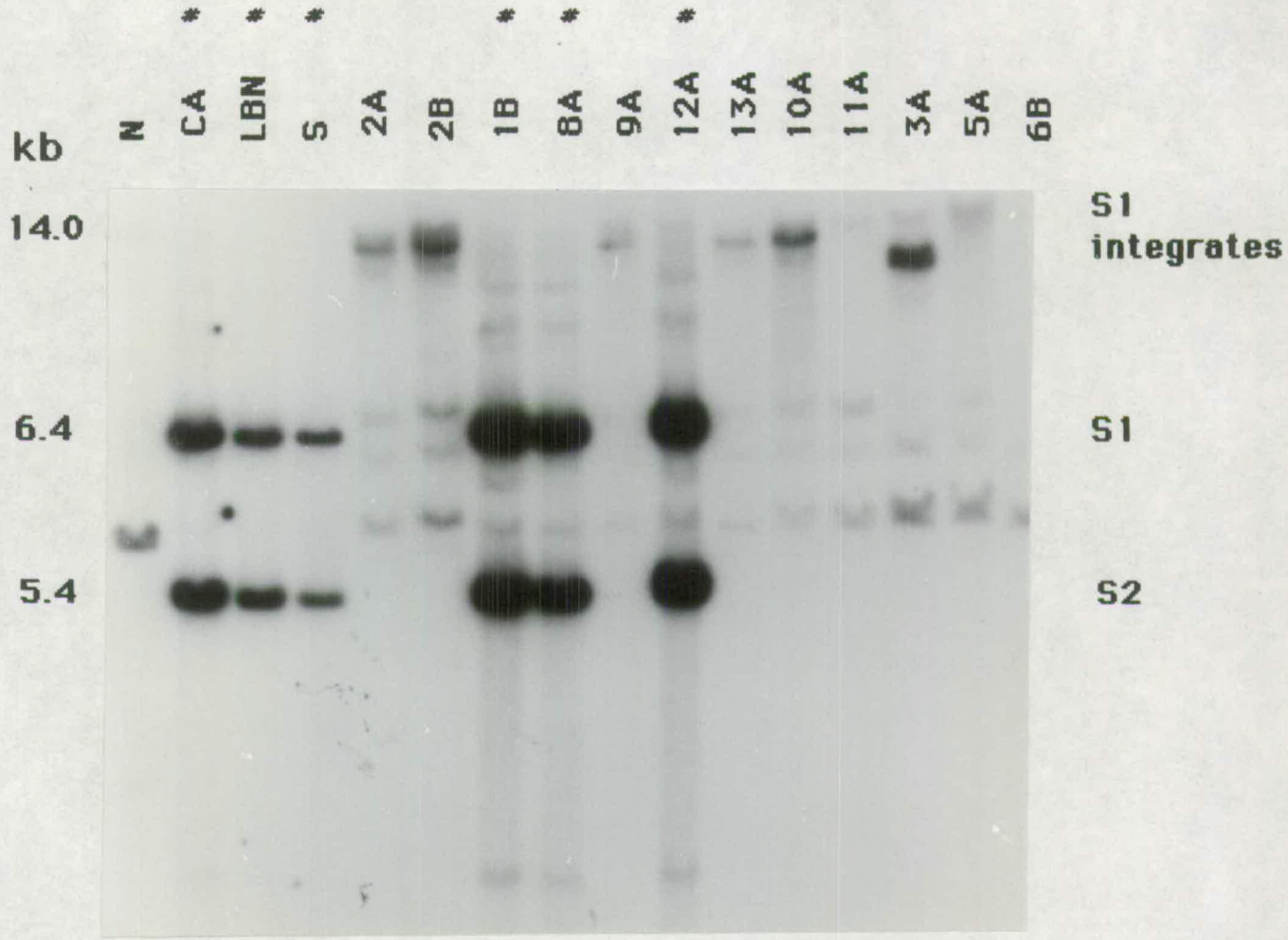
Differences in hybridization signal intensity are due to inconsistencies in the amount of mitochondrial DNA loaded per lane.

* denotes fragments derived from the termini of linear chromosomes



# denotes male sterile lines

Fig. 4.4 Hybridization of S2342 (homologous to both S1 and S2) to a Southern blot of BamHI digested mitochondrial DNA from the W182BN regenerant lines compared with fertile (N) and sterile parental (CA, LBN, S) mitochondrial DNA. The filter is the same as that used for fig. 4.3.
The sterile regenerants 1B, 8A and 12A show a pattern indistinguishable from that of the parental CMS-S lines (the faint signals appearing in the sterile regenerants are only apparently absent from the parental lines because of the lower DNA loadings in these lanes). The large (c. 14kb) fragments visible in the DNA from 2A, 2B, 9A, 13A, and 10A are coincident with those in the previous probing (fig. 4.3), confirming that they represent the φ-S1-φ, σ-S1-φ and σ-S1-σ double integration products. 3A exhibits an anomalous smaller hybridizing fragment.
The numerous faint bands hybridizing in most lanes are due to the small amount of STIR homology present in this probe.



* denotes male sterile lines

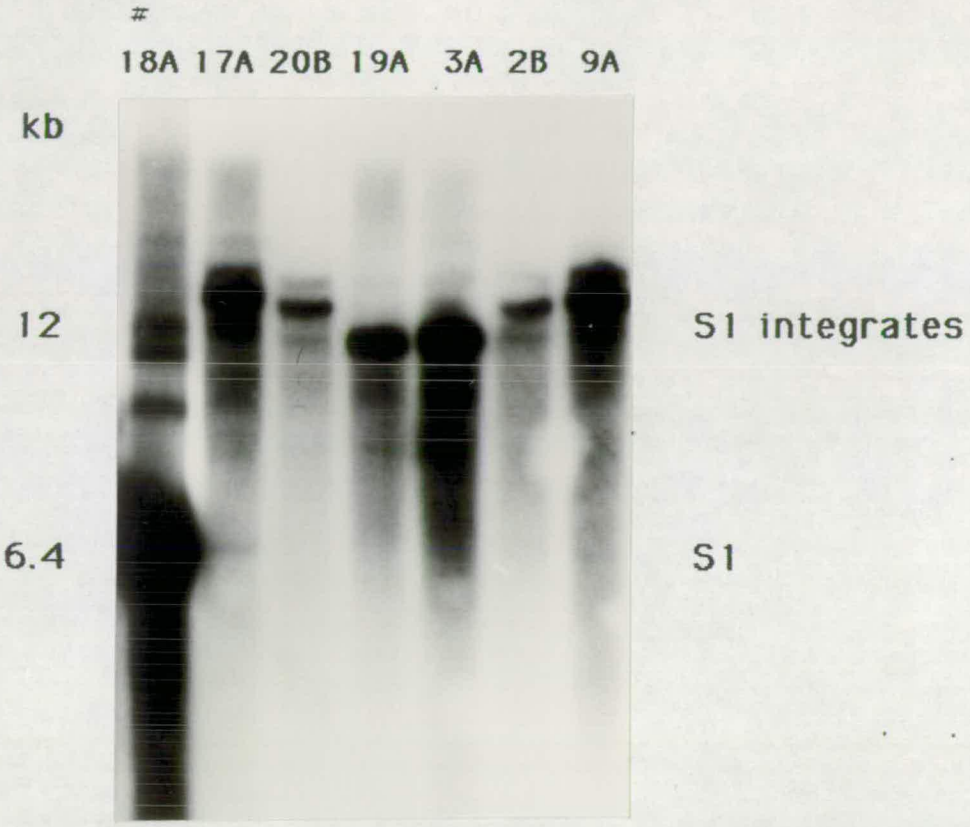
probe specific for S1 (S1B5) on mtDNA from other regenerant lines (fig. 4.5). This is as predicted if these fragments are complete S1 integrates (the three fragments being φ-S1-φ, φ-S1-σ/σ-S1-φ and σ-S1-σ). The fertile regenerants 3A and 19A show a smaller S1 hybridizing fragment (figs. 4.4, 4.5), which does not hybridize to M2C1 (fig. 4.3). It seems likely therefore that in these regenerants there has been a rearrangement in an integrated S1 sequence which may have involved the loss of attached σ sequence. This rearrangement probably included the loss of one STIR, or the original arrangement would be able to reform by recombination with the **σ-R/φ-R** targets which are still present in these revertants.

Not all the mtDNAs from the fertile regenerants exhibit the S1 integrate fragments to the same extent. The presence of these fragments in a regenerant line appears to depend on the age of the callus at the time when the original plant was regenerated from it (see table 4.1). Plants regenerated from cultures less than five months old contain high levels of S1 integrates, whilst those regenerated from cultures over a year old have apparently lost these integrates, implying that these structures are unstable or selected against in culture. The exception is the novel recombinant arrangement found in 3A and 19A which is present in plants regenerated after 14-15 months in culture, and the line 9A which shows these S1 integrates despite being regenerated from a 12 month old culture. However, this culture (460, table 4.1) seems anomalous as it was the only culture capable of producing sterile regenerants after 12 months (e.g. 8A), and thus may be considered 'slow'.

The striking observation about these independent reversion events seen in these regenerant plants is that they are so similar. No differences were seen between revertants with the cytoplasmic genotypes S, CA or LBN in this study even though the mitochondrial genome in these

Fig. 4.5 Hybridization of S1B5 (specific to S1) to a Southern blot of BamHI digested mitochondrial DNA from the W182BN regenerant lines compared with fertile (N) and sterile parental (CA, LBN, S) mitochondrial DNA.

The three high molecular weight fragments detectable with the S1/S2 probe S2342 (fig. 4.4) hybridize equally well to this S1-specific probe, as does the anomalous smaller fragment of the lines 19A and 3A. Interesting comparisons to note are that 18A is a sterile regenerant line obtained from the same culture at the same time as the fertile regenerant line 17A. 19A was derived from the same culture as 20B, though it was regenerated nearly a year later.



# denotes male sterile line

cytoplasms can be differentiated on the basis of restriction endonuclease patterns, and the cytoplasms are phenotypically distinguishable in the presence of some restorer genes (table 3.1, Sisco et al. 1985). To achieve my objective of a thorough study of mtDNA rearrangements during CMS-S reversion, I compared the mtDNA from these fertile regenerant lines with that from examples of revertants with the nuclear backgrounds M825/Oh07, WF9 and 38-11. These revertants were all examples of spontaneous reversion in field grown plants.

4.4 Cytoplasmic revertants from other sources

Figure 4.6 shows mtDNA from revertants with the nuclear genotypes WF9, M825/Oh07, 38-11 and W182BN probed with the S plasmid probe S2342. The hybridization pattern of each of the revertant mtDNAs is easily distinguishable, which when contrasted with the basic similarity between all the independent W182BN revertants discussed earlier, implies a nuclear background effect on the mtDNA alterations occurring during reversion. The most strikingly different revertant pattern is that of the WF9 revertant, which has retained S1 and S2 in high copy number, as previously reported (Escote et al. 1986, Ishige et al. 1985), and in fact, with this general S plasmid probe, is difficult to distinguish from its sterile parent.

To gain useful information on which mtDNA alterations are affected by nuclear background, more specific hybridization probes were employed. Figure 4.7 shows mtDNA from the same revertants again cut with BamHI, but on this occasion probed with the R-specific probe IS1E5. As expected, the M825 and W182BN revertants lack the 1.75kb BamHI **R** linear end fragment, as does the 38-11 revertant. This would be expected if the mtDNA organisation has reverted to a circular form during fertility

Fig. 4.6 Hybridization of S2342 to a Southern blot of BamHI digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mitochondrial DNA.

The revertants shown are :

(i) WF9-S 85:6838

(ii) M825/Oh07-VG 801-8

(iii) 38-11-S 81-115-2

(iv) W182BN-S fertile regenerant 10A

This general S plasmid probe highlights the free S1 and S2 present in the WF9 revertant mtDNA but lacking from the other revertant mtDNAs. The other three revertants are easily distinguishable by their hybridization patterns, implying different rearrangements in their integrated plasmid sequences. As three out of four of the revertants had the same original cytoplasm, it is likely that the differences are due to nuclear gene effects.

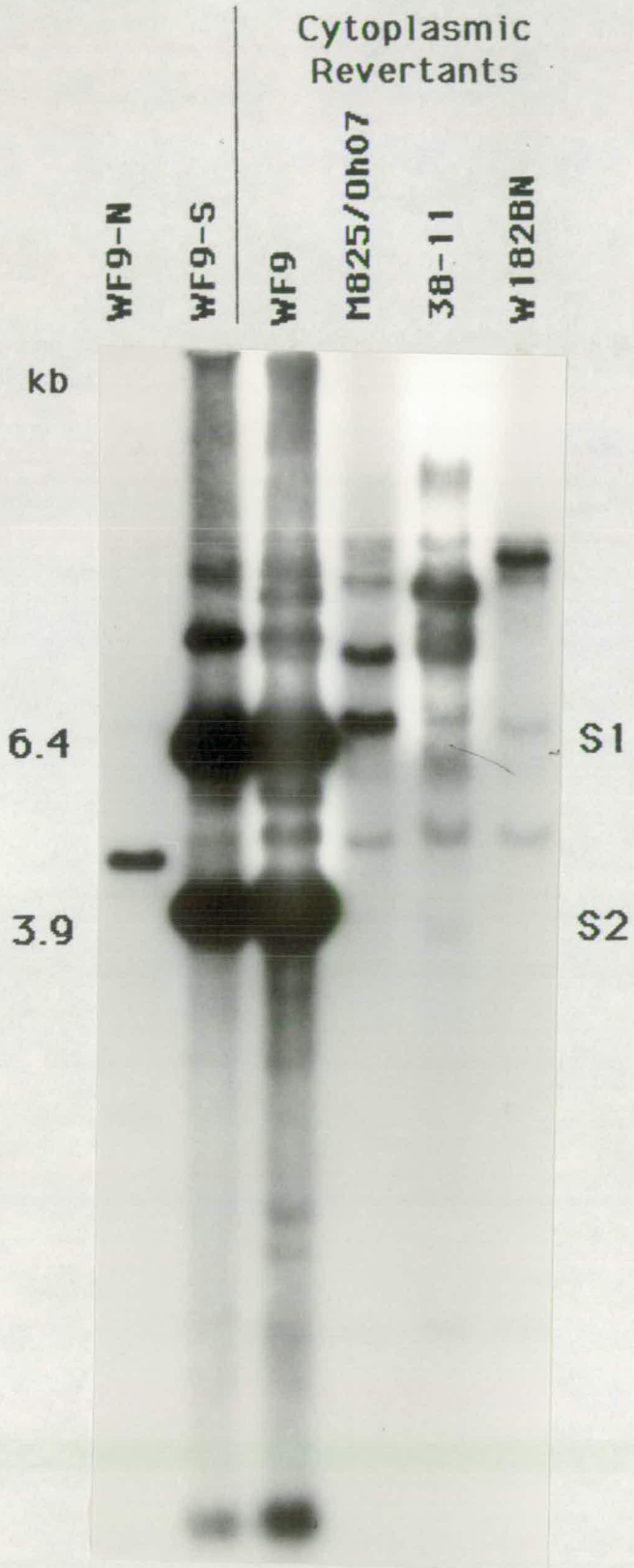

Cytoplasmic Revertants
WF9-N
WF9-S
WF9
M825/Oh07
38-11
W182BN
kb
6.4
3.9
S1
S2

Fig. 4.7 Hybridization of IS1E5 to a Southern blot of BamHI digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mitochondrial DNA.

The revertants shown are :

(i) WF9-S 85:6838

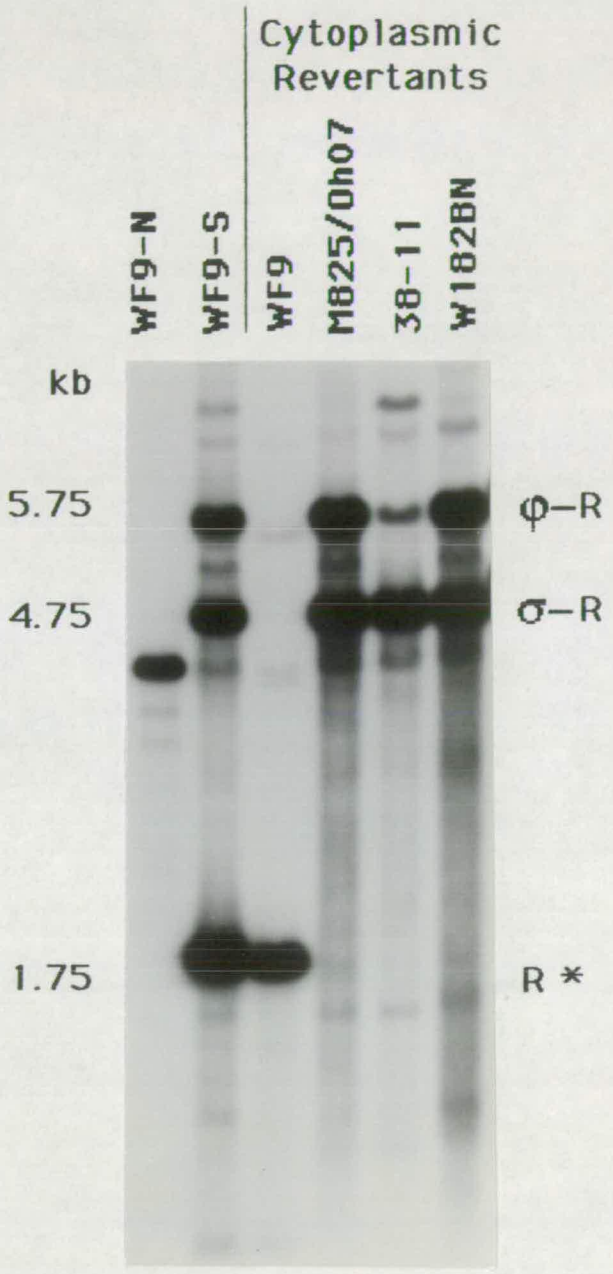
(ii) M825/Oh07-VG 801-8

(iii) 38-11-S 81-115-2

(iv) W182BN-S fertile regenerant 10A

All the revertants lack at least one of the fragments typical of the sterile mitochondrial DNA. The WF9 revertant lacks both target fragments; the other three revertants lack the **R** chromosome terminus.

* denotes fragments derived from the termini of linear chromosomes


Cytoplasmic Revertants
WF9-N
WF9-S
WF9
M825/Oh07
38-11
W182BN
kb
5.75
4.75
1.75
φ-R
σ-R
R *

reversion. In complete contrast, the WF9 revertant mtDNA does contain this fragment. Therefore, WF9 revertant mtDNA, in addition to retaining free plasmids (Fig. 4.6), also retains the linear chromosomes characteristic of the CMS-S parent. However, the IS1E5 probe does unambiguously separate the WF9 revertant from its sterile parent (and also from the other cytoplasmic revertants) by highlighting the conspicuous lack of STIR target fragments (**σ-R** and **φ-R**) in this revertant. Similarly, differences between the WF9 revertant and the others emerges after probing SalI digested mtDNA from the same revertants with the S2-specific probe S2341 (fig. 4.8). The WF9 revertant closely resembles the typical S pattern, including the 7.9kb σ-S2 linear end and the 12.4kb φ-S2 linear end. Of the other mitochondrial DNAs, the W182BN revertant lacks any detectable homology to this probe, the 38-11 revertant shows only very faint hybridization (at positions not corresponding to any predictable recombination products), and the M825 revertant shows a single prominent band, also with a mobility different from that of any of the bands in the standard S track- this is the rearranged integrated form of S2 previously reported (Schardl et al. 1985).

To show further that independent reversion events within any one nuclear background are similar, I compared several revertants from within each of the backgrounds WF9 and M825/Oh07 (fig. 4.9). These mtDNAs (probed with the σ-specific probe M2C1) confirm earlier observations. All the WF9 revertants retain linear chromosomes (e.g. the 9.4kb σ-S1/S1-σ fragment) but lose STIR targets (the 4.75kb **σ-R** fragment), whilst the M825/Oh07 revertants lose fragments characteristic of linear chromosomes.

Fig. 4.8 Hybridization of S2341 to SalI digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mitochondrial DNA.

The revertants shown are :

(i) WF9-S 85:6838

(ii) M825/0h07-VG 801-8

(iii) 38-11-S 81-115-2

(iv) W182BN-S fertile regenerant 10A

The WF9 revertant closely resembles the standard WF9-S parent, with both fragments characteristic of linearised chromosomes detectable, as well as free S2. The 38-11 and W182BN revertants in contrast lack any significant homology to the probe at all. The M825/0h07 revertant shows one prominent band with an altered mobility to that of the typical S pattern; this is the partially deleted rearrangement described by Schardl et al. (1985).

* denotes fragments derived from the termini of linear chromosomes

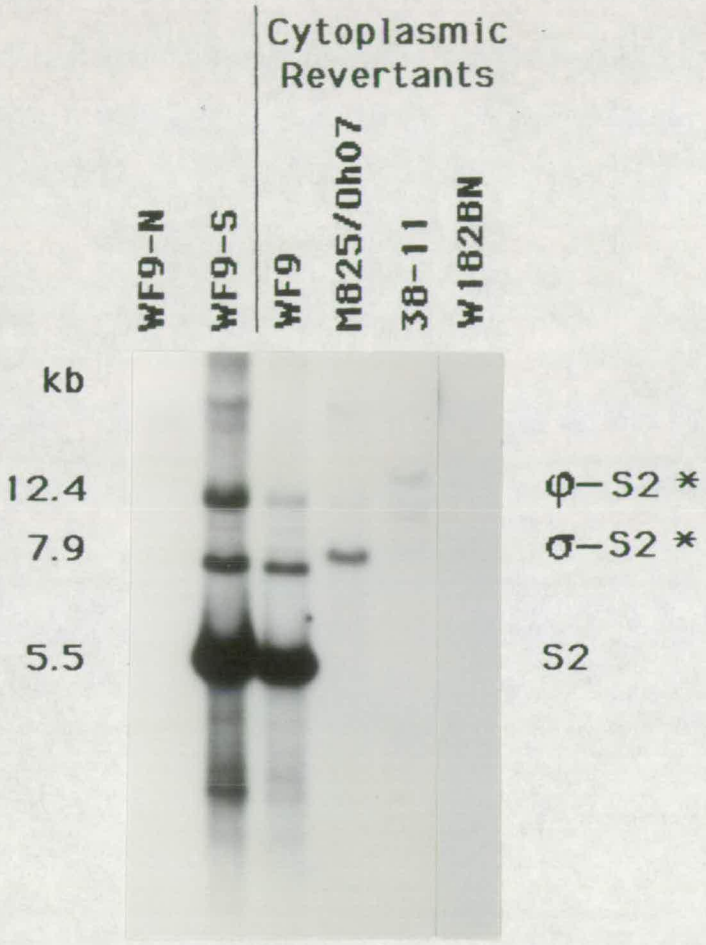

Cytoplasmic Revertants
WF9-N
WF9-S
WF9
M825/Oh07
38-11
W182BN
kb
12.4
7.9
5.5
φ-S2 *
σ-S2 *
S2

Fig. 4.9 Hybridization of M2C1 to a Southern blot of BamHI cut mitochondrial DNA from WF9 and M825/Oh07 revertants and corresponding sterile parental lines.

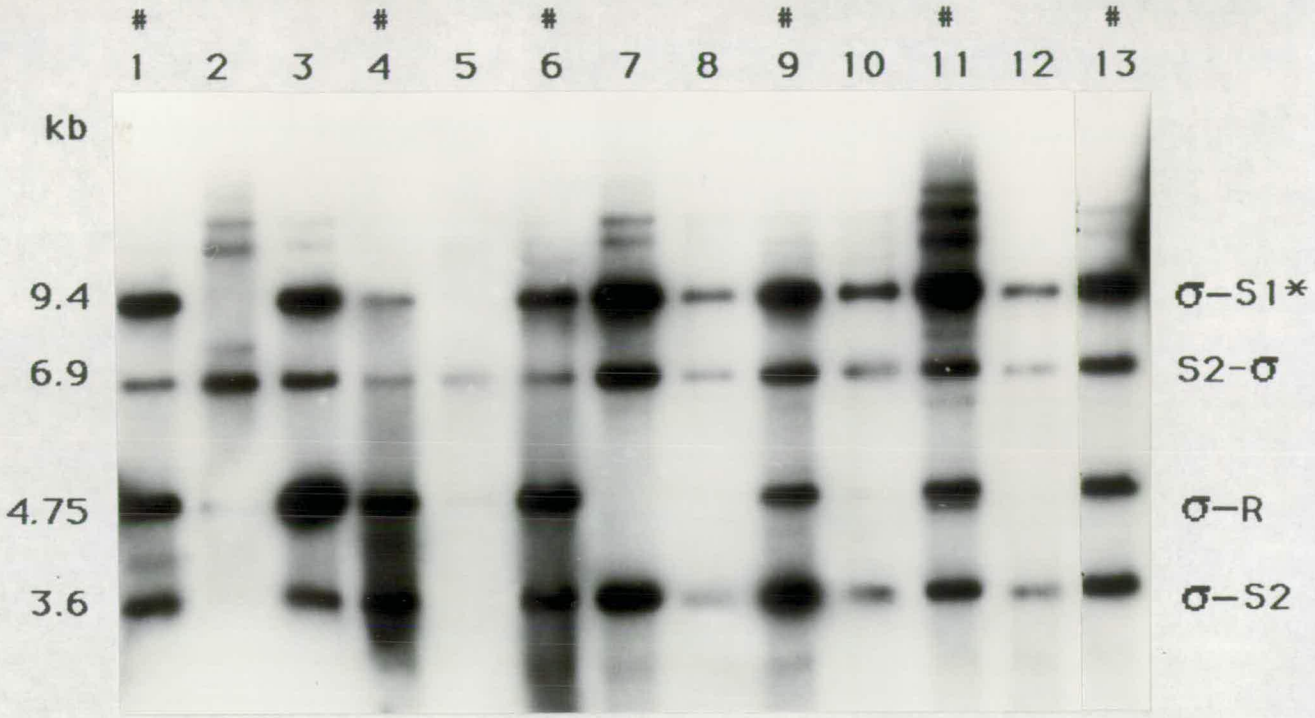
1	M825/Oh07-VG	7	CR from #9
2	CR from #1	8	CR from #9
3	NR from #4	9	WF9-RD
4	M825/Oh07-VG	10	CR from #11
5	CR from #6	11	WF9-RD
6	M825/Oh07-VG	12	CR from #13
		13	WF9-ML

CR= cytoplasmic revertant; NR= nuclear revertant

All four of the cytoplasmic revertants from WF9 parents, with either RD or ML cytoplasm show similar patterns. The only change from the parental pattern is the loss of the 4.75kb **σ-R** target fragment. The 9.4kb σ-S1/S1-σ linear chromosome end is retained.

The M825/Oh07 cytoplasmic revertants have lost this 9.4kb fragment, and also the 3.6kb S2 containing fragment; this fragment corresponds to the end of S2 deleted in these revertants (Schardl et al. 1985). The **σ-R** fragment is apparently reduced in quantity. This is probably an artefact of loading inequalities, and if compensated for, the 6.9kb S2-σ fragment would be considerably more abundant in the revertant as opposed to the parental mtDNAs.

* denotes fragments derived from the termini of linear chromosomes



# denotes male sterile lines

4.5 Discussion

In summary, it can be seen that all the revertants studied, whether derived from field-grown plants or plants regenerated from culture, lack some STIR-linked fragments found in their sterile progenitors (table 4.3). In some cases the loss of certain fragments is linked with the appearance of new fragments, presumably generated by recombinational rearrangements. Beyond these general similarities, the revertants from each source differ (table 4.3). The mtDNA alterations that I have described for the fertile W182BN regenerant lines are superficially similar to those previously reported for M825 and M825/Oh07 revertants, involving a loss of free plasmids coupled with an increase of integrated plasmid sequence relative to the rest of the genome (Levings et al. 1980, Kemble and Mans 1983, Schardl et al. 1985). However, in the case of W182BN revertants the integrated plasmid is S1, whereas the previously examined M825 revertant mtDNAs had accumulated integrated S2. Furthermore, this integrated S2 sequence contained a deletion of its left TIR. This may be analogous to the deleted version of S1 present in the W182BN revertants 3A and 19A, which appears to be stable unlike the apparently intact S1 integrates of 'young' cultures, which are lost with increasing age in culture. The 38-11 revertant I have included in my comparison most closely resembles the W182BN pattern, having lost S2 homologous sequence, but retaining integrated S1 sequences. These integrated S1 sequences are more abundant in the revertant than in the sterile parent (a common feature for integrated plasmid sequences in all the cytoplasmic revertants) and have undergone a deletion of one end (Escote-Carlson, Laughnan and Gabay-Laughnan, personal communication). This again is analogous to the case of integrated plasmid sequences in M825 revertants and to the W182BN regenerant lines 3A and 19A. WF9 revertants, which retain the free episomes of their parents but lack the

Table 4.3. Comparison of cytoplasmic revertants from different CMS-S sources

		Nuclear genotype of revertants to fertility			
Features	CMS-S	WF9	M825/Oh07	38/11	W182BN
free S1, S2	+	+	-	-	-
σ - σ' target	+	-	+	+	+
φ - σ' target	+	-	+	+	+
S1 linear ends	+	+	-	-	-
S2 linear ends	+	+	-	-	-
R linear ends	+	+	-	-	-
integrated intact S1	(+)	(+)	(+)	(+)	+
integrated rearranged S1	-	-	-	+	-
integrated rearranged S2	-	-	+	-	-

\+ present
\- absent
(+) present at low levels

σ-R and φ-R target sequences, can be considered to be in a class of their own.

These results strongly suggest a link between the type of molecular events during reversion and the nuclear genotype. This link may be connected with other observed effects of the nuclear genotype on reversion (see **4.1**), but the pattern of differences between the revertants suggests instead a connection with nuclear effects on mitochondrial genome organisation. The genotypes M825 and 38-11 influence the ratio of free S1 to S2 (Laughnan and Gabay-Laughnan 1983; see **3.5**) and their integration products, and this may be reflected in which integrated sequences are predominant in the revertants; sterile 38-11 plants contain more free and integrated S2 than S1, whereas the revertant retains integrated (but rearranged) S1 sequences but loses integrated S2 sequences. In general, for M825 plants the situation is reversed. The most suggestive link, however, is the the fact that the mitochondrial genome in WF9 plants, though normal with respect to the free episomes, exhibits unusual stoichiometries of the STIR recombination products; specifically a larger than normal proportion of linear products (fig. 3.8; Leaver et al. 1985). Circumstantially this seems likely to have a bearing on the anomalous molecular events involved with reversion in plants with this genotype. Thus whatever the mechanism of reversion, it must involve one or more nuclear gene products, which are likely to be involved in replication and/or recombination of S episomal sequences.

Reversion appears to occur after loss or rearrangement of STIR-linked sequences in the mitochondrial genome. In the one study that has examined the recombination points of these rearrangements, the extent of the homology between recombining sequences is small (Schardl et al. 1985), implying that such recombinations would be rare, and would require selection and/or amplification to become fixed in the genome.

Such fixation of rare recombinant products has been shown in the maize genome (see chapters 5 and 6; Small et al. 1987), but what is remarkable during reversion is the number of such apparently 'rare' alterations which occur within a single plant generation (or within 3 months of cell culture) and the fact that many occur in a similar or identical fashion in a number of independent plants.

The lesion responsible for pollen infertility in CMS-S maize is as yet unidentified. Cytoplamic revertants seem an ideal system for studying the molecular events underlying the CMS phenotype, as they provide a number of independent comparisons to be made to their sterile progenitors. In addition, the mtDNA from all known revertants resembles that of their sterile progenitors much more closely than that from fertile plants with N cytoplasm. Revertants from cultures with the CMS-T cytoplasm proved invaluable in identifying the apparent underlying cause of T-type male sterility. In these revertants a 6.6kb Xhol fragment present in the sterile progenitor plants has been replaced by a 6.3kb Xhol fragment (Umbeck and Gengenbach 1983). This rearrangement is due to a recombination event between two 55bp direct repeats (Rottman et al. 1987) which has led to the deletion of the urf13-T gene encoding the 13kd polypeptide deeply implicated as a cause of sterility in T-type mitochondria (see **1.3.1**). Unfortunately, the situation in CMS-S mitochondria appears to be more complicated. Numerous mtDNA alterations occur on reversion to fertility and no fragment has been identified which is lacking from all the revertants. Furthermore, although CMS-S mitochondria do synthesize variant polypeptides encoded by the S1 and S2 (Manson et al. 1986, Zabala and Walbot, 1987) these are present in mitochondria both from fertile WF9 cytoplasmic revertants and from fertile RU cytoplasms (Liddell and Leaver unpublished). Revertants that have not retained S plasmids lose the CMS-S specific variant polypeptides

(Lidell and Leaver, unpublished). In conclusion, it can be said that reversion to fertility in CMS-S revertants occurs by the same general mechanism as in CMS-T revertants (i.e. fixation of recombinant molecules which have portions of the progenitor genome rearranged or missing) but the particular alteration responsible for the restoration to fertility in these plants remains unresolved.

Chapter 5- Stoichiometry differences in atpA gene types in maize cytoplasms

5.1 Introduction

As has been shown in the previous two chapters, the number and complexity of rearrangements in the CMS-S cytoplasm makes anything but descriptive study difficult at this stage. Therefore, in order to broaden the study to incude cytoplasms other than CMS-S, whilst at the same time narrowing the field of the investigation to allow more focused experiments, I decided to concentrate on a particular region of the maize genome. I chose the region around the atpA gene, which had been partially characterized and showed a surprising amount of variation between cytoplasms.

5.2 The maize mitochondrial atpA gene

The maize atpA gene, encoding the α-subunit of the F_1 ATP synthase complex, has been cloned and sequenced (Braun and Levings 1985, Isaac et al. 1985a). In the mitochondrial genome of fertile N maize the gene lies entirely within a 12kb repeat (repeat-6, fig. 1.1), with the 3' terminus of the gene positioned 650 bases from one end of the repeat (Isaac et al. 1985a). Consequently, two copies of the atpA gene can be represented on the 570kb maize master circle with different 3' flanking sequences (Dawson et al. 1986; fig. 1.2). In contrast, the C and T cytoplasms of maize have been reported to contain only a single copy of the atpA gene (Braun and Levings 1985, Isaac et al. 1985a). The atpA arrangement in C mtDNA appears identical to one of those in N mtDNA, whereas in T mtDNA the 3' flanking sequences of the gene are different from those of either of the two copies of the gene in N mtDNA (Isaac et al. 1985a). CMS-S mtDNA has

been reported to contain either one copy of the gene (Isaac et al. 1985a) or two (Braun and Levings 1985). Thus the region of the maize mitochondrial genome around the atpA gene(s) appears to vary considerably between cytoplasms, both in copy number and in sequence organization. This variation seemed worthy of further study, as the 12kb repeats have been reported to be involved in inter- and intra-molecular recombination (Lonsdale et al. 1984) and thus would be expected to be important in determining the structure of the mitochondrial genome.

5.3 Distribution of atpA types in maize cytoplasms

MtDNA from a number of maize lines was screened with atpA-specific probes to examine the variability of the organization of the 12kb repeat region. ALXR18, which covers the 5' end of the atpA gene, hybridized to a 3.5kb BamHI fragment in every mtDNA studied (fig. 5.1A), whereas the probe BLSC1, covering the 3' end of the gene and the immediately adjacent flanking sequence, distinguished a total of four different atpA arrangements specific to different cytoplasms. These atpA arrangements were designated as atpA types 1-4 (fig. 5.1B). atpA types 1 and 2 are characteristic of N mtDNA, type 3 is specific to CMS-S mtDNA and type 4 specific to CMS-T mtDNA. CMS-C mtDNA contains only the type 1 arrangement of N mtDNA.

Using the probe BLSC1, I examined the distribution of these four atpA types in 44 different nuclear-cytoplasmic combinations (data summarised in table 5.1). The nuclear genotype appeared to have no influence over the atpA types present in the associated cytoplasm. However, the pattern of atpA types present did correlate strongly with the cytoplasmic designation. In fact, the probe BLSC1 proved a sensitive indicator of cytoplasmic origin, distinguishing three separate subgroups of fertile N-like cytoplasms, and also distinguishing between the S and CA

Fig. 5.1. Location of the gene for the α-subunit of F_1 ATPase (atpA) in the mitochondrial DNA of N, C, S and T cytoplasms of maize. hybridization of **A**) ALXR18 and **B**) BLSC1 atpA probes to BamH1 digested maize mitochondrial DNA from N, C, S and T cytoplasms.


region covered by probes


approximate extent of homology to Type 1 clone

B BamH1 restriction site


atpA coding region

The probe BLSC1 distinguishes the 4 major atpA gene arrangements found in maize mitochondria. The same DNA probed with ALXR18 shows that all four arrangements are identical at the 5' end . I have designated the four atpA arrangements as types 1-4 as shown.

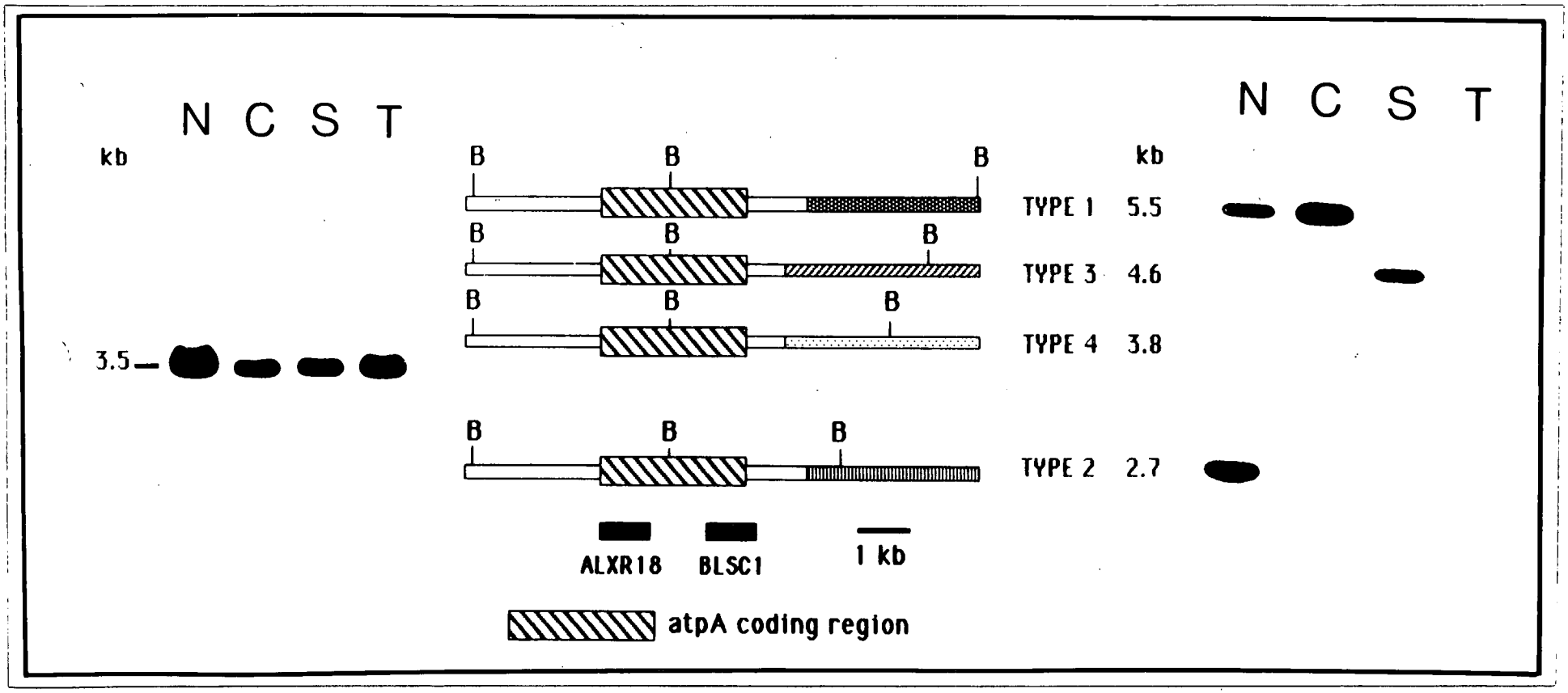

N C S T
kb
3.5
B
B
B
kb
TYPE 1 5.5
TYPE 3 4.6
TYPE 4 3.8
TYPE 2 2.7
N C S T
ALXR18
BLSC1
1 kb
atpA coding region

Table 5.1. Predominant atpA types in different maize cytoplasms

atpA TYPES	MAIZE LINES (written as nuclear genotype-cytoplasmic genotype)
1 and 2 :	B37-N, B73-N, WF9-N, M825-N, 38/11-N, C0192 x WJ-N, C0192 x WJ-234, C0192 x WJ-LF, R181-N (N cytoplasms)
2 only :	ECU321[a], ECU398[a], CUN443[a], C0192 x WJ-SG, C0192 x WJ-OY (N-like cytoplasms)
1 only :	B37-C, WF9-C, C0192 x WJ-F (C cytoplasms)
3 only :	B37-S, 38/11-S, M825-S, WF9-S, WM13-S, C0192 x WJ-J (S cytoplasms)
2 and 3 :	WF9-ML, WF9-RD, M825-Vg, C0192 x WJ-MY, C0192 x WJ-H, C0192 x WJ-PS, C0192 x WJ-G, C0192 x WJ-I, C0192 x WJ-B, C0192 x WJ-SD, C0192 x WJ-L, C0192 x WJ-D, C0192 x WJ-CA, WI82BN-CA (S-like cytoplasms)
4 only :	PUN6[a], WI82BN-181, C0192 x WJ-181 (N-like cytoplasms); WI82BN-T, B37-T, WF9-T, C0192 x WJ-Q (T cytoplasms)

[a] ECU321, ECU398, CUN443 and PUN6 are male-fertile South American accessions with restriction enzyme cleavage profiles similar to commercially used N cytoplasms. CUN443 and ECU398 contain R plasmids (Weissinger et al., 1982).

subgroups of CMS-S (tables 3.1, 5.1). A number of fertile N-like cytoplasms, including those from the primitive South American accessions ECU321, ECU398 and CUN443 lack the type 1 arrangement present in typical N cytoplasm. This difference in copy number amongst fertile cytoplasms suggests that there is no functional requirement for two atpA genes.

A particularly intriguing finding is that the fertile N-like cytoplasms 181 and that from the Peruvian accession PUN6 contain the same predominant atpA type as that found in T cytoplasm. This implies that these N-like cytoplams may be (or be related to) the progenitors of T cytoplasm, although it should be stressed that the restriction endonuclease patterns of these two fertile lines closely resemble that of N mtDNA, and differ noticeably from that of CMS-T mtDNA (not shown).

As shown for the N group of maize cytoplasms, the CMS-S group can also be divided into cytoplasms with one or two copies of the atpA gene. The CA subgroup (table 3.1), in addition to the CMS-S specific type 3 arrangement, also contain the type 2 arrangment of N mtDNA. The S subgroup contain only the type 3 arrangement. Interestingly, J cytoplasm, placed in the CA subgroup by Sisco et al. (1985) (with reservations), lacks the type 2 arrangement, and thus by this criterion resembles the S subgroup. Given the differences in fertility restoration phenotype and restriction endonuclease patterns between J and other members of the S subgroup, it should probably now be considered as a subgroup by itself.

5.4 Sub-stoichiometric levels of atpA gene types

Most, but not all, N and S cytoplasms were found to contain two predominant atpA types (table 5.1). In those N and S cytoplasms containing only one predominant atpA type, the other expected type could always be

detected at sub-stoichiometric levels provided that blotting and hybridization conditions were optimal (see **2.2.3**). For example, in fig. 5.2 the N-like cytoplasms OY and SG show low levels of the type 1 atpA arrangement typical of true N cytoplasm, and the S-like cytoplasm J contains low levels of the type 2 arrangement present in most CMS-S cytoplasms. In addition, I could detect anomalous atpA arrangements in N mtDNA, not accounted for by the published map of the N genome, and not explainable as recombination products of any of the known repeats in the genome. Some of these atpA arrangements appeared to be identical to those characteristic of CMS lines. For example, in fig. 5.2 mtDNAs from the fertile N-like cytoplasms SG, OY, 234 and N all show low levels of the type 3 arrangement characteristic of CMS-S mtDNA, and 234 mtDNA also shows sub-stoichiometric levels of the type 4 atpA arrangement characteristic of CMS-T mtDNA. A summary of all the results I obtained is shown in table 5.2.

It seemed unlikely that differences in hybridization intensity of the same fragment in different cytoplasms was due to differences in homology to the probe, because in many cases the cytoplasms were closely related, and higher plant mtDNAs show low rates of sequence drift (see **1.4**). If the low hybridization signals were due to low probe homology, one would not expect restriction sites to be preserved, and yet mtDNA cut with a number of different restriction enzymes gave identical results (fig. 5.3).

Trivial explanations of the data, in particular cross-contamination between samples, were carefully considered, and largely ruled out by performing mitochondrial purification of samples from different cytoplasmic groups on different days in rigorously cleaned equipment. DNA contamination from bacteria, fungi and plastids within the seedling samples is unlikely to explain the results as it would require the

Fig. 5.2. Sub-stoichiometric restriction fragments containing the atpA gene in maize mitochondrial DNA.

A. One day exposures of autoradiographs of BamH1 digested maize mtDNA (nuclear genotype CO192 x WJ, 5µg/track) probed with BLSC1. **Lane 1**, 181 cytoplasm; **Lane 2**, SG cytoplasm; **Lane 3**, OY cytoplasm; **Lane 4**, 234 cytoplasm; **Lane 5**, N cytoplasm; **Lane 6**, H cytoplasm; **Lane 7**, PS cytoplasm; **Lane 8**, J cytoplasm. Lanes 1-5 are fertile 'N-like' cytoplasms, 6-8 are CMS-S cytoplasms. Faint bands of hybridization can be seen in all tracks, some of which correspond in size to predominant bands found in other cytoplasmic types. All the cytoplasms shown, except 181, contain atpA types 1, 2 and 3 in detectable amounts. The cytoplasm 234 also contains type 4 in low levels. Special care was taken to avoid cross-contamination of separate samples; mitochondria and mtDNA from fertile and CMS-S lines were prepared on different days, and the N and S samples were run as separate blocks on gels, divided by empty tracks to eliminate carry-over of samples during loading. Similar results were obtained with a wide range of other nuclear/cytoplasmic combinations (table 5.2).

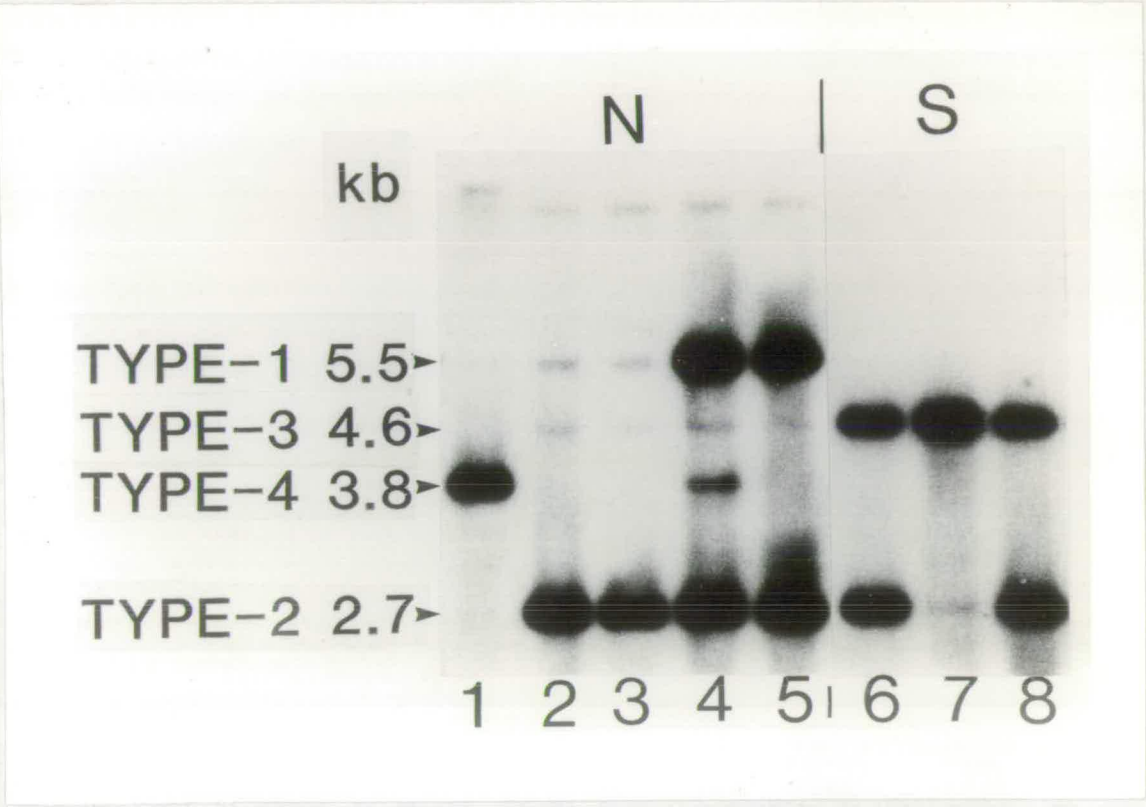

N
S
kb
TYPE-1 5.5
TYPE-3 4.6
TYPE-4 3.8
TYPE-2 2.7
1 2 3 4 5 6 7 8

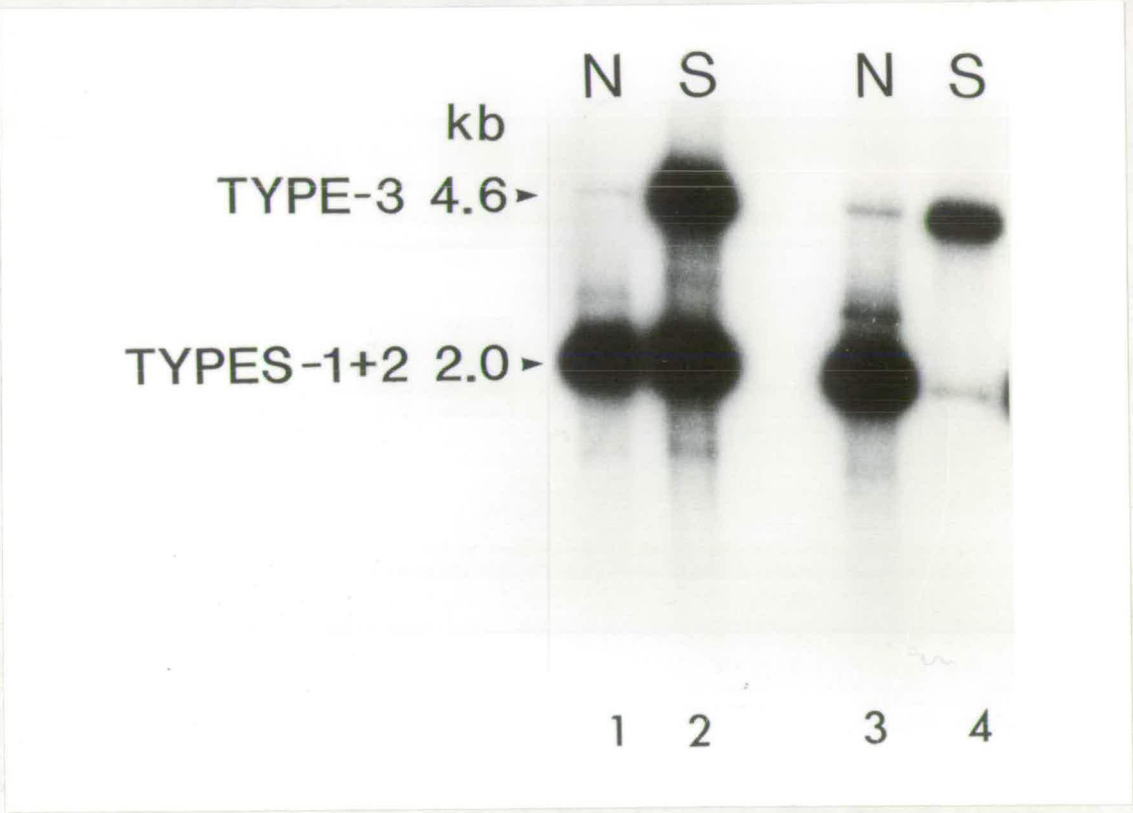
Table 5.2. Stoichiometries of atpA types in the mitochondrial genomes of N-like and S-like maize cytoplasms

	atpA types detected			
N-like cytoplasms	1	2	3	4
B73-N	+++++	+++++	+	
WF9-N	+++++	+++++	+	
38/11-N	+++++	+++++	+	
C0192 x WJ-N	+++++	+++++	+	
C0192 x WJ-234	+++++	+++++	+	+
C0192 x WJ-OY	+	+++++	+	
C0192 x WJ-SG	+	+++++	+	
ECU321[a]	+	+++++	+	
ECU398[a]	+	+++++	+	
CUN443[a]	+	+++++	+	
PUN6[a]	+			+++++
W182BN-181	+		+	+++++
S-like cytoplasms	1	2	3	4
B37-S		+	+++++	+
B73-S		+	+++++	+
38/11-S		+	+++++	+
WF9-S		+	+++++	+
M825/0h07-Vg	+	+++++	+++++	+
W182BN-CA		+++++	+++++	
C0192 x WJ-MY	+	+++++	+++++	
C0192 x WJ-H	+	+++++	+++++	
C0192 x WJ-PS	+	+++++	+++++	
C0192 x WJ-G	+	+++++	+++++	
C0192 x WJ-I	+	+++++	+++++	

Number of crosses(+) represents approximate relative abundance. Designations are written as nuclear genotype-cytoplasmic genotype (where both are known).

[a] ECU321, ECU398, CUN443 and PUN6 are male-fertile South American accessions with restriction enzyme cleavage profiles similar to commercially used N cytoplasms. CUN443 and ECU398 contain R plasmids (Weissinger et al., 1982).

Fig. 5.3. Two day exposure of an autoradiograph of maize mtDNA digested with EcoRI and probed with BLSC1. **Lane 1**, M825/0h07-N; **Lane 2**, M825/0h07-VG; **Lane 3**, WF9-N; **Lane 4**, WF9-S. atpA types 1 and 2 are inseparable in an EcoRI digest. The two N mtDNAS show small amounts of type 3, the two S mtDNAs show either a high level of type 1 + type 2 (VG cytoplasm, **lane 2**) or a low level (S cytoplasm, **lane 4**).


N S N S
kb
TYPE-3 4.6►
TYPES-1+2 2.0►
1 2 3 4

contaminating organism or organelle to contain sequences identical to those of maize mitochondria. The atpA genes of bacteria, fungal mitochondria and chloroplasts differ considerably from those of maize mitochondria, and would be extremely unlikely to contain the same pattern of restriction endonuclease sites. Although transfer of DNA sequences has occurred from chloroplasts to mitochondria during the evolution of higher plants (see **1.2.3**), there is no evidence for transfer in the other direction (Schuster and Brennicke 1987a). Finally, under the purification conditions I use for maize mitochondria, maize chloroplast DNA is largely lost or degraded.

A conceivable explanation for these observations was that the seed stocks I had used for these experiments contained heterogenous mixtures of cytoplasms, and the apparently sub-stoichiometric fragments were due to a small subgroup of seeds containing the anomalous atpA types at normal abundant levels. I investigated this possibility by extracting mtDNA from immature cobs of individual plants and probing this mtDNA with BLSC1 (fig. 5.4 and data not shown). These experiments revealed no significant differences between the atpA hybridization patterns of mtDNA from single plants and those from seedling coleoptiles. Therefore I conclude that mitochondria from many maize cytoplasms contain (in addition to their predominant atpA type(s)) one or more atpA-containing fragments at markedly sub-stoichiometric levels which are identical to predominant atpA types present in other cytoplasms. These results have been published (Small et al. 1987).

5.5 Discussion

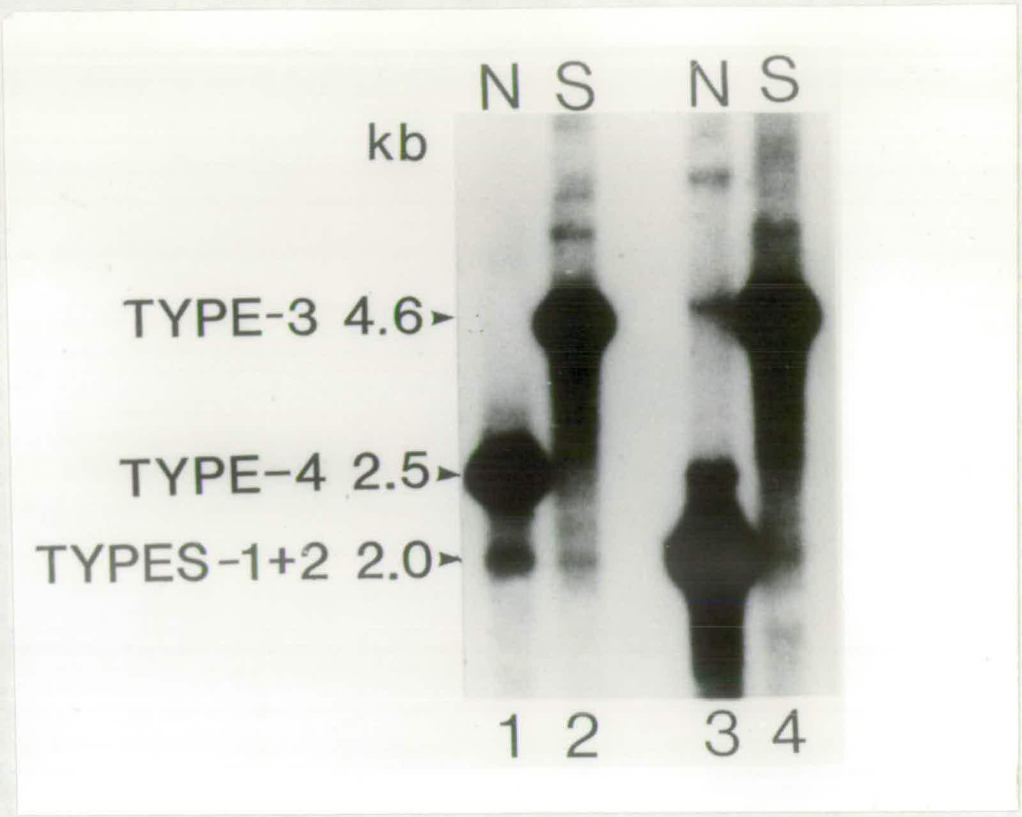
My observations show that the atpA gene (and therefore at least part of the 12kb repeat) is located in at least four genomic environments (referred to as atpA types) in different maize cytoplasms. The copy

Fig. 5.4. Sub-stoichiometric atpA hybridizing fragments exist in mitochondrial DNA from single plants.

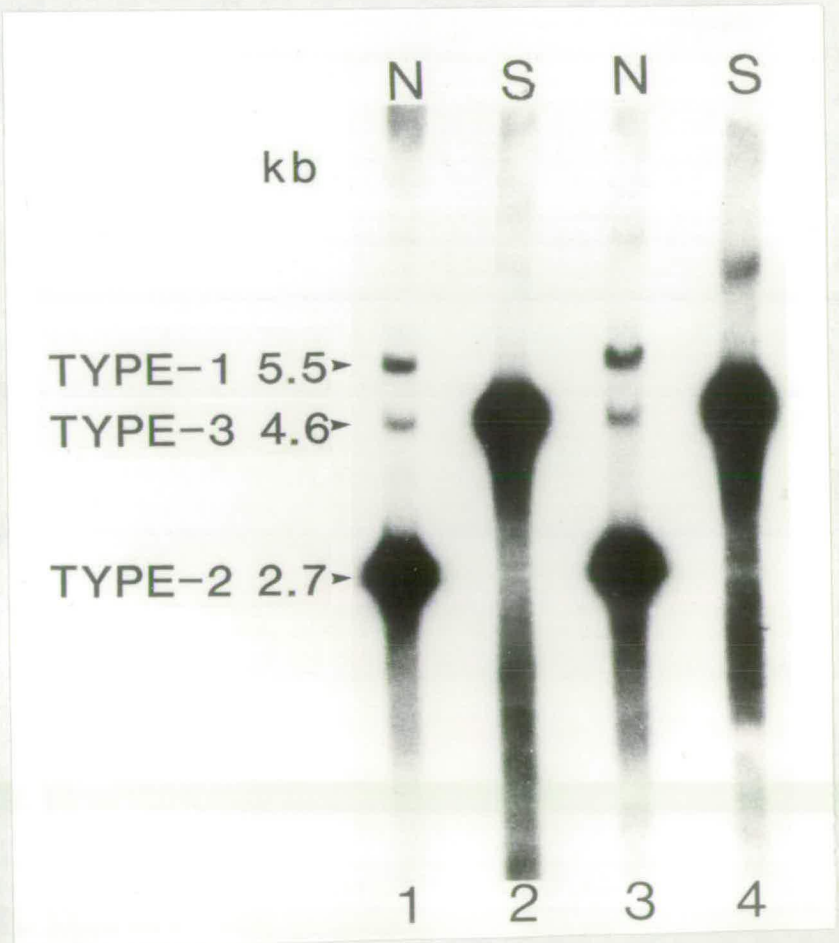
A Three-day exposure of EcoRI digested maize mtDNA probed with BLSC1. **Lane 1**, W182BN-181; **lane 2**, W182BN-S; **lane 3**, WF9-N; **lane 4**, WF9-S. The mtDNAs used in this experiment were purified from single immature cobs. A faint band representing type 1 and/or 2 can be seen in **lane 1** (181 cytoplasm), and also but less clearly in **lanes 2** and **4** (S cytoplasm). **Lane 3** (N cytoplasm) contains faint bands representing the type 3 and type 4 arrangements. Similar results were obtained after cleaving the mtDNA from other individuals of the same lines with BamHI (data not shown). Large fragments hybridizing in **lanes 2, 3** and **4** probably represent partial digestion products.

B Three-day exposure of BamHI digested maize mtDNA probed with BLSC1. **Lanes 1** and **3**, CO192 X WJ-SG; **lanes 2** and **4**, CO192 X WJ-J. Each lane contains the mtDNA of a single immature cob. The SG mtDNA shows low levels of types 1 and 3 clearly, as predicted by the same experiment on pooled coleoptile mtDNA (fig. 5.2, lane 2). The J mtDNA shows no clear faint bands, but the expected low levels of type 2 (fig. 5.2, lane 7) may be obscured by the smear of degradation products from the abundant type 3 fragment.

A


N S N S
kb
TYPE-3 4.6
TYPE-4 2.5
TYPES-1+2 2.0
1 2 3 4

B


N S N S
kb
TYPE-1 5.5
TYPE-3 4.6
TYPE-2 2.7
1 2 3 4

number of the gene appears to vary between one and two copies per master circle even in closely related cytoplasms. This loss and creation of repeated sequence may be an important part of mitochondrial genome evolution. Clark-Walker et al. (1985) have shown that novel mitochondrial genomes can be generated in yeast by crossing two different petite strains, each containing a non-functional mitochondrial genome encoding only a portion of the genetic information of the wild-type genome. Provided that the sum total of the two sub-genomes includes all the wild-type genome, and that there is some overlap between them, recombination between the two progenitor genomes can give rise to a single circular genome containing a direct repeat of the sequence common to both of the original sub-genomes. This recombinant genome is unstable (and requires continual selection pressure to maintain it) because of high frequency reversion to the original two-circle configuration. This situation, although contrived in yeast, is directly analogous to the multipartite genomes found in plant mitochondria. The unstable yeast configuration can resolve into a stable form by deletion of one of the repeats, preventing reversion to a two-circle form. Such events occur at low frequency by recombination between short regions of homology scattered throughout the yeast genome, and result in a stably maintained novel genome organization. It is intriguing that such creation and deletion of repeated sequences during genome evoloution is now being shown to be a feature of plant mitochondrial DNAs (Small et al. 1987, Pring et al. 1987, 1988).

A particularly interesting feature of the observations described in this chapter is the detection of sub-stoichiometric fragments, (I shall refer to these sub-stoichiometric fragments as 'sublimons') present in most maize mtDNAs, which correspond in size to abundant fragments in other cytoplasms. It seems tempting to suggest that the abundant fragments

arose by fixation and amplification of their corresponding sublimons. Based on this assumption, the observed pattern of atpA stoichiometry differences can be used to develop an evolutionary tree of maize cytoplams (fig. 5.5), which agrees well with those postulated on the basis of other evidence (Lonsdale et al. 1987), but due to the hypervariability of the atpA region, distinguishes several more possible intermediate cytoplasms than have hitherto been suggested.

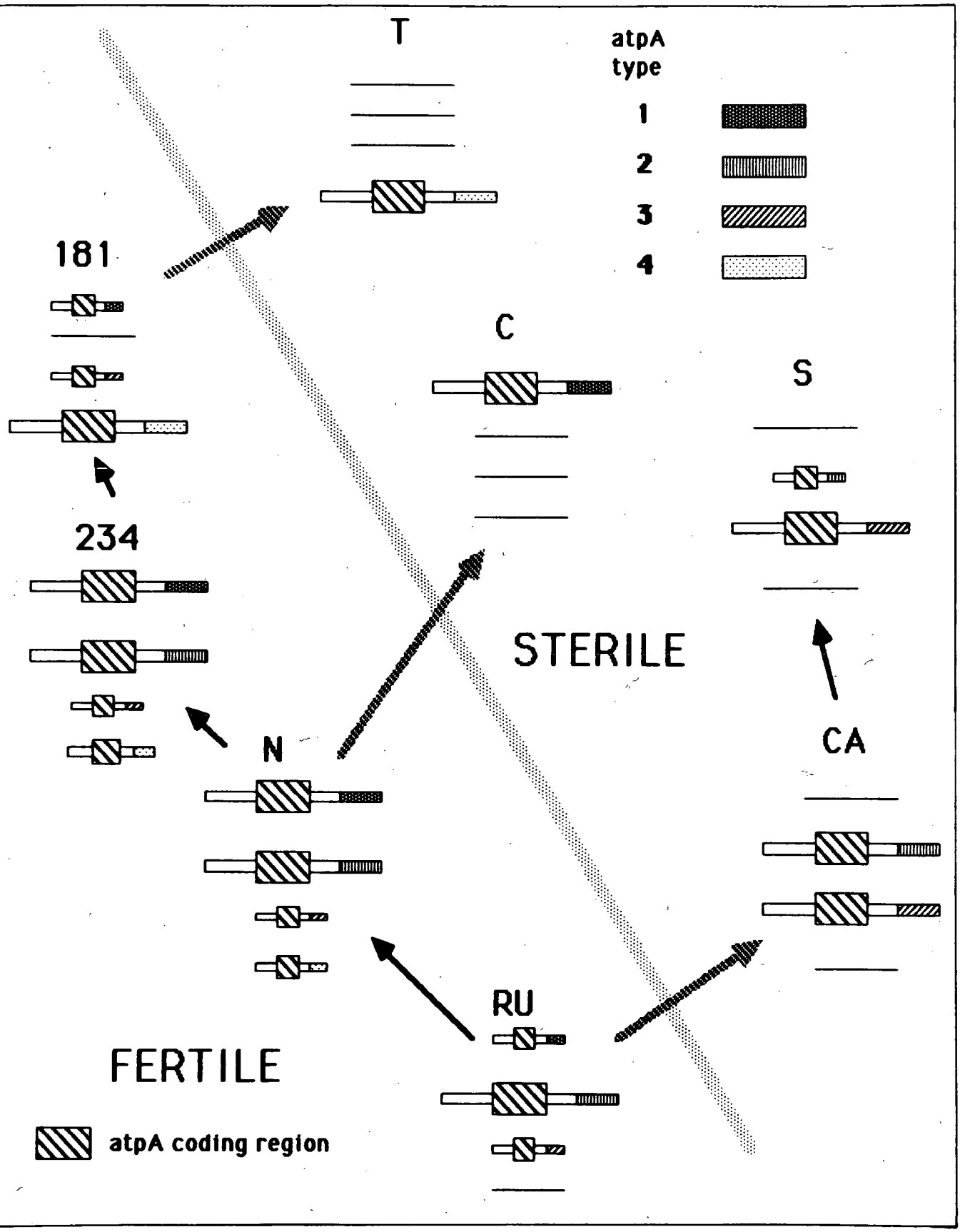
It seemed to be worth considering at this point whether these results could be used to postulate general mechanisms of genome evolution in higher plant mitochondria. It is possible that the sub-stoichiometric atpA types I have described here are examples of a whole class of rare recombinant molecules present in plant mitochondria. Rice chloroplasts have recently been shown to contain a minor rearranged genome at low levels in addition to their major genome (Moon et al. 1987). Poorly hybridizing mtDNA fragments have been observed on many occasions by other workers with other probes in many species, but are often attributed (on little or no evidence) to small repeats containing partial probe homology. Sublimons, given certain circumstances, could provide a reservoir of sequence rearrangements which on amplification could give rise to novel genome organizations. This process has been observed in the mitochondrial genome of cultured soybean and Brassica cell lines (Morgens et al. 1984, Morgan and Maliga 1987).

An important problem to be considered is the formation of these sublimons. By analogy with the STIR recombination system discussed in the previous chapters, the most likely suggestion appears to be that sublimons are created by infrequent recombination across small repeated sequences of 200bp or less, similar to the process responsible for petite formation in yeasts. An equally important and related problem is the retention of sublimons in the genome in the face of repeated segregation

Fig. 5.5 Possible interrelationships of maize cytoplasms based on atpA-type stoichiometries.

The relative stoichiometries of different atpA types can be used to predict possible evolutionary interrelationships between the mitochondrial genomes of different cytoplasms. This evolutionary tree uses RU cytoplasm as the progenitor cytoplasm, a choice which is generally accepted (Lonsdale 1987).

The size of the boxes represents approximate relative abundance of the atpA types in the various genomes (data from table 5.2). The large arrows indicate the gross differences in mitochondrial genome organization, as revealed by restriction endonuclease patterns, separating fertile from sterile cytoplasms.


T
atpA type
1
2
3
4
181
C
S
234
STERILE
N
CA
RU
FERTILE
atpA coding region

during mitosis. In relation to this problem, it is worth noting that mtDNA from both seedling coleoptiles and immature cobs showed sublimon sequences, indicating a certain degree of stability during plant development. To investigate these matters, rather than describing and proving the existence of a range of other sublimon sequences, I concentrated on investigating in more detail the formation of the atpA sublimons of maize mtDNA.

Chapter 6- Formation of sub-stoichiometric recombinant atpA arrangements in maize mitochondria

6.1 The hypotheses to be tested

On considering the observations and discussion in the previous chapter, it seemed possible that sub-stoichiometric components in the genome (sublimons) may play a part in genome evolution. The extent to which this may be true depends on the mechanism of formation and maintenance of these molecules. I have considered two (not necessarily exclusive) possibilities:-

(i) Infrequent recombination between short repeats (not large enough to be detected during the construction of the genome map) generates novel rare recombinant arrangements. These sublimons are segregated out during seed formation by stochastic processes thus preventing their fixation in the genome. Rarely, freak segregation results in the passage of a recombinant arrangement into the next plant generation in sufficient quantity to become fixed, creating a novel repeat. Duplication of obligate sequences (e.g. the atpA gene) creates redundancy, allowing the later loss of one repeat without adverse selection pressure. The main tenet of this hypothesis is that sublimons are reformed de novo by recombination between abundant sequences during every generation.

(ii) A second possibility is that the sublimons are formed by very rare or unique aberrant recombination events, and are maintained by replication rather than recombination. This hypothesis requires that sublimons are not lost by segregation during zygote formation.

6.2 Comparison of mitochondrial genome organizations in OY, N and C cytoplasms

To test these two possibilities, I first chose to attempt to discover the events leading to the formation of the type 1 atpA sublimon I had detected in those N-like cytoplasms which possess only the type 2 atpA arrangement at abundant levels (see table 5.2). Such cytoplasms include OY, SG and those of the South American accessions ECU321, ECU398 and CUN443. The reasons for this choice were twofold; firstly, as Zea mays originated in South America, the atpA organization typified by the South American accessions is probably ancestral to that of N. Therefore it seemed likely that the standard N arrangement rose by fixation of the type 1 atpA sublimon detectable in these South American lines, and comparison of the two might reveal how this occurred. The evolutionary link between other cytoplasms and the sublimons within them are not as clearly evident. Secondly, as it would be necessary to compare the sequence organization around the atpA gene in the chosen cytoplasms with that of standard N mtDNA, the experiments would be facilitated by choosing a cytoplasm that was closely related to standard N, thus limiting the number of superfluous arrangements not connected with the rearrangement under study. This would only be possible by selecting a cytoplasm from within the same cytoplasmic group as N, and not for example a male-sterile cytoplasm.

For these reasons I chose to compare the regions surrounding the atpA genes in mtDNA from the cytoplasms LF and OY. LF has typical N mtDNA containing both type 1 and type 2 atpA arrangements in equal amounts, but OY mtDNA lacks the type 1 atpA arrangement (table 4.1). Apart from this difference the two have virtually identical restriction endonuclease profiles, and are easily distinguished from F (a member of the CMS-C group) mtDNA (fig. 6.1A), which lacks the type 2 atpA arrangement. Dr. D.

M. Lonsdale (PBI, Cambridge) kindly supplied two cosmid clones (G4 and G5) spanning the two 12kb repeats from WF9-N (figs. 1.2, 6.1D). The cosmid clone G5 covers the type 1 atpA region lacking from OY; G4 covers the type 2 atpA repeat lacking from F mtDNA. Using G5 and G4 as probes on XhoI cut mtDNA from LF, OY and F shows that F mtDNA contains numerous rearrangements in these regions of the genome (fig. 6.1 B, C). Fragments C (the type 2 atpA containing fragment), E and F of G4 are missing from F mtDNA, and as the intervening fragments D and A are intact, a simple deletion cannot explain the data. The large A fragments of both G4 and G5 are present, so although C mtDNA has been shown to contain only a single atpA gene, the rest of the 12kb repeat is still present as a large duplication. In addition, two prominent new fragments hybridize, presumably containing the points of rearrangement.

OY mtDNA lacks three of the fragments present in these cosmid clones (fig. 6.1B, C). The missing 3.65 kb XhoI fragment (G5 fragment F) contains the type 1 atpA arrangement and thus was expected to be absent (fig. 6.1B). OY mtDNA also lacks the A and E XhoI fragments of G4 (figs. 6.1C, 6.2), which are the 5' end and 5' flanking sequences of the type 2 repeat as represented on the N mtDNA master circle. Therefore the region including the 12kb repeat homology in OY mtDNA is unlike either of those represented on the WF9-N master circle. Instead it is one of the recombinant forms, flanked by 5' type 1 sequence and 3' type 2 sequence.

The same fragments are missing from mtDNA of Latin American accessions (with RU-type cytoplasms) lacking the type 1 repeat (fig. 6.2 and data not shown). Thus it seemed likely that the N mtDNA arrangement is derived from that found in the OY and RU cytoplasms. If this is the case, the OY and RU cytoplasms must contain fragments with homology to the missing fragments, such that the N-like organization can be created by recombination between these progenitor fragments. Unfortunately, no

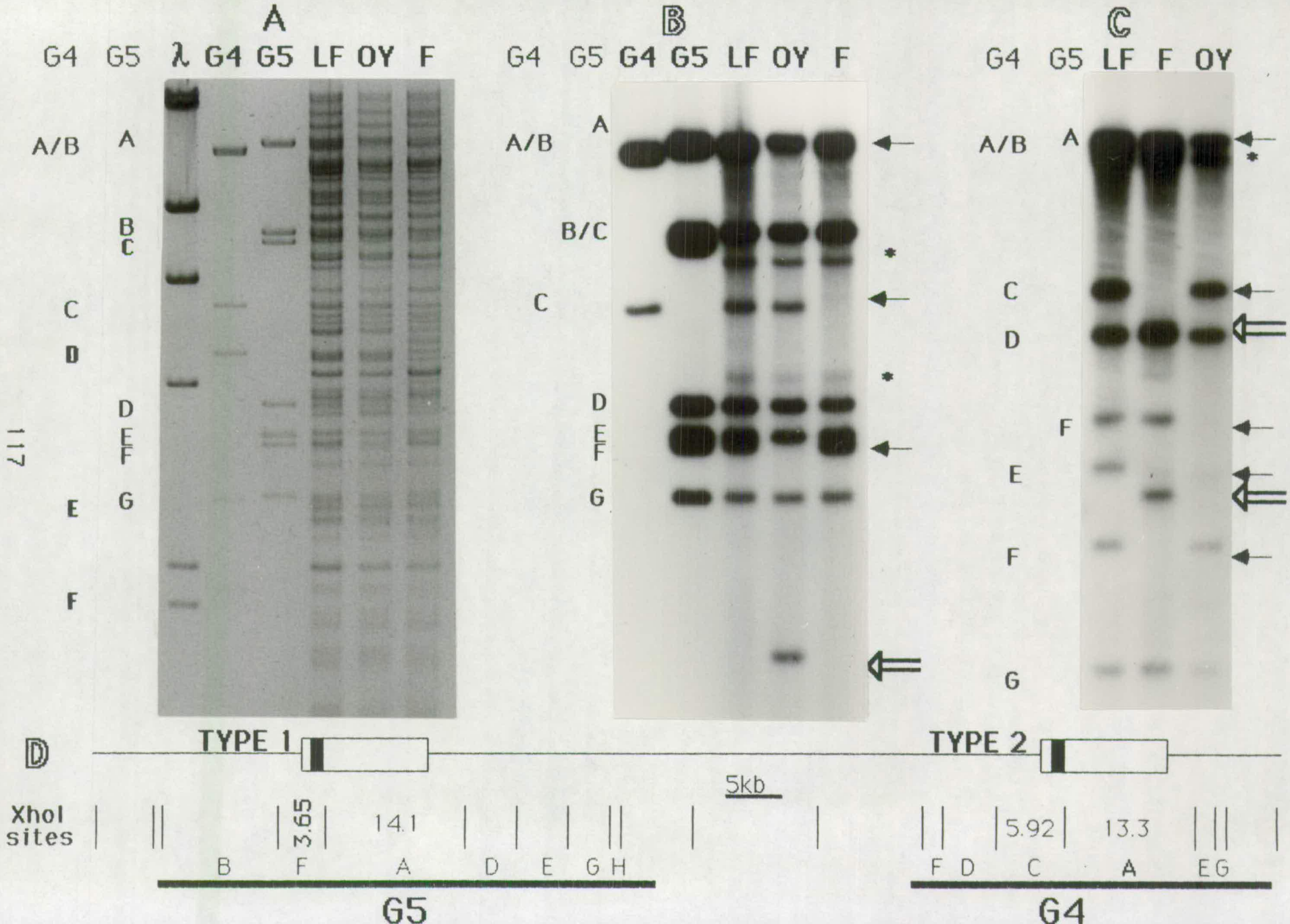
Fig. 6.1. Hybridization of the cosmids G4 and G5 to mtDNA from LF, OY and F mtDNA.

A 0.8% agarose gel (stained with ethidium bromide, and photographed under ultra-violet light) of XhoI cut mtDNA from LF, OY and F compared with the cosmid clones G4 and G5, also cut with XhoI. HindIII digested λ DNA is included to give size comparisons. The XhoI fragments of G4 and G5 are lettered according to size (see **D**, below). Where two bands are inseparable it is indicated as A/B. The restriction profiles of LF and OY mtDNA are virtually indistinguishable but F mtDNA differs from the other two in a number of locations.

B Overnight exposure of a Southern blot of the gel in **A** probed with G5, labelled by nick translation. Arrows mark fragments that are not present in all the mtDNAs. Open arrows mark novel fragments specific to one of the mtDNAs. Asterisks mark fragments hybridizing in the mtDNAs that are not present in the cosmids; these bands are due to hybridization of the cloned mtDNA included within the vector-containing fragment of the cosmids (i.e. fragment B of G4, fragment C of G5). Three fragments of G4 hybridize to the G5 probe; B (because of vector homology) plus A and C as these include the 12kb repeat present in both cosmid inserts.

C Overnight exposure of a Southern blot of a gel similar to that in **A** probed with G4. Arrows and asterisks mark fragments as above.

D XhoI restriction map of the region in N mtDNA including the 12kb repeats (see fig. 1.2 for the full N genome map). The 12kb repeats are shown by open boxes; the position of the atpA genes are marked by vertical black bars. The areas covered by the cosmid inserts of G4 and G5 are indicated by heavy lines. The XhoI fragments of G4 and G5 are indicated by letters. Missing letters represent vector containing fragments.


A
G4 G5 λ G4 G5 LF OY F
A/B
A
B
C
C
D
D
E
F
G
E
F
B
G4 G5 G4 G5 LF OY F
A/B
A
B/C
C
D
E
F
G
C
G4 G5 LF F OY
A/B
A
C
D
F
E
F
G
117
D
TYPE 1
TYPE 2
5kb
XhoI sites
3.65
14.1
5.92
13.3
B F A D E G H
F D C A E G
G5
G4

unexplained fragments hybridize to G4 in OY mtDNA (fig. 6.1C). This may be because either OY mtDNA lacks entirely the sequences on the missing fragments, or because novel homologous fragments are inseparable from other hybridizing fragments. However, OY mtDNA does contain a 1.65kb XhoI fragment homologous to G5 not present in N or C mtDNA. I presumed that this fragment was the progenitor of the missing type 1 atpA arrangement and thus would contain homology to type 1 specific DNA. To prove this I used the clone AN6, containing a 3.4 kb EcoRI insert, as a type 1-specific probe on the same filter as used in fig. 6.1B, after stripping the previous probe with 0.4M NaOH (fig 6.3A). To characterize in detail the events leading to the creation of the type 1 arrangement, both as a sublimon in OY mtDNA and as an amplified sequence in N mtDNA, it was necessary to clone this 1.65kb sequence.

6.3 Formation of the sub-stoichiometric type 1 atpA fragment in OY mtDNA

I constructed an XhoI cut OY mtDNA library in pUC18 and probed this with a 3.4 kb EcoRI fragment specific to the type 1 atpA flank. As the clone AN6 (containing the same EcoRI fragment) could not be used as a probe because of the polylinker and lacZ homology between mp18 and pUC18, the 3.4kb EcoRI fragment was gel-purified on DEAE paper (see **2.2.8**) from a PvuI/EcoRI digest of pIII, a pBR328 clone containing the type 1 atpA gene. A single hybridizing clone (pAB5), containing a 1.65kb insert, was identified and its restriction endonuclease sites mapped in comparison to pIII (fig. 6.3B). The point of divergence between the two appeared to occur close to the EcoRI site of the pAB5 insert, so I made EcoRI/HindIII M13mp19 subclones of pAB5 to allow DNA sequencing progressing from the EcoRI site towards the point of divergence. The sequence of clone RHAB59 showed 55bp perfect homology to part of the

Fig. 6.2. Hybridization of cosmid clone G5 to mitochondrial DNA from three South American cytoplasms compared with that of LF, OY and F cytoplasms.

These XhoI cut mtDNAs were separated on a 0.6% agarose gel for 40 hours before blotting. The extra separation compared to fig. 6.1B allows the mtDNA fragments homologous to the large A fragments of G4 and G5 to be distinguished. The mtDNAs from OY and the South American accessions ECU321, ECU398 and CUN443 lack the G4 A fragment (arrowed) found in LF and F mtDNA. ECU398 and CUN443 contain R plasmids (Weissinger et al 1982). F mtDNA lacks the type 2 atpA-containing fragment (C, arrowed) of G4. The asterisk marks a mtDNA fragment homologous to sequence within the G5 vector-containing fragment (G5 C).

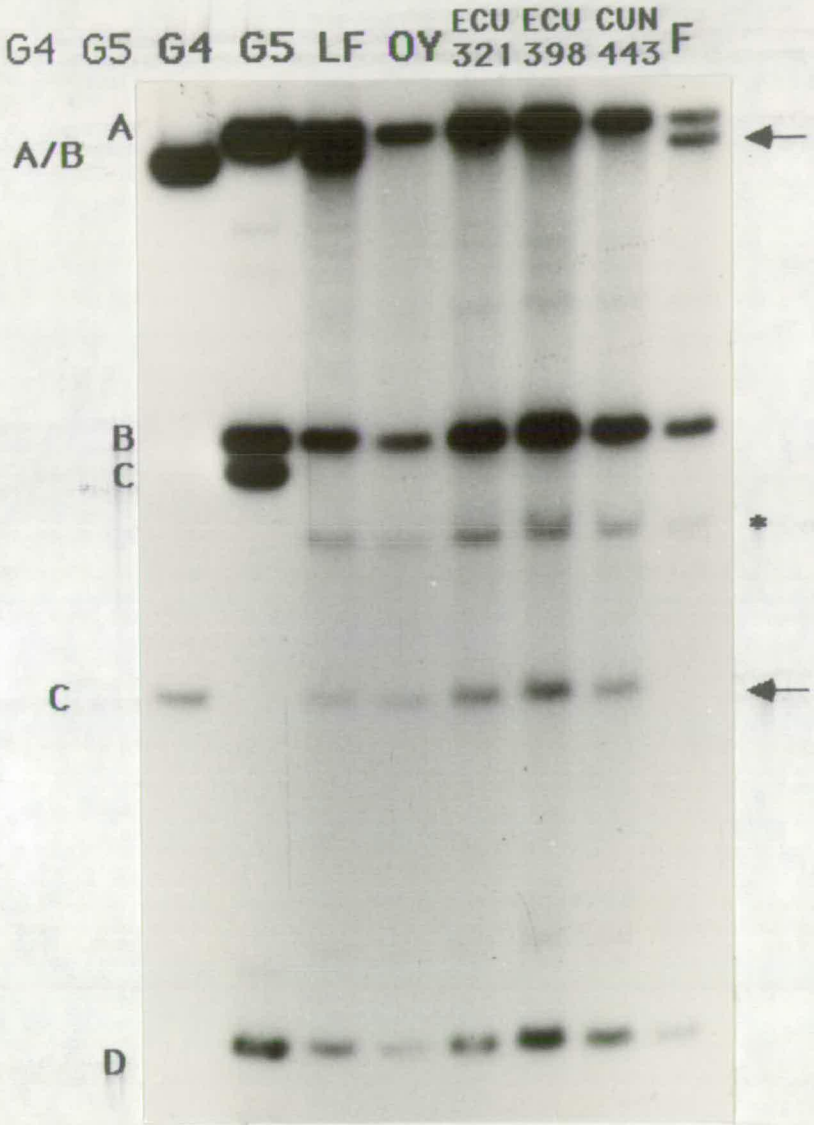
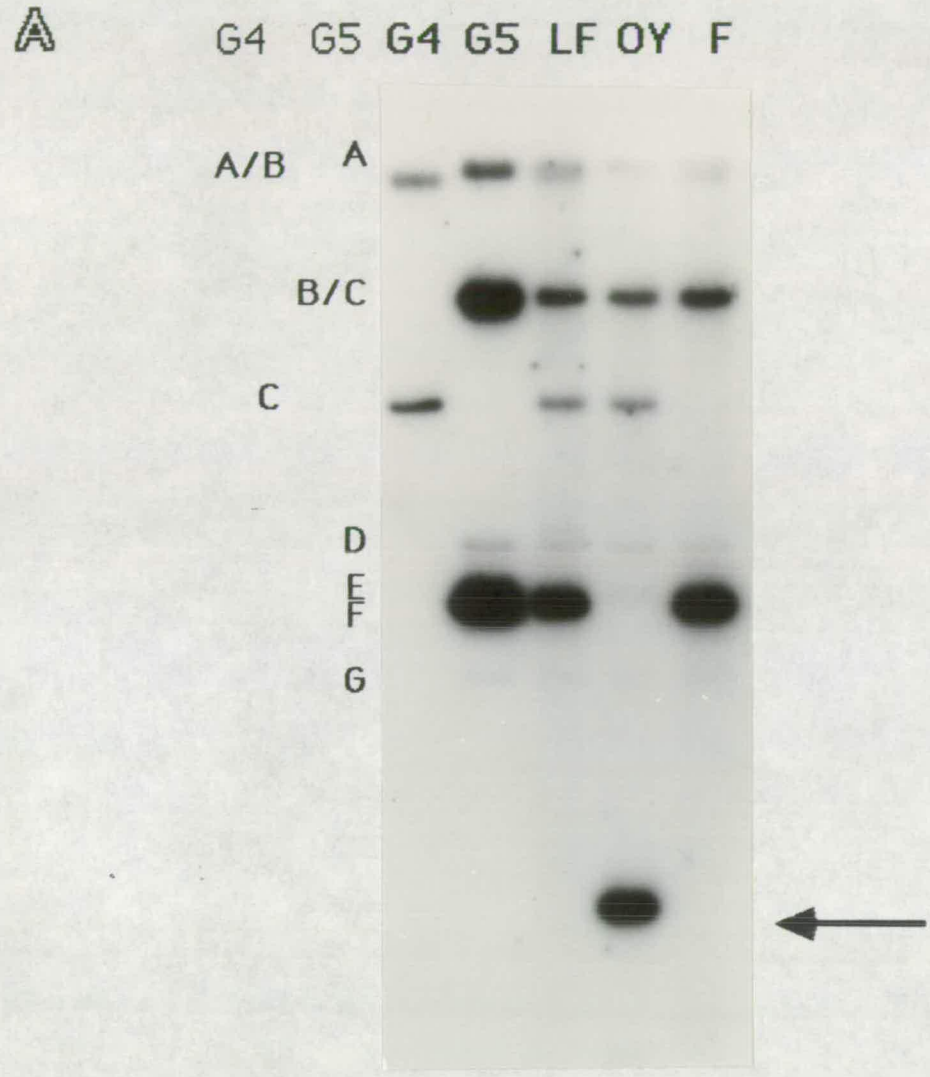

G4
G5
G4
G5
LF
OY
ECU 321
ECU 398
CUN 443
F
A/B
A
B
C
C
D
*

Fig. 6.3. Identification and restriction mapping of a novel 1.65kb XhoI fragment from OY mtDNA.

A Hybridization of AN6 to the cosmid clones G4 and G5, and to LF, OY and F mtDNA. The filter used in this experiment is that shown in fig. 6.1B after removal of the previous probe with 0.4M NaOH. The probe AN6 contains a 3.4kb EcoRI fragment specific to the 3' flank of the type 1 atpA arrangement (see **B**). This probe contains substantial homology to fragments B and F of G5. The EcoRI site closest to the atpA gene lies just within the 12kb repeat, so the probe also contains slight homology to fragment C of G4. Other hybridization signals are probably due to incomplete removal of the previous probe, with the exception of the novel 1.65kb fragment of OY mtDNA which hybridizes strongly (arrowed), indicating it contains a considerable amount of type 1 flank sequence.

B Restriction maps of the type 1 atpA region and the insert of pAB5, a clone of OY mtDNA selected by hybridization to the 3.4kb EcoRI fragment marked. The insert of pAB5 is the novel 1.65kb XhoI fragment from OY mtDNA shown in **A**. Restriction digests of pIII (a clone of the type 1 atpA region from Dr. P. Isaac) and pAB5 with XhoI (X), EcoRI (E), BamHI (B) and SalI (S) revealed that the pAB5 insert was identical to the corresponding region from pIII except for about 300bp at one end. The arrow shows this region, which was subcloned into M13mp19. The direction of the arrow indicates the direction of sequencing of one selected subclone, RHAB59.


A
G4 G5 G4 G5 LF OY F
A/B
A
B/C
C
D
E
F
G
B
probe used to isolate pAB5
atpA type 1
X E BS
E B S X
E
atpA
1kb
X E B S X
pAB5 insert
RHAB 59

12kb repeat, implying that the 1.65kb XhoI fragment cloned into pAB5 contains 181bp homology with the type 2 atpA sequence (fig. 6.4). This extent of homology is similar to the 185bp homology between STIR sequences in CMS-S mtDNA, and so might be expected to be sufficient for homologous recombination. Recombination between the pAB5 sequence and the type 2 atpA arrangement would be predicted to give two products- the type 1 atpA arrangement and a larger XhoI fragment composed of type 2 atpA flank and the novel sequence of RHAB59 (fig. 6.5A). Using RHAB59 as a probe on N and RU mtDNA detects both these products as sublimons (fig. 6.5B). Confirmation for the reciprocal product as well as for the atpA type 1 already known to be present was obtained by hybridizing BM4 (a HindIII/EcoRI clone specific to the 3' flank of the type 2 atpA arrangement) to the same blot (fig. 6.5C). Interestingly, RHAB59 detects no novel fragments in N mtDNA, implying that the non-repeated sequence of RHAB59 is unique to OY/RU mtDNA, and not present in N mtDNA.

6.4 Conversion of OY mtDNA organization into N mtDNA organization

These results suggest that the type 1 atpA sublimon fragment in OY and RU mtDNA is generated by infrequent homologous recombination between a 181bp sequence situated on both the novel 1.65kb XhoI fragment and the type 2 flank of the atpA gene in these cytoplasms. The size of the repeat and the presence of the reciprocal product in comparable amounts suggest that de novo recombination is sufficient for these observations, thus favouring the first hypothesis in **6.1**. Based on these observations, a possible sequence of events can be postulated for the conversion of an OY/RU-type genome organization into an N-type form (fig. 6.6).

Fig. 6.4 DNA sequence of the clone RHAB59 compared to the sequences of the 3' borders of the two 12kb repeats from N mitochondrial DNA.

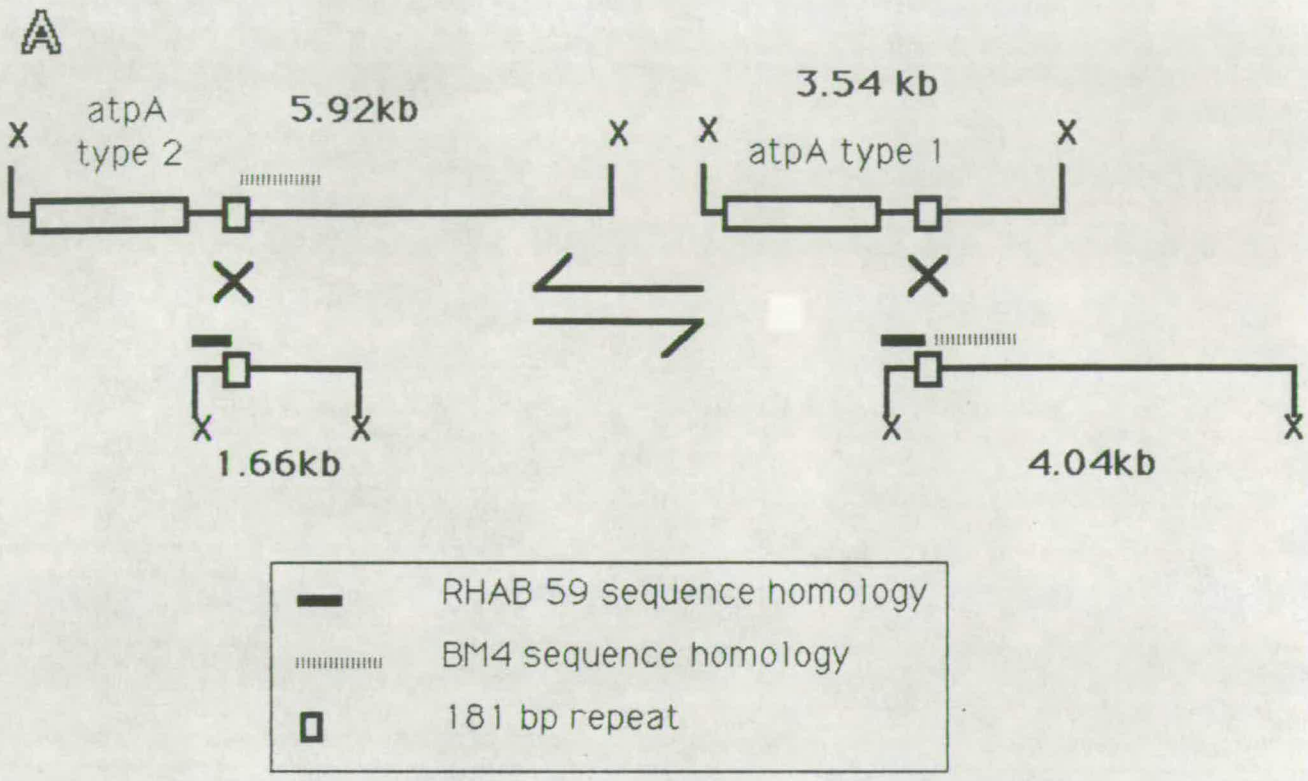
Homologous sequences are marked by vertical bars. Regions homologous between all three sequences are printed in bold type. The underlined sequence represents the predicted sequence of the pAB5 insert considering the similarity of the restriction maps between the pAB5 insert and the type 1 atpA region. The insert of pAB5 thus probably contains 181bp of perfect homology to the type 2 atpA sequence present in OY mtDNA.

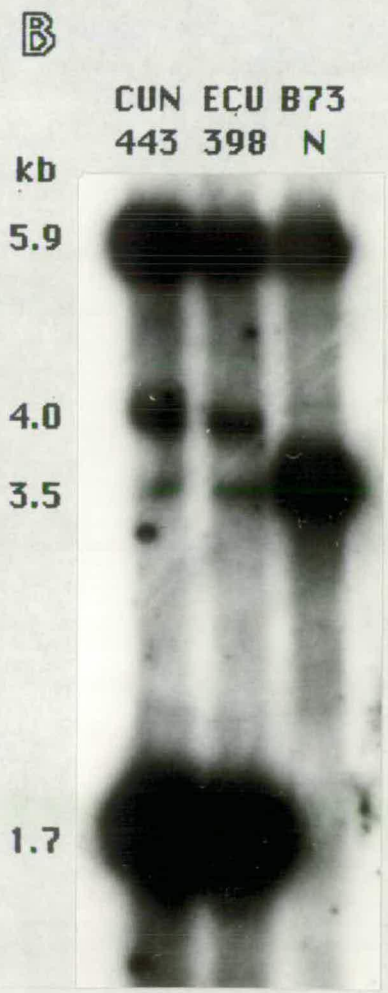
Fig. 6.5. Two sublimons are formed as the result of reciprocal homologous recombination between the type 2 atpA arrangement and the novel AB5 fragment in RU mtDNA.

A Predicted recombination products between the type 2 atpA arrangement and the novel AB5 fragment. Reciprocal homologous recombination across the 181bp repeat common to these sequences should give rise to fragments of 3.5kb (the type 1 atpA arrangement) and 4.0kb. To test this prediction I used the probes RHAB59 and BM4 (as shown) on RU mtDNA. Both probes include some sequence homologous to the 181bp repeat, so could theoretically hybridize to all four fragments.

B Hybridization of RHAB59 to XhoI cut mtDNA from CUN443 and ECU398 compared to N mtDNA. Sublimon fragments can be detected of 4.0kb and 3.5kb in the CUN443 and ECU398 mtDNA, fitting well with the predicted results. The 3.5kb sublimon fragment corresponds to the type 1 atpA arrangement clearly hybridizing in the N mtDNA. The difference in intensity of the two sublimon bands is probably due to the difference in the length of their sequence homology to the probe.

C Hybridization of BM4 to a similar gel to that of B. The 4.0kb sublimon fragment is clearly detectable with this probe as predicted.


A
atpA
type 2
5.92kb
3.54 kb
atpA type 1
1.66kb
4.04kb
RHAB 59 sequence homology
BM4 sequence homology
181 bp repeat


B
CUN ECU B73
443 398 N
kb
5.9
4.0
3.5
1.7

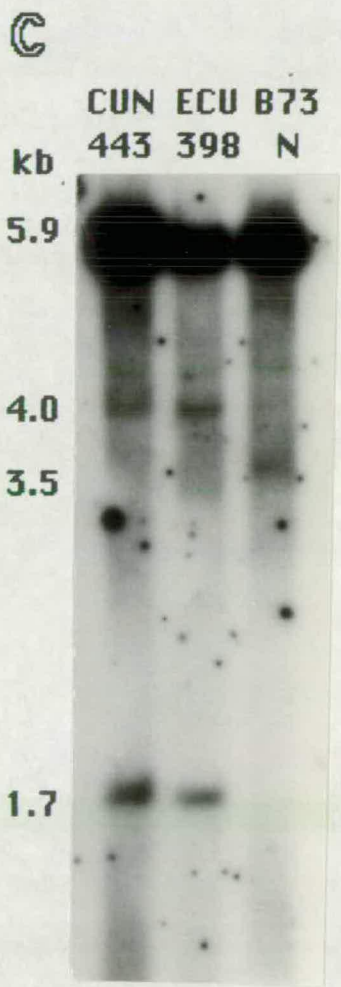
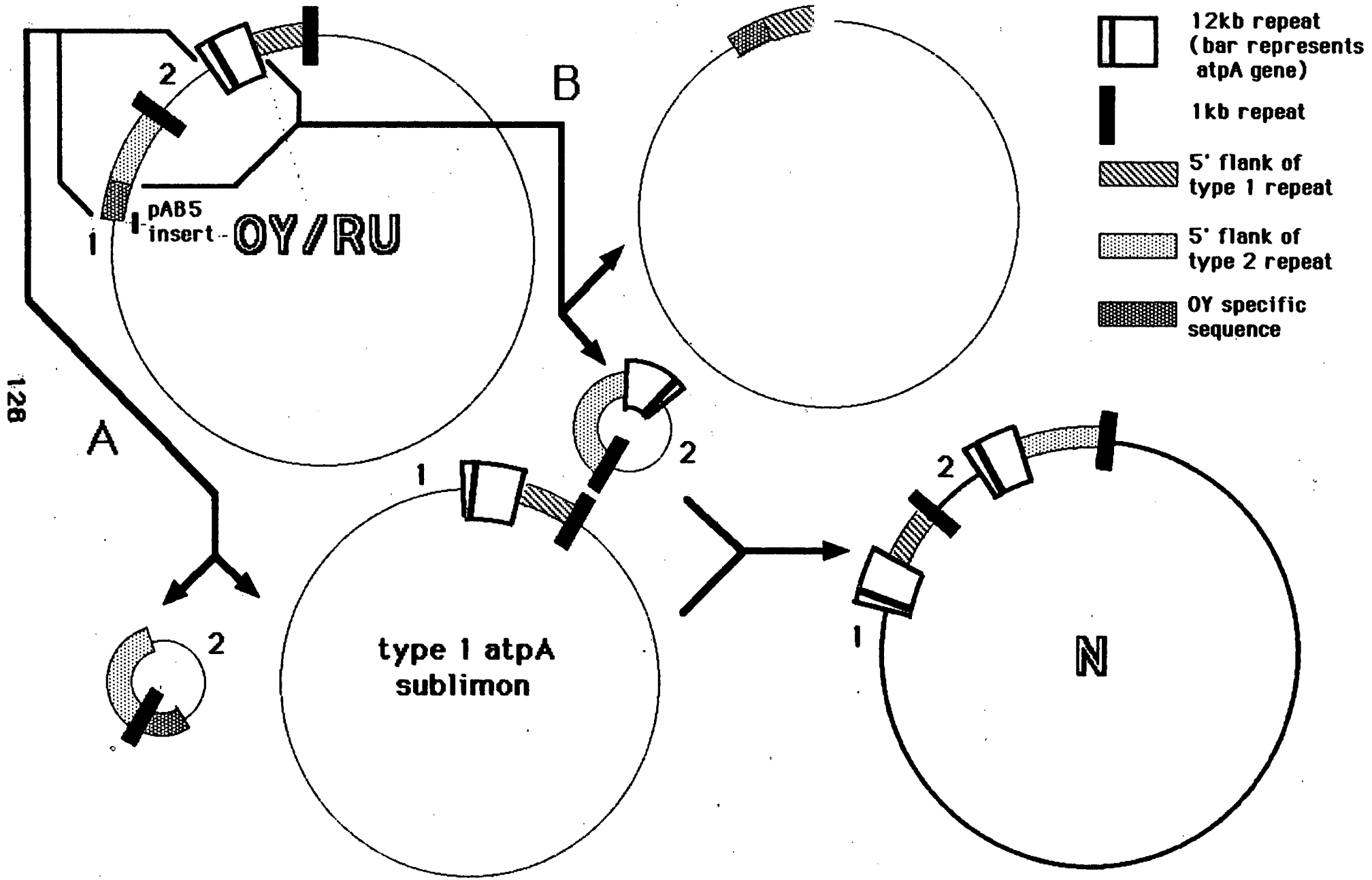

C
CUN ECU B73
443 398 N
kb
5.9
4.0
3.5
1.7

Fig. 6.6. Scheme for the conversion of the OY/RU-type genome organization to an N-type organization.

I have calculated the organization of the atpA and 12kb repeat associated sequences in OY/RU. Conversion to an N-type genome can be achieved via a two-stage process requiring initially two rare recombination events, labelled **A** and **B**.

A represents the generation of the type 1 atpA sublimon by recombination with the homologous 181bp sequence at the 3' flank of the 12kb repeat sequence. **1** and **2** refer to the type 1 and type 2-specific sequences respectively.

B represents a second rare recombination event required to generate another pair of circles, one of which, when recombined with the type 1 atpA sublimon circle (via a pair of 1kb repeats situated on both circles, see fig. 1.2) gives rise to the N genome organization. There is no evidence for this recombination event (**B**); it is indicated merely to suggest a possible way in which the OY/RU genome organization could be rearranged to give an N-type genome organization. This hypothesis requires one to postulate that there is homology between the sequences indicated on the OY/RU genome map.


12kb repeat
(bar represents
atpA gene)
1kb repeat
5' flank of
type 1 repeat
5' flank of
type 2 repeat
OY specific
sequence
pAB5
insert
OY/RU
A
B
type 1 atpA
sublimon
N
1
2
128

6.5 Investigation of the type 3 atpA sublimon in N mtDNA

In order to confirm the generality of the observations above, I decided to repeat a similar procedure of investigations on the type 3 atpA sublimon of N mtDNA (see **5.3**). An XbaI-EcoRI fragment specific to the type 3 atpA flank was gel-purified from the CMS-S atpA clone HS3C5 (fig. 6.7C). This fragment was used as a probe on libraries of EcoRI cut B37-N mtDNA cloned into pBR328. The clone pZmE67 was identified by the probe, but further investigation revealed four inserts after EcoRI digestion, including two of 1.7kb, only one of which hybridized strongly to the probe (not shown). These two 1.7kb EcoRI fragments were gel-purified, recloned into pUC18, and again screened with the type 3 specific probe. The pUC18 clone T3H4 showed best homology to the probe, and subsequently its restriction endonuclease sites were mapped in comparison to the type 3 atpA clone HS3C5 (fig. 6.7). Surprisingly, the insert of clone T3H4 does not contain all the sequence specific to the type 3 flank, and thus cannot give rise to the type 3 atpA arrangement by recombination with either the type 1 or type 2 arrangements. To discover whether this missing type 3 sequence was present elsewhere in the N genome, the two small (c. 200bp) KpnI fragments of HS3C5 were used to probe N and S mtDNA cut with KpnI (fig. 6.8A). These fragments, which must be an integral part of the type 3 atpA sublimon of N mtDNA, are lacking in easily detectable amounts from the N genome. There is some hybridization to high molecular weight fragments in the N mtDNA. The T3H4 insert does contain one of the three KpnI sites delimiting the two fragments comprising the probe, so this insert probably contains some homology to the probe. Therefore the poorly hybridizing N mtDNA fragments in fig. 6.9A may be KpnI fragments containing the T3H4 insert sequence. The amount of radioactive probe used in this experiment may have been too low to detect sublimon levels of the 200bp KpnI fragments,

Fig 6.7. Restriction mapping of the plasmid T3H4 in comparison with the type 3 atpA clone HS3C5.

A Ethidium bromide stained agarose gel of T3H4 and HS3C5 digested with the restriction enzymes shown (RI=EcoRI, RV=EcoRV).

B Southern blot of the gel in **A** probed with the XbaI-EcoRI fragment used to isolate T3H4 from the clone library. The positon of the probe is shown on the map below (R1=EcoRI, R5=EcoRV, X=XbaI, K=KpnI). Thick lines indicate insert DNA, thin lines indicate vector DNA. The extent of the homology between the two plasmids is shown. The point of divergence between HS3C5 and the two atpA arrangements of N mtDNA occurs between the end of the atpA gene and the EcoR5 site marked. Therefore, T3H4 lacks about 1kb of the type 3 specific sequence.

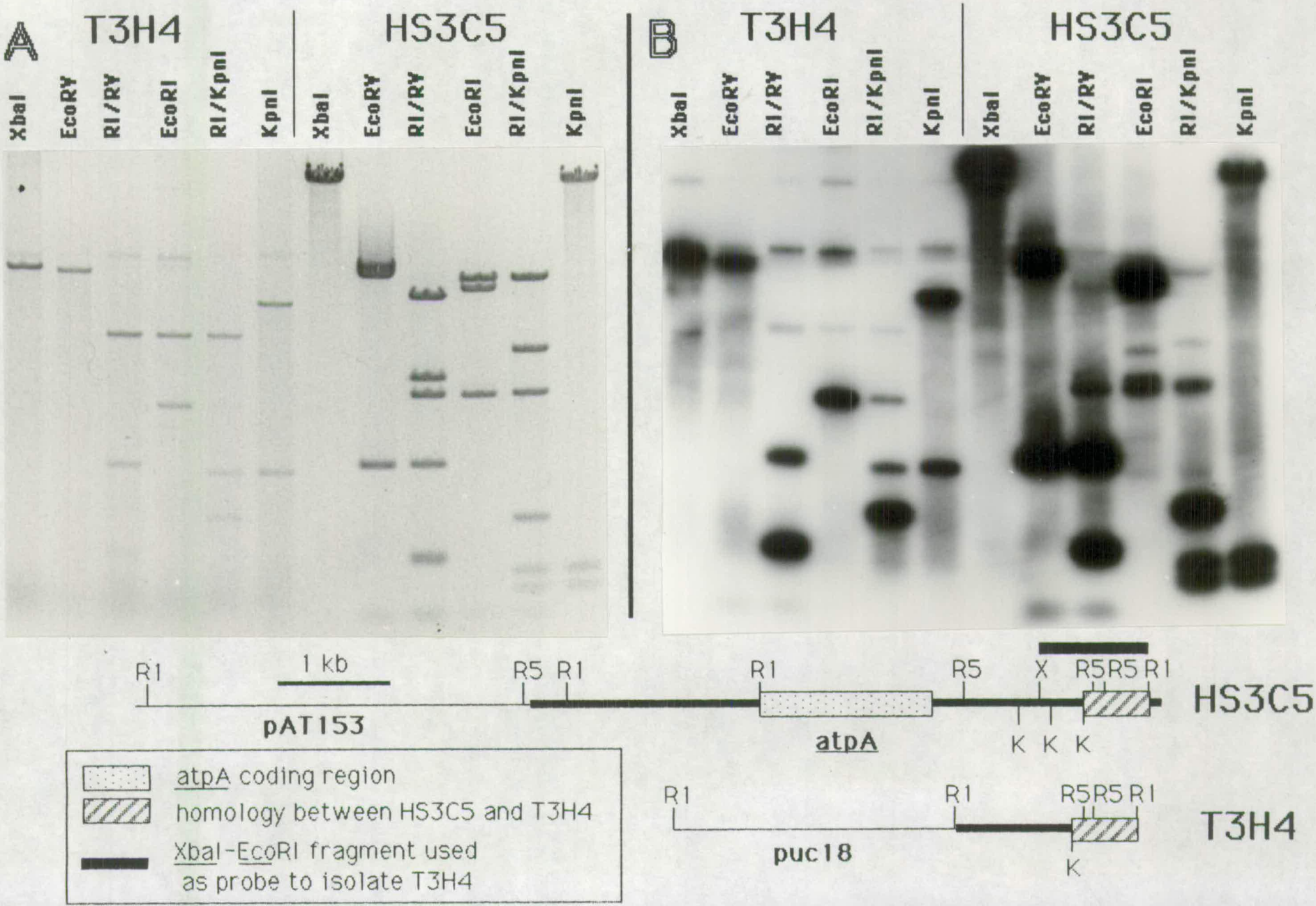
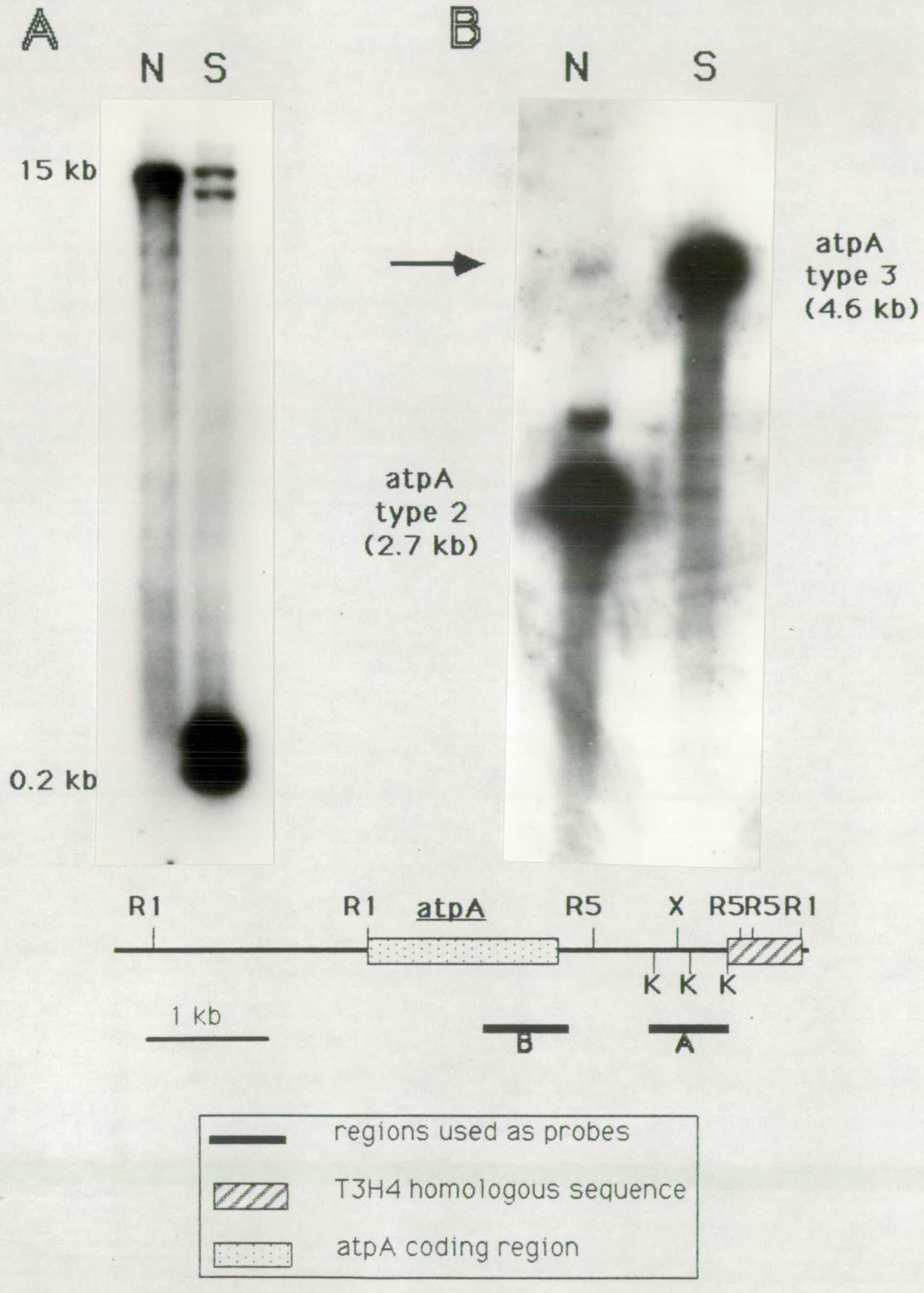

A
T3H4
HS3C5
XbaI
EcoRV
RI/RV
EcoRI
RI/KpnI
KpnI
B
T3H4
HS3C5
XbaI
EcoRV
RI/RV
EcoRI
RI/KpnI
KpnI
R1
1 kb
R5
R1
pAT153
R1
R5
X
R5R5
R1
K K K
atpA
HS3C5
atpA coding region
homology between HS3C5 and T3H4
XbaI-EcoRI fragment used
as probe to isolate T3H4
R1
R1
R5R5
R1
K
puc18
T3H4

Fig. 6.8. The genome of N mitochondria does not contain sequences necessary for the formation of the type 3 sublimon.

Hybridization of **A**, the two 200bp KpnI fragments of HS3C5 and **B**, BLSC1 to **A**, KpnI cut and **B**, BamH1 cut N and S mtDNA. The locations of the sequences used as probes are shown below. **A** is a one week exposure; **B** was exposed overnight. The arrow indicates the position of the type 3 sublimon detected with the BLSC1 probe in N mtDNA. No sublimons are visible corresponding to the small KpnI fragments in **A**, but this may be due to differences in probe strength or amount of DNA loaded in the two experiments.


A
N S
15 kb
0.2 kb
B
N S
atpA
type 2
(2.7 kb)
atpA
type 3
(4.6 kb)
R1
R1
atpA
R5
X
R5R5R1
K K K
1 kb
B
A
regions used as probes
T3H4 homologous sequence
atpA coding region

but the type 3 atpA sublimon is present in this N mtDNA, as shown by probing the same N and S mtDNAs with BLSC1 (fig. 6.8B).

6.6 Discussion

At the start of this chapter, two conflicting, but not necessarily exclusive hypotheses were postulated to explain the presence of sublimons in plant mitochondrial genomes. The experiments described in this chapter were intended to allow informed judgement on which of the two most convincingly explained the situation in vivo. The study of the type 1 atpA sublimon of OY and RU mtDNAs suggested that recombination de novo was sufficient to explain the results. However, the observations on the type 3 atpA sublimon of N mtDNA cannot be explained in this fashion. Part of the sequence comprising this sublimon is lacking from the N mitochondrial genome, and thus this sublimon cannot be generated de novo by recombination. It seems the only explanation is that the type 3 sublimon in N mtDNA is an evolutionary relic, implying that rare recombinant molecules can be maintained in the genome for long periods without being continuously reformed by recombination. This has important repercussions when considering the potential for genome change in plant mitochondria, as will be discussed in the following chapter.

Chapter 7- Discussion and speculation on a possible role for sublimons in the evolution of plant mitochondrial genomes

7.1 Recombination in the maize mitochondrial genome

The base substitution rate in higher plant mtDNA is low (see **1.5**), and thus any consideration of genome evolution in plant mitochondria must predominantly concentrate on the organizational differences between related mitochondrial genomes. The observed organizational diversity appears to have arisen by the fixation of molecules created by rare recombination events between short repeats scattered throughout the genome, a feature of many plant mtDNAs (Lonsdale 1987). These events may be catalysed by the same recombination system as that operating on the large repeats described in **1.2**. Recombination in plant mitochondria is thought to be due to a general sequence independent homologous recombination system; there is no evidence of any site-specific system active in plant mitochondria (Lonsdale et al. 1988). No direct observations have been made on the activity or substrate requirements of these putative 'recombinases', but tentative conclusions can be drawn after comparisons with other systems, and by using the indirect evidence at our disposal.

The RecBC and RecF recombinase systems from E. coli show a linear increase in recombination frequency with increasing length of homology between the two substrate sequences above a threshold of 23-27bp or 44-90bp respectively (Shen and Huang 1986). The frequency of recombination in these systems decreases 40 fold with a decrease in substrate homology from 100% to 90%. In vitro studies with RecA show heteroduplex formation between substrates with 151bp homology, but not

between substrates of only 30bp homology (Gonda and Radding 1983).

The two putative recombination substrates studied in detail in this thesis are of similar lengths, 186bp for the STIR sequence in CMS-S mtDNA and 181bp for the atpA linked repeat in OY mtDNA. Based on the data above, these sequences would theoretically act as efficient substrates for the E. coli enzymes, although they would probably not be large enough to form substrates for high rates of recombination. These substrates and their predicted recombination products are detected in markedly non-equimolar ratios in maize mitochondria, implying equilibrium has not been achieved, and suggesting that recombination is slow in comparison with other contributory processes, such as the relative rates of replication of the molecules containing these repeats. In contrast, most or all large repeats (those greater than 1kb) in plant mitochondrial genomes are apparently at or near recombinational equilibrium, as all four recombinant possibilities are equally represented in the genome (Palmer and Shields 1984, Stern and Palmer 1984b, Lonsdale et al. 1984, Falconet et al. 1984, Hiesel et al. 1987, Lonsdale et al. 1988). At the other extreme, no recombinant products have been detected (in mtDNA extracted from seedlings) resulting from recombination across a small 55bp direct repeat present in CMS-T mitochondria (Rottman et al. 1987). Recombination must occur across this repeat at low levels, however, as fixation of one of the recombinant products is responsible for fertility reversion in cultured CMS-T cells. Thus one can postulate that the recombinase system in maize mitochondria has similar characteristics to those of E. coli recombinases, and one can assume frequent recombination between larger repeats (over 500bp), less frequent recombination between repeats of 100-200bp, and very infrequent recombination between repeats of 50bp and less.

The activity of this recombinational system would directly affect the

ratios of subgenomic circles to each other within the multipartite mitochondrial genome. In addition it also has implications for copy number control of sequences present on different sub-genomic circles, as inter-molecular recombination could compensate for differences in the efficiency of different replication origins. Circles lacking replication origins may be able to gain access to a replication origin by recombining with a circle that does possess one. This may allow the survival of sub-genomic circles that would otherwise be lost.

7.2 Maintenance of mitochondrial genome structure

The complex multicircular mitochondrial genomes of higher plants remain structurally stable for many generations (Brennicke and Schwemmle 1984, Oro et al. 1985), although on an evolutionary timescale they appear to evolve very rapidly in structure (Palmer and Herbon 1987b). It is still not entirely clear whether these genomes should be regarded as a master circle, with sub-circles created by recombination, or as a collection of independently replicating circular molecules with some recombinational interchange. The minicircular plasmids of maize (see **1.2.1**) are examples of independently replicated circular DNAs maintained in the genome. Others have been described in the Oenothera mitochondrial genome, where numerous large circular molecules (5-20kb+), at widely varying stoichiometries, have been identified in addition to the main mitochondrial genome by gel electrophoresis of undigested mtDNA (Brennicke and Blanz 1982). The study of some of these circular molecules has revealed that they were formed by 'looping out' of main genome sequences lying between short direct repeats. The repeats involved in at least one of these events were extremely short (ten nucleotides) suggesting a unique or extremely rare event (Manna and Brennicke 1986). Furthermore, the reciprocal junction fragments of these recombination

events are not found, strongly implying that these circles have been maintained and amplified by independent replication, and are not generated by de novo recombination. The least abundant of these circular molecules would appear to be analogous to the 'sublimons' I have described in Chapters 5 and 6. My results with the type 3 atpA sublimon of N mtDNA show that rare recombinant molecules can apparently be stably maintained in maize mitochondria in the absence of continuing recombination.

7.3 Possible implications of sublimons in the evolution of the plant mitochondrial genome

I have defined sublimons as molecules containing novel recombinant arrangements present at sub-stoichiometric levels in the genome. Amplification and fixation of a sublimon at stoichiometric levels would create a novel genome structure, and my observations with atpA probes suggests this has happened during the evolution of the maize mitochondrial genome. This amplification event could be passive (by segregation) or active (by selective replication).

If these sublimons are retained through several generations without a significant contribution from recombination events (as suggested by the study of the type 3 atpA sublimon in N mtDNA), then there is the possibility of subsequent rearrangements and sequence drift in comparison to the original progenitor sequences. Sublimons might be expected to show rapid molecular evolution because:

(a) given that these molecules are present at relatively low levels, further mutational events in one of these molecules will affect a relatively high proportion of them. Mutations occurring under such circumstances would become more rapidly fixed than mutations in highly abundant sequences.

(b) there would be no selection pressure against mutation as sublimons cannot be expected to have any direct phenotypic significance.

Consequently one might expect sublimons to progressively accumulate mutational events, both base substitutions and further recombinational rearrangements. Occasionally, one of these rearrangements may lead to the amplification of such a molecule to a 'normal' stoichiometry and this amplification event would create a novel mtDNA restriction endonuclease pattern.

Sublimons may be involved in the phenomenon of cytoplasmic reversion to fertility, described in chapter 4. In cytoplasmic revertants, rearranged mtDNA molecules have become fixed in the genome that appear to have been generated by recombination between short regions of homology (Schardl et al. 1985, Rottman et al. 1987). It seems possible that these rearranged molecules existed as sublimons in the genome of the sterile parent. However, an attempt to find the fertile revertant mtDNA configuration as a sub-stoichiometric component of the CMS-T genome has failed (Pring et al. 1988). Lonsdale et al. (1988) have shown the presence (in field-grown CMS-S revertants) of sub-stoichiometric mtDNA arrangements corresponding to the abundant novel arrangements of other CMS-S revertants. This observation could be explained by differential amplification of several pre-existing sublimons.

One can speculate that if widespread, the existence of sublimons in a genome allows the possibility of a sudden drastic reorganization by the amplification of these pre-existing rare recombinant molecules. Although this may seem unlikely, such catastrophic changes are implied by a recent report of a transition from N-type cytoplasm to S-type cytoplasm induced by the recessive nuclear mutation iojap (ij) (Lemke et al. 1985). This phenotypic switch was accompanied by the appearance of the S1 and S2 DNAs in the mtDNA of the new CMS line (designated cms ij-1). A similar

event, from N to CMS-T has also been observed (Lemke, personal communication). This second novel CMS line has been designated cms ij-2.

I have examined the mtDNA from these plants, their parents and related progeny from the same crosses. Hybridization patterns of gene specific probes e.g. atpA (fig. 7.1) to these mtDNAs confirm that the plant cms ij-1 (from the cross R181-N x ijijEv-N, the male parent being homozygous for iojap) has CMS-S like mtDNA, and the plant cms ij-2 (from the cross W182BN-181 x ijijEv-N) has CMS-T-like mtDNA. The female parent of the second cross has a fertile cytoplasm (181) with mtDNA similar to that of N in most respects, but in the region of the atpA gene it resembles CMS-T mtDNA (fig. 7.1). This suggests that the 181 cytoplasm might be expected to be predisposed to a transformation to T cytoplasm, and circumstantially suggests the validity of the observation. Protein synthesis by isolated mitochondria (in organello) from both cms ij-1 and cms ij-2 confirm that they are of the CMS-S and CMS-T groups respectively (Liddell and Leaver, unpublished).

The results of Lemke et al. have not been widely accepted by molecular biologists in this field because of the difficulty in explaining the molecular events needed to effect such a transformation. The mitochondrial genomes of N, S and T cytoplasms differ at a large number of loci, with multiple insertions, deletions, point mutations and rearrangements of sequences (Leaver et al. 1985, Dewey et al. 1986). It is inconceivable that such widespread and apparently random rearrangements could occur identically on a number of independent occasions. However, my results with atpA probes implies that at least some sequence arrangements previously shown to be characteristic of male-sterile lines can be detected in fertile lines at low levels. If a high proportion of the rearranged sequences characteristic of male-sterile lines were already present at low levels in fertile lines then the transformations reported by

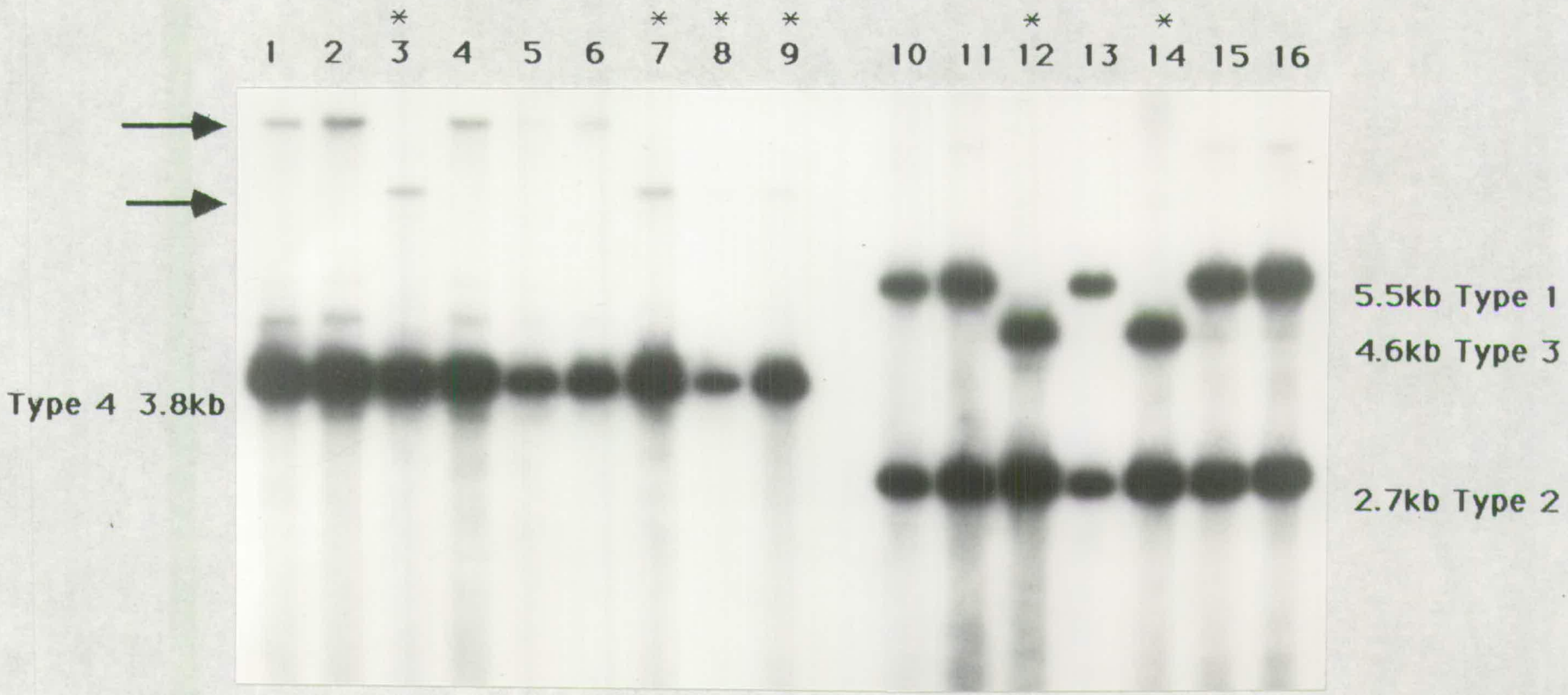
Fig. 7.1. Hybridization of an atpA probe to mtDNA from cms ij-1 and 2 compared with related lines.

1. 1984: 1589	W182BN-181	10. 1985: 6392	ijij Ev-N
2. 1985: 6096	W182BN-181	11. 1985: 6103	R181-N
3. 1982: 691	restored CMS-T	12. 1985: 5973	**cms ij-1**
4. 1980: 1540	fertile	13. 1980: 1529	fertile
5. 1982: 707 x 688	partial sterile	14. 1985: 6908	W182BN-CA
6. 1983: 13903LB	partial sterile	15. 1984: 1883	partial sterile
7. 1984: 1908	**cms ij-2**	16. 1985: 6438A	partial sterile
8. 1985: 6101	W182BN-T		
9. 1984: 1260	W182BN-T		

The atpA probe BLSC1 was hybridized to a Southern blot of BamHI digested mtDNAs from the lines listed above. atpA types are labelled as defined in chapter 5.

1-10 represents lines related to the novel CMS source cms ij-2, which resulted from a cross of W182BN-181 (1 and 2) with a male parent homozygous for iojap (10). The 181 cytoplasm resembles T in its atpA organization, but the two can be distinguished by large faintly hybridizing fragments (arrowed, compare lines 2 and 3). Lines 4, 5 and 6 are siblings retaining the parental hybridization pattern from the same cross that produced cms ij-2.

Lines 10-16 represent lines related to the novel CMS source cms ij-1. 10 and 11 are the male and female parents of the cross repectively. 14 is a CMS-S cytoplasm as a comparison to cms ij-1; 13, 15 and 16 are siblings to cms ij-1 but show the parental hybridization pattern.



* denotes mtDNAs from cytoplasms conferring male-sterility

Lemke et al. become much more plausible.

To investigate the possibility that sublimons could be involved in these alleged transitions, I chose as a marker the urf13-T gene of CMS-T. This gene encodes the 13kDa polypeptide thought to be responsible for CMS in this cytoplasm (see **1.3.1**). The degree of conservation of the component sequences in this region with their homologous sequences in N mtDNA varies (fig. 7.2), implying temporal separation of the formative recombination events. No intermediates between the N and CMS-T organizations in this region are known, and thus this sequence arrangement fits well with the hypothesis of rapidly evolving low abundance constituents of mtDNA. The complexity and apparent randomness of this sequence arrangement strongly suggest that for an N to CMS-T transition to occur, this sequence must pre-exist in the progenitor genome. I used an synthetic oligonucleotide as a probe on mtDNA from N, 181, PUN6 and CMS-T cytoplasms (fig 7.3). No evidence of any sublimons containing this sequence were observed in any of the fertile cytoplasms, but this may be due to the low sensitivity of oligonucleotide probes.

If independently maintained and evolving sublimons are to be suggested as vehicles of genome change, then serious consideration has to be given to the inheritance and segregation of mtDNA molecules during both mitosis and meiosis, as the initial expectation would be that rare recombinant molecules would tend to be lost from cell lineages during repeated rounds of cell replication.

7.4 Segregation of heterogeneous or multipartite genomes

Most organelle genomes segregate rapidly during successive mitotic divisions from a mixed (heteroplasmic) to a pure (homoplasmic) population (reviewed in Birky 1978, 1983). This has been shown for

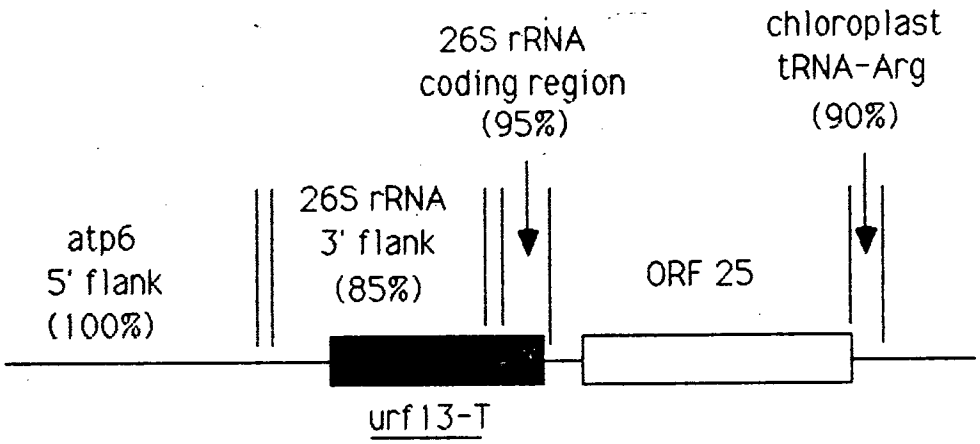


Fig. 7.2 The homology between various portions of the chimaeric urf13-T region of CMS-T mtDNA and their presumed progenitor sequences.

Vertical lines indicate the sites of the recombination points involved in the formation of this region. The urf13-T gene is shown by a solid box, the open reading frame encoding a putative 25kDa polypeptide (ORF 25) is shown by an open box. The major homologous sequences from N mtDNA are indicated, and their percentage base sequence homology with sequences of the urf13-T region are given. The figure given for the tRNA-Arg gene is percentage base sequence homology to the homologous gene from tobacco chloroplast DNA.

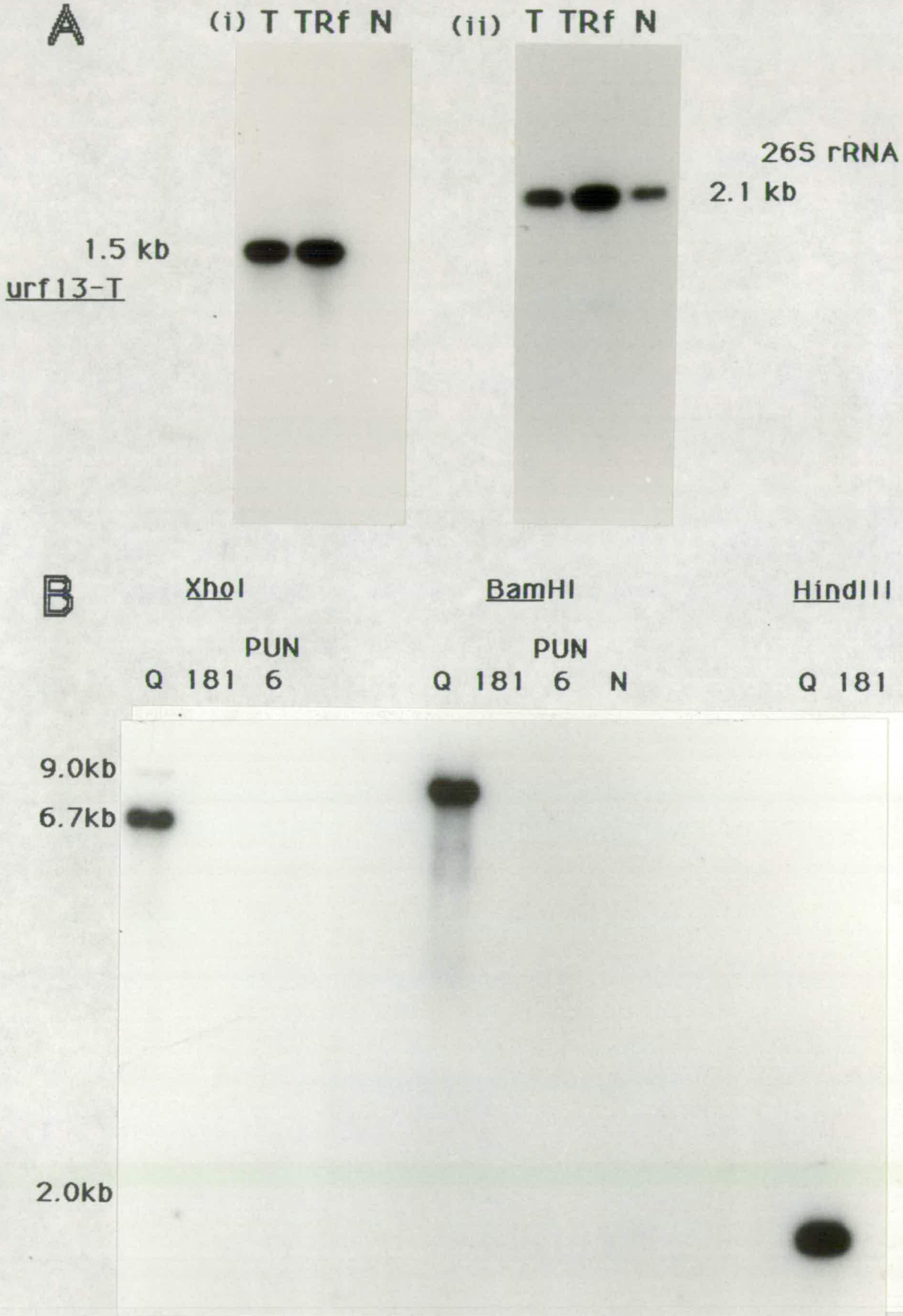
Fig. 7.3. Hybridization of a synthetic oligonucleotide probe for the urf13-T gene to maize mitochondrial DNA from fertile cytoplasms.

A 20-base oligodeoxynucleotide sequence identical to a portion of the urf13-T gene was synthesized (see below, marked in bold type). The sequence shown was selected because of its low homology to its corresponding sequence in the 3' flank of the 26S rRNA gene from N mtDNA (see below). The 20-base sequence in the corresponding region of the 3' flank of the 26S rRNA was also synthesized (see below, marked in bold type). Both oligodeoxynucleotides were end-labelled with ^{32}P for use as hybridization probes.

```
PHE LEU CYS ILE LEU LEU ILE LYS GLY TYR
TTT TTG TGC ATA TTA TTG ATA AAG GGA TAT  urf13-T
 ||  ||  |  |  ||     |  ||  ||  |||  |||
ATT TTT TTC ATT AGG CAG ATT AAA GGA TAT  3'flank of 26S rRNA
```

A Hybridization of (i) the urf13-T probe and (ii) the 26S rRNA probe to T, TRf and N mtDNA digested with AvaI (TRf mitochondrial DNA is identical to T mtDNA, but the nucleus of this line carries the CMS-T restorer genes). The two probes are shown to be specific for their own complementary sequences under the hybridization conditions used. N mtDNA does not contain any detectable homology to the urf13-T probe.

B Hybridization of the urf13-T probe to mtDNA from cytoplasms Q (a member of the CMS-T group), 181, PUN6 and N. Only Q mtDNA shows any detectable homology to the probe. The mtDNAs 181 and PUN6 resemble T mtDNA in the arrangement of their atpA gene, but otherwise resemble N mtDNA (see **5.3**).


A
(i) T TRf N
(ii) T TRf N
26S rRNA
2.1 kb
1.5 kb
urf13-T
B
XhoI
BamHI
HindIII
PUN
Q 181 6
PUN
Q 181 6 N
Q 181
9.0kb
6.7kb
2.0kb

chloroplast genomes either after crossing plants containing genetically marked plastids (in those species exhibiting biparental inheritance) (Adams et al. 1976, Birky 1983) or after fusion of protoplasts containing similarly marked plastids (Nagy et al. 1981, Fluhr et al. 1983, Pelletier et al. 1983). The same is true for mitochondria in yeast. Crosses between yeast strains containing different alleles of a marker gene to form a heteroplasmic zygote segregate to homoplasmy in around 20 mitotic divisions. This segregation is partially statistically predictable by modelling replication and partitioning of mtDNA molecules as stochastic processes (Birky 1978, 1983). However, to achieve the rapidity of segregation observed with chloroplasts and yeast mitochondria it is necessary to assume non-random mixing of mitochondrial genomes in cells; specifically, like genomes must tend to associate with like, and thus are more likely to cosegregate into the same daughter cell (VanWinkle-Swift 1980, Birky 1983).

The study of the segregation of plant mitochondrial genomes is complicated by recombination between parental mtDNAs. Regeneration of cybrids created by the fusion of protoplasts containing mtDNAs distinguishable by their restriction endonuclease patterns results in plants which have mtDNA unlike that of either parent (Belliard et al. 1979, Galun et al. 1982, Boeshore et al. 1983,1985, Fluhr 1983, Nagy et al. 1981,1983, Chetrit et al. 1985, Kemble et al. 1986). These novel mtDNA genomes contain fragments from both parents (Rothenberg et al. 1985) as well as novel fragments generated by recombination between the parental mtDNAs (Rothenberg and Hanson 1987, Morgan and Maliga 1987). This inter-genomic recombination must be preceded by mitochondrial fusion. Fusion of mitochondria is probably normal in plant cells, and is not an artefact of the protoplast fusion procedure. Otherwise it is difficult to

see how complex plant mitochondrial genomes could arise or be maintained intact when calculations show that in many cell types the number of master circles per mitochondrion is of the order of one (discussed in Lonsdale et al. 1988). If this is the case then the mtDNA complement of a plant cell (chondriome) can probably be considered as a single entity, providing that the cell generation times are sufficient for complete mixing of the mitochondria. Dividing plant meristematic cells contain 5-10 large reticulate mitochondria, each presumably containing many mitochondrial genomes (Bendich and Gauriloff 1984). Mitochondrial mixing and fusion would reduce the rate of segregation, and add to the stability of the genome.

However, although the novel mtDNA organizations of cybrids are stable, each cybrid shows a unique pattern. This suggests rapid segregation to homoplasmy after an initial heterogenous state when a large number of possible mtDNA conformations coexist in the initial fused cell. It is difficult to reconcile rapid genome segregation with the maintenance of sublimons which are not continuously recreated recombinogenically. However, there is considerable evidence that the segregation of plant mitochondrial genomes observed in cultured cells is not entirely applicable to the normal plant life cycle. Plant mitochondrial genomes often undergo alterations, especially deletions during periods in culture (Morgens et al. 1984, Morgan and Maliga 1987, Rode et al. 1987). The best examples are probably cytoplasmic revertants to fertility in maize. During cytoplasmic reversion, rare recombination products are fixed in the genome within (in some cases) a single sexual generation (Schardl et al. 1985), requiring a considerable degree of segregation, probably coupled with positive selection pressure. Reversion to fertility is greatly enhanced in cultured CMS material, in fact revertants from CMS-T and from the CMS-S source W182BN (see Chapter 4) have only been obtained

from cultured cells (and never found in field-grown plants), perhaps because of a greater opportunity for segregation (Brettell et al. 1980, Earle et al. 1987). Interestingly, I have found that intermediate mtDNA organizations evolving in culture along a predictable path are fixed in plants regenerated from culture (the S1 integrate fragments of the 'young' W182BN revertants are stable for several generations in plants, but are lost if the cells are kept in culture; see **4.3**). This further evidence also implies that the same rate of segregation is not present in the normal plant life-cycle.

Mammalian mitochondrial genomes segregate to homoplasmy very rapidly after heteroplasmy has arisen during the usual sexual lifecycle (Hauswirth and Laipis 1985). This is due to several special features of mammalian development. For example, multiple rounds of mitochondrial fission occur without genome replication during oogenesis, and there is no evidence for mitochondrial fusion to prevent segregation during this process. In addition, mammalian embryos are partitioned at a very early stage into different tissue types, including a segregation between primordial germ cells and somatic cells, further dividing up the original complement of mitochondrial genomes. Hauswirth and Laipis (1985) have estimated that only five mitochondrial genomes serve to define the cytoplasmic genotype of the next generation. These five genomes can be considered as the unit of inheritance for mammalian mtDNA.

Unfortunately, in plants there is a lack of relevant information with which to make analogous calculations. Little is known of the mechanism or developmental timing of plant mtDNA replication. Studies of mtDNA synthesis in maize using immature cob and coleoptile tissue (Carlson et al. 1986, Bedinger and Walbot 1986) and in wheat using young germinated embryos (Ricard et al. 1983) have failed to demonstrate any significant mtDNA replication, only mtDNA repair. Estimates of mitochondrial genome

number per cell are limited to differentiated, non-dividing cells (a typical value, from curcurbit hypocotyl cells, is 100-140 (Ward et al. 1981)). Meristematic cells, from which the tissues of the cob will eventually develop, may contain a larger number of mitochondrial genomes per cell. In summary, it can be said that no reliable estimate can be placed on the unit of inheritance for plant mitochondrial genomes, but the value could be higher than for mammalian genomes.

7.5 Conclusions

The mitochondrial genome organization of higher plants is extremely complex and yet relatively invariant from generation to generation. However, comparisons between related species, and often between cytoplasms within species, occasionally reveals large differences in sequence organization. Thus a kind of 'punctuated equilibrium' can be envisaged for plant mitochondrial DNA evolution, with periods of stability interspersed by sudden widespread rearrangements of the genome. Sublimons, rare recombinant molecules retained in the mitochondrial genome for long periods, would play a part in this hypothetical mode of mtDNA alteration. However, the evidence is as yet still inconclusive, and much work remains to be done, particularly with respect to the characteristics of replication and inheritance of mitochondrial DNA in higher plants, if this hypothesis is to become accepted.

7.6 Future work

The work in this thesis leaves several questions unanswered. Chapters 3 and 4 discussed and compared the mtDNA of CMS-S cytoplasms and cytoplasmic revertants thereof, but failed to discover any correlation between the mtDNA and the phenotype that could account for the

molecular basis for CMS-S, such as has been demonstrated for CMS-T. It seems likely, therefore, that to make progress in this field, the problem should be approached from the other side; the physiology of pollen abortion in the anthers of CMS-S plants should be examined in order to discover the biochemical malfunction responsible, and only then may the mtDNA mutation be identified.

The investigations and speculations on plant mitochondrial genome evolution have also been hampered by a lack of basic biochemical and physiological knowledge. It is essential for significant further progress that the mechanisms responsible for mtDNA replication and recombination (and their developmental control) are characterized. Some specific problems, however, could and should be answered rapidly by standard molecular biological techniques. The putative scheme for the conversion of the OY/RU-type genome to the N-type genome organization (fig. 6.6) is currently being tested, with the intention of completing the description of this evolutionary transition. Further observations on the type 3 atpA sublimon of N mtDNA (see **6.5**) are also planned, as the validation of these results is crucial to the most far-reaching speculations of this discussion. In a similar vein, it is intended to rigorously examine the alleged novel CMS sources produced by Lemke et al. (see **7.4**) in order to prove or discount the claimed cytoplasmic transitions these plants represent. Confirmation of the results of Lemke et al. would require a rethinking of the conventional views of plant mitochondrial genome, but could be explained by some of the processes discussed in this chapter.

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Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize

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Four genomic arrangements of the maize mitochondrial *atpA* gene (encoding the α subunit of the F_1 ATPase), have been characterized. Most N (fertile) and S (male-sterile) cytoplasms contain two *atpA* arrangements of equal abundance. Prolonged exposure of blots of maize mitochondrial DNA probed with *atpA*-specific sequences show that cytoplasms previously reported to lack one of the *atpA* arrangements do contain the second arrangement but at low levels. Similarly, restriction fragments containing the *atpA* gene previously thought unique to male-sterile S and T cytoplasms are present in low abundance in fertile cytoplasms. These observations suggest that fertile and male-sterile cytoplasms of maize may be more closely related than previously thought, and suggest possible mechanisms to explain the observed mitochondrial genome diversity.

Key words: cytoplasmic male-sterility/DNA recombination/maize mitochondria/selective amplification/sublimons

Introduction

Physical and genetic maps of the mitochondrial genome of N (fertile) maize have recently been published (Lonsdale *et al.*, 1984; Dawson *et al.*, 1986). The mitochondrial DNA (mtDNA) can be represented as a 'master circle' of 570 kb, which can give rise to smaller sub-genomic circles by recombination across direct repeats. Differences in replication and recombination rates between different DNA circles gives rise to a complex multipartite genome (Lonsdale *et al.*, 1984). Restriction endonuclease cleavage patterns of maize mtDNA from different cytoplasms reveal many characteristic differences between fertile (N) cytoplasms and each of the three different male-sterile cytoplasms (CMS-C, T and S; Pring and Levings, 1978), whilst cytoplasms within any one group show only slight differences (Levings and Pring, 1977; Sisco *et al.*, 1985). Variation in the relative stoichiometry of different DNA fragments has also been reported (Borck and Walbot, 1982). The male sterile cytoplasms, C,T and S, were initially distinguished on the basis of the specific nuclear fertility restorer genes (Rf) which restore the ability of the mature plant to produce functional pollen. The designations N,C,T and S are also used to refer to the different mitochondrial genome organizations found in each of these cytoplasmic groups. These different genomic organizations have apparently arisen by multiple widespread mtDNA rearrangements, some of which have generated novel open reading frames (Dewey *et al.*, 1986), which may encode the characteristic variant polypeptides synthesized by mitochondria from the three CMS cytoplasms (Forde *et al.*, 1978).

Location of the atpA gene in the mitochondrial genome of different maize cytoplasms

The maize mitochondrial *atpA* gene has been sequenced (Braun and Levings, 1985; Isaac *et al*, 1985) and its locations on the physical map of mtDNA from N cytoplasm in a WF9 nuclear background identified (Dawson *et al.*, 1986). In this fertile cytoplasm of maize the gene lies entirely within a 12-kb repeat, with the 3′ terminus of the gene positioned 650 bases from one end of the repeat (Isaac *et al.*, 1985). Consequently, two copies of the *atpA* gene can be represented on the 570-kb 'master circle' with different 3′-flanking sequences. In contrast, C,T and some S cytoplasms of maize have been reported to contain only a single copy of the *atpA* gene (Braun and Levings, 1985; Isaac *et al.*, 1985). Mitochondrial DNA from C cytoplasms contains an *atpA* arrangement indistinquishable from one of those found in mtDNA from N cytoplasms. However, novel DNA sequences are found 3′ to the gene in mtDNA from S and T cytoplasms, so that the *atpA* arrangements in these cytoplasms can be distinguished from one another and from the forms found in the N genome (Braun and Levings, 1985; Isaac *et al.*, 1985). Thus the region of the maize mitochondrial genome around the *atpA* gene(s) appears to vary considerably between cytoplasms, both in copy number and in sequence organization. This variation seemed worthy of further study, as the 12-kb repeats (including the *atpA* gene) have been reported to be involved in inter- and intra-molecular recombination (Lonsdale *et al.*, 1984), and thus would be expected to be important in determing the genome structure.

We report in this paper that this region of the mitochondrial genome exhibits considerable variation within the cytoplasmic groups N and S. These variations are apparently due to large differences in stoichiometry of existing genomic arrangements rather than to the *de novo* creation of novel sequences by recombination. We also report that similar variations in stoichiometry account for some of the previously reported diversity in this region of the genome between the cytoplasmic groups N,T and S, and discuss the implications of these results in our understanding of the mechanisms involved in the evolution of the mitochondrial genome organizations of the various classes of maize cytoplasm.

Results

In this study we have screened mtDNA from a number of different maize cytoplasms in a variety of nuclear backgrounds with *atpA*-specific gene probes to examine the variability in the organisation of the 12-kb repeat region (containing the gene). A DNA probe (ALXR18) covering the 5′-end of the *atpA* gene hybridized to a 3.5-kb *Bam*HI fragment in each case (see Figure 1A), whereas the probe BLSCI, covering the 3′ end of the gene and the immediately adjacent flanking sequence, distinguished four *atpA* arrangements, designated types 1–4 (see Figure 1B). The predominant types of the gene found depended on the

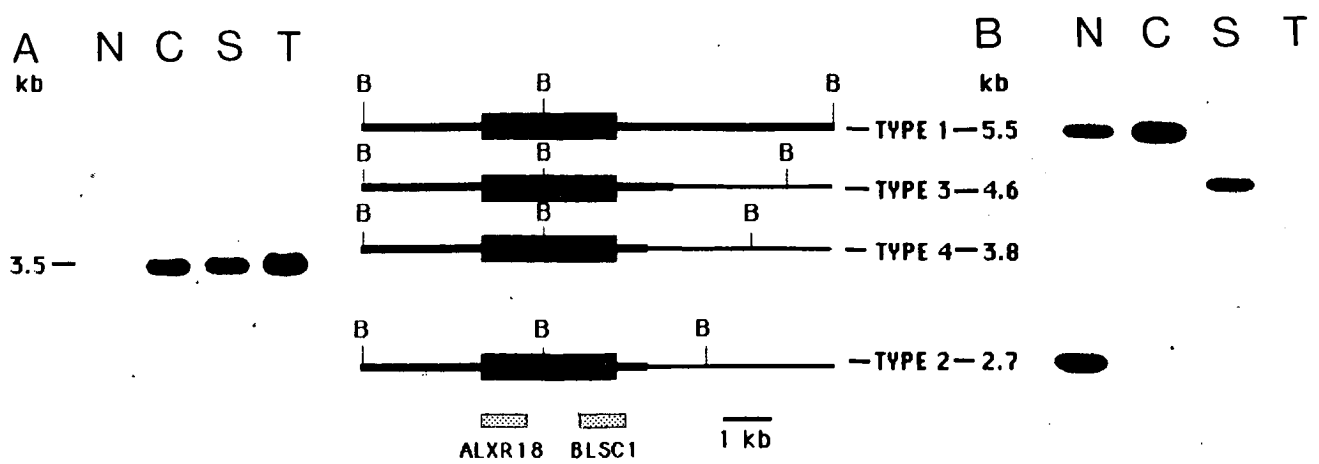

Fig. 1. Location of the gene for the α-subunit of F_1 ATPase (*atpA*) in the mitochondrial DNA of N, C, S and T cytoplasms of maize. Hybridization of (**A**) ALXR18 and (**B**) BLSC1 *atpA* probes to *Bam*HI-digested maize mitochondrial DNA from N, C, S and T cytoplasms.

▒ region covered by probes.
▬ approximate extent of homology to Type 1 clone.
B BamHI restriction site.
atpA coding region.

The probe BLSC1 distinguishes the four major *atpA* gene arrangements found in maize mitochondria. The same DNA probed with ALXR18 shows that all four arrangements are identical at the 5′ end. We have designated the four *atpA* arrangements as types 1–4 as shown.

cytoplasm of the line studied, and varied even within the classification of N,T,C or S (see Table I). A survey of many different nuclear/cytoplasmic combinations indicated that the nuclear genotype did not appear to affect which of the *atpA* types were predominant in any cytoplasm (see Table I).

Sub-stoichiometric levels of atpA gene types in different cytoplasms

Most, but not all, N and S cytoplasms were found to contain two predominant *atpA* types (see Table I). In those N and S cytoplasms containing only one predominant *atpA* type, the other expected type could always be detected, albeit at very low levels (see Figure 2, lanes 2, 3 and 7, and Table II). In addition, we could detect anomalous *atpA* gene arrangements in N mtDNA, not accounted for in the published map of the N mitochondrial genome, and not explainable as recombination products between any of the known repeats in the genome. Some of these rearrangements were characteristic of CMS lines, and were again present only at low stoichiometry (see Figure 2 and Table II). Mitochondrial DNA cut with restriction enzymes other than *Bam*HI gave similar results (see Figure 3), indicating these faint hydridization signals are truly due to sub-stoichiometric levels of the *atpA* types, rather than to hybridization to sequences showing only partial homology to the probe. Other faint hybridization signals have been seen reproducibly in these experiments which do not correspond to any of the designated *atpA* types. These signals may be other sub-stoichiometric *atpA* arrangements which have not been found in an amplified form in any cytoplasm, or may represent regions of the genome at 'normal' stoichiometry which contain only partial homology to the probe.

One possible explanation of these observations was that the seed stocks we had used for these experiments contained heterogeneous mixtures of cytoplasms, and the apparently sub-stoichiometric fragments were due to a small subgroup of the seeds containing the anomalous *atpa* types at normal, abundant levels. We investigated this possibility by extracting mtDNA from immature cobs of individual plants and probing this mtDNA with BLSC1 (Figure 4). Such experiments revealed no differences between the *atpA* hybridization patterns of mtDNA from single plants and those from pooled seedling coleoptiles.

Table I. Predominant *atpA* types in different maize cytoplasms

atpA types	Maize lines (written as nuclear genotype-cytoplasmic genotype)
1 and 2	B37-N, B73-N, WF9-N, M825-N, 38/11-N, Co192×WJ-N, CO192×WJ-234, CO192×WJ-LF, R181-N (N cytoplasms)
2 only	ECU321[a], ECU398[a], CUN443[a], CO192×WJ-SG, CO192×WJ-OY (N-like cytoplasms)
1 only	B37-C, WF9-C, CO192×WJ-F (C cytoplasms)
3 only	B37-S, 38/11-S, M825-S, WF9-S, WM13-S, CO192×WJ-J (S cytoplasms)
2 and 3	WF9-ML, WF9-RD, M825-Vg, CO192×WJ-MY, CO192×WJ-H, CO192×WJ-PS, CO192×WJ-G, CO192×WJ-I, CO192×WJ-B, CO192×WJ-SD, CO192×WJ-L, CO192×WJ-D, CO192×WJ-CA, WI82BN-CA (S-like-cytoplasms)
4 only	PUN6[a], WI82BN-181, CO192×WJ-181 (N-like cytoplasms); WI82BN-T, B37-T, WF9-T, CO192×WJ-Q (T cytoplasms)

[a]ECU321, ECU398, CUN443 and PUN6 are male-fertile South American accessions with restriction enzyme cleavage profiles similar to commercially used N cytoplasms. ECU321 and ECU398 contain R episomes (Weissinger *et al.*, 1982).

Discussion

Our observations show that the *atpA* gene is located in at least four genomic environments (referred to as *atpA* types) in different maize cytoplasms. This region of the genome is a sensitive indicator of evolutionary relationships, separating several sub-groups of cytoplasms within the usual groupings of N and S. The *atpA* probe used in this study has also separated cytoplasms (e.g. CA versus J) previously placed in the same CMS-S subgroup on the basis of fertility restoration and restriction endonuclease digestion patterns of mtDNA (Sisco *et al.*, 1985). The most interesting finding, however, is that the N-like cytoplasms 181 and PUN6 have *atpA* hybridization patterns unlike

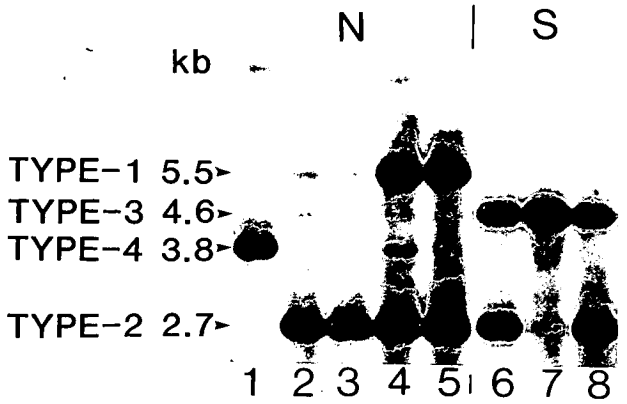

Fig. 2. Sub-stoichiometric restriction endonuclease fragments containing the *atpA* gene in maize mitochondrial DNA. One-day exposures of autoradiographs of *Bam*HI-digested maize mtDNA (nuclear genotype CO192 × WJ, 5 μg/track) probed with BLSC 1 (see Figure 1). **lane 1**, 181 cytoplasm; **lane 2**, SG cytoplasm; **lane 3**, OY cytoplasm; **lane 4**, 234 cytoplasm; **lane 5**, N cytoplasm; **lane 6**, D cytoplasm; **lane 7**, J cytoplasm; **lane 8**, CA cytoplasm. **Lanes 1–5** are fertile 'N-like' cytoplasms, **lanes 6–8** are CMS-S cytoplasms. Faintly hybridizing bands can be seen in all tracks, some of which correspond in size to predominant bands found in other cytoplasmic types. All the N cytoplasms shown, except 181, appear to contain *atpA* types 1, 2 and 3 in detectable amounts. The cytoplasm 234 also contains type 4 in low levels. The S-like cytoplasm J contains only a low level of type 2, the other two S cytoplasms shown have equal amounts of types 2 and 3. Similar results were obtained with a wide range of other nuclear/cytoplasmic combinations (Table II). Special care was taken to avoid cross-contamination of samples by preparing mitochondria and mtDNA from fertile and male-sterile lines on different days.

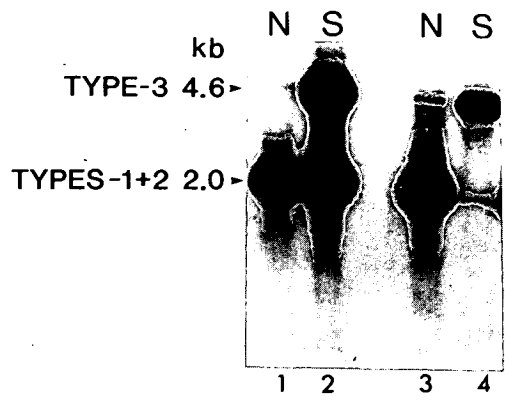

Fig. 3. Two-day exposure of an autoradiograph of maize mitochondria DNA digested with *Eco*RI and probed with BLSC1 (see Figure 1). **lane 1**, M825/Oh07-N; **lane 2**, M825/Oh07-Vg; **lane 3**, WF9-N; **lane 4**, WF9-S. *atpA* types 1 and 2 are inseparable in an *Eco*RI digest. The two N mtDNAs show small amounts of type 3, the two S mtDNAs show either a high level of type 1 + type 2 (Vg cytoplasm, **lane 2**) or a low level (S cytoplasm, **lane 4**). All DNAs are loaded at 4 μg/track. Similar results were seen with the enzymes *Pst*I and *Hin*dIII.

Table II. Stoichiometries of *atpA* types in the mitochondrial genomes of N-like and S-like maize cytoplasms

N-like cytoplasms	*atpA* types detected			
	1	2	3	4
B73-N	+++++	+++++	+	
M825/Oh07-N	+++++	+++++	+	+
WF9-N	+++++	+++++	+	+
CO192×WJ-N	+++++	+++++	+	
CO192×WJ-234	+++++	+++++	+	++
CO192×WJ-OY	+	+++++	+	
CO192×WJ-SG	+	+++++	+	
ECU321[a]	+	+++++	+	
CUN443[a]	+	+++++	+	
PUN6[a]	+			+++++
W182BN-181	+			+++++
CO192×WJ-181				+++++
S-like cytoplasms				
B73-S		+	+++++	
WF9-S		+	+++++	
CO192×WJ-J		+	+++++	
W182BN-S		+	+++++	
W182BN-CA		+++++	+++++	
M825/Oh07-Vg		+++++	+++++	
CO192×WJ-CA		+++++	+++++	
CO192×WJ-D		+++++	+++++	

Number of crosses represents approximate relative abundance. Designations are written as nuclear genotype-cytoplasmic genotype (where both are known).

[a]ECU321, CUN443 and PUN6 are male-fertile South American accessions with restriction enzyme cleavage profiles similar to commercially used N cytoplasms. ECU321 contains R episomes (Weissinger *et al.*, 1982).

all other N cytoplasms examined (see Figures 2 and 4 and Table II), but similar to that of mtDNA from T mitochondria, implying that they may be related to the progenitor of T cytoplasm.

The *atpA* types described in this paper are present at different stoichiometries in a wide range of maize cytoplasms. As it is unlikely that all the maize plants we have studied are chimaeras of cell types containing different mitochondrial genomes, the sub-stoichiometric *atpA* types presumably form parts of larger circular or linear molecules present in low copy number relative to the rest of the mitochondrial genome in the same cell. These sub-stoichiometric molecules ('sublimons') probably originated as a result of infrequent recombination events between very short regions of homology not marked on the published map of the WF9N mitochondrial genome. Our data suggest that these recombinant molecules are retained through many plant generations, as we can find sub-stoichiometric *atpA* types in most of the maize lines we have examined. Hence these molecules must be maintained by normal replication or are continually being formed by *de novo* recombination. If these rare recombinant molecules are being continuously formed by *de novo* recombination then one would expect the proportion of these molecules in the genome to rise if they were replicated at normal rates as well, until an equilibrium is reached with the reverse reaction. Given that the very low levels we found for some *atpA* types are far from the expected equilibrium levels one must assume lower net replication rates for these sub-stoichiometric molecules if *de novo* recombination is the primary mode of formation. However, these sub-stoichiometric *atpA* types include at least part of the 12-kb repeat, which has been shown by circumstantial evidence to be recombinationally active (Lonsdale *et al.*, 1984), and rapid intermolecular recombination would be expected to compensate for inequalities in the efficiency of replication origins on different molecules. The simplest assumption, therefore, is that the sub-stoichiometric *atpA* types are maintained by replication and the contribution by *de novo* recombination is small. There is also the possibility that the mechanics of cell division could preferentially keep sub-stoichiometric molecules in germ-line cells.

Evolution of mitochondrial genome diversity in maize

The evolutionary relationships between the different maize cytoplasms are obscure. The fertile N and three CMS cytoplasms

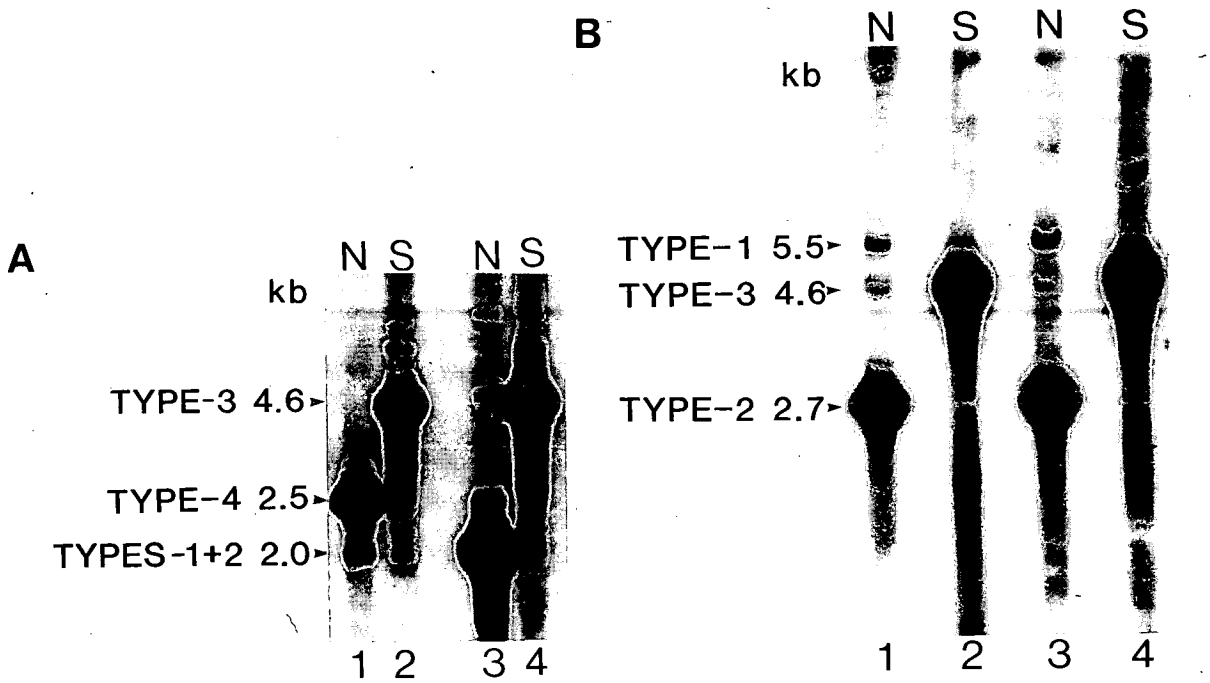

Fig. 4. Sub-stoichiometric *atpA* hybridizing fragments exist in the mitochondrial DNA from single plants. (**A**) Three-day exposure of *Eco*RI-digested maize mtDNA probed with BLSC1 (see Figure 1). **lane 1**, W182BN-181; **lane 2**, W182BN-S; **lane 3**, WF9-N; **lane 4**, WF9-S. The mtDNAs used in this experiment were purified from single immature cobs. A faint band representing type 1 and/or 2 can be seen in **lane 1** (181 cytoplasm), and also but less clearly in **lanes 2** and **4** (S cytoplasm). **Lane 3** (N cytoplasm) contains faint bands representing the type 3 and type 4 arrangements. Similar results were obtained after cleaving the mtDNA from other individuals of the same lines with *Bam*HI (data not shown). These observations rule out heterogenous seed populations as a major factor in explaining our results. Higher mol. wt fragments hybridizing in **lanes 2, 3** and **4** probably present partial digestion products. (**B**) Three-day exposure of *Bam*HI-digested maize mtDNA probed with BLSC1 (see Figure 1). **lanes 1** and **3**, CO192 × WJ-SG; **lanes 2** and **4**, CO192 × WJ-J. Each lane contains the mtDNA of a single immature cob. The SG mtDNA shows low levels of types 1 and 3 clearly, as predicted by the same experiment on pooled coleoptile mtDNA (Figure 2, lane 2). The J mtDNA shows no clear faint bands, but the expected low levels of the type 2 fragment (Figure 2, lane 7) may be obscured by the smear of degradation products from the abundant type 3 fragment.

differ widely in mtDNA organizsation, and it is commonly thought that the different cytoplasms diverged in pre-history. CMS cytoplasms can be maintained in natural populations by the presence of 'fertility restorer' genes present in the nucleus of many natural maize populations (Duvick, 1965), and thus may well be ancient. However, no obvious intermediates between the various cytoplasmic groups N,C,S and T have been identified, and in fact analysis of mtDNA from ancient maize lines indigenous to Latin America have revealed remarkable similarity between different fertile accessions and the cytoplasms of modern inbred lines (Weissinger *et al.*, 1982; Kemble *et al.*, 1983). Either the evolutionary intermediates of the different maize cytoplasms have become extinct, and/or the evolution of the different cytoplasms occurred by sudden catastrophic events. Such sudden genome alterations in maize have been reported after the introduction of the nuclear recessive mutation *iojap*, which apparently induced a change from N to S cytoplasm (Lemke *et al.*, 1985). However, the results of Lemke *et al.* have not been widely accepted because of the difficulty in explaining the molecular events needed to effect such a transformation. The genomes of N,S and T differ at a large number of loci, with multiple insertions, deletions, point mutations and rearrangements of sequences (Schardl *et al.*, 1984; Leaver *et al.*, 1985; Dewey *et al.*, 1986). It appears inconceivable that such widespread, apparently random rearrangements could occur identically to generate the S mtDNA organization on a number of independent occasions.

Further evidence of rapid mitochondrial genome reorganization is available in other species following somatic hybridization of protoplast fusion. These hybrids frequently contain mtDNA restriction fragments not representative of either parent, apparently indicating widespread recombination and reorganization within a single plant life-cycle (Belliard *et al.*, 1979; Boeshore *et al.*, 1983). An alternative explanation for sudden genome reorganizations is selective amplification of pre-existing sub-stoichiometric (possibly undetectable) molecules (perhaps together with a reduction of previously abundant molecules). Amplification of sub-stoichiometric sequences has been previously suggested to explain some changes in mtDNA organization (Morgens *et al.*, 1984). Our data suggest that such sub-stoichiometric DNA molecules exist in maize mitochondria. The extent of such apparent reorganization obviously depends on the number of novel sequence arrangements present at very low levels in plant mitochondria. As yet we only have evidence for sub-stoichiometric molecules including the maize *atpA* gene region, though others may exist.

Our data imply that the products of rare or unique recombination events may in some cases be retained in the genome for many generations at low levels. These rare recombinant molecules might be expected to show rapid molecular evolution; firstly, because mutational events would be more easily 'fixed' in such a small population, and secondly, because there would be no selection pressure against mutation on these low abundance molecules as they cannot be expected to have any direct functional significance. [It has recently been shown that the rice chloroplast genome may be heterogeneous, consisting of a major functional genome and a minor rearranged genome, in a similar manner to the situation we describe (Moon *et al.*, 1987)]. Thus one would expect these molecules to progressively accumulate mutations and rearrangements more rapidly than the rest of the genome. Occasionally one of these rearrangements

may lead to the amplification of a previously sub-stoichiometric molecule to 'normal' stoichiometry (possibly with concomitant reduction of other abundant molecules). The pattern of *atpA* types found in various N and S cytoplasms suggests that this can happen. If this previously sub-stoichiometric molecule had accumulated mutational events and rearrangements the resulting genome would exhibit a novel restriction pattern. One prediction of this hypothesis would be that the amplication of the sub-stoichiometric molecule would be expected to generate large new repeats in the genome. A possible example of this phenomenon is the TURF2H3 region of the T genome (Dewey *et al.*, 1986). This region of DNA appears to have been generated by at least seven recombination events, involving flanking and coding sequences of the maize mitochondrial 26S ribosomal RNA gene, the ATPase subunit 6 gene, and the chloroplast tRNA-Arg gene. The degree of conservation of the component sequences in this region with their homologous sequences in N mtDNA varies, implying temporal separation of the formative recombination events, and yet no evolutionary intermediates have been discovered. The 5′ region of the *atp6* gene, which forms part of this region, is present as a large repeat in the T genome. Thus this chimaeric region of the T genome fits all the criteria expected of a sequence which has evolved as a low abundance constituent of mitochondrial DNA.

Materials and methods

Mitochondria were isolated from 4-day-old coleoptiles or immature cobs as described previously (Leaver *et al.*, 1983), except that purification of mitochondria from coleoptiles was carried out on a discontinuous sucrose gradient (Boutry and Briquet, 1982). Mitochondrial DNA was isolated by solubilization of the mitochondria in 0.5% *N*-lauroyl sarcosine (Sigma), 100 mM EDTA, 100 mM Tris−Cl (pH 8.0) 0.1 mg/ml Proteinase K (Boehringer) for 1 h at 60°C, followed by CsCl density gradient centrifugation in the presence of 75 μg/ml ethidium bromide as described by Fox (1979). After centrifugation, the ethidium bromide was removed by extraction with butan-1-ol, and the CsCl was removed by two ethanol precipitations followed by washing with cold 70% ethanol. 2−5 μg of mtDNA from each maize line was digested to completion with the chosen restriction endonuclease, fractionated on a 0.8% agarose (Sigma type II) gel and transferred to nylon filters (Hybond-N, Amersham). These filters were pre-hybridized for 30−60 min in hybridisation buffer (0.6 M NaCl, 60mM Na_3citrate (4 × SSC), 0.1% (w/v) SDS, 50 mM sodium phosphate (pH 5.5), 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinyl pyrrolidone (mol. wt 40 000) and 200 μg/ml denatured herring sperm DNA) at 65°C. Single-stranded M13 sequencing clones of the *atpA* gene (Isaac *et al.*, 1985) were used to generate ^{32}P-labelled probes (Hu and Messing, 1982), which were hybridized to the nylon filters under stringent conditions (65°C in hybridization buffer) overnight. The filters were then washed in one or two changes of fresh hybridization buffer (lacking herring sperm DNA) at 65°C, followed by washing in 2 × SSC for 30 min at room temperature. The filters were then exposed to pre-flashed Curix RP1 X-ray film (Agfa-Gevaert)(Figure 1) or Amersham Hyperfilm (Figures 2, 3 and 4) for 1−7 days at −80°C.

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