The integrated forms of the S1 and S2 DNA elements of maize male sterile mitochondrial DNA are flanked by a large repeated sequence

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ABSTRACT

The mitochondrial DNA of maize was cloned using the cosmid, Homer I. Recombinants carrying sequences homologous to the S1 and S2 DNA elements of male sterile maize have been analysed. Restriction endonuclease maps for Sac II, Sma I and Bam HI have been constructed. The S1 and S2 sequences are single copy sequences occurring at unique sites; each is flanked by a 26 kb repeated sequence. The repeated sequence has been shown not to contain the mitochondrial ribosomal RNA genes.

INTRODUCTION

Cytoplasmically inherited male sterility in maize (*Zea mays* L.) has been correlated with alterations in the organisation and expression of the mitochondrial (mt) genome (1,2). In maize, four genetically different cytoplasms are known; N: normal, allowing normal fertile pollen development and T, C and S which cause pollen sterility. MtDNA from S-cytoplasm is distinguished by two free replicating DNA species, S1 (6.2 kb) and S2 (5.2 kb) which are not seen in N, T or C cytoplasms (1). Sequences cloned from the S1 and S2 DNA elements hybridize to high-molecular-weight mtDNA in N cytoplasm, suggesting that the S1 and S2 DNA elements exist in an integrated state. In contrast it would appear that sequences homologous to the S1 and S2 DNA elements are essentially absent from the mt genomes of C and T cytoplasms (3). The apparent reintegration of the S1 and S2 DNA elements into high-molecular-weight mitochondrial DNA in S cytoplasm is correlated with restoration of fertile pollen production (4). In all cases the hybridisation patterns appear complex and are not easily explained by a single excision or integration event (3,4).

The mtDNA of N-cytoplasm has been cloned into the cosmid vector, Homer I. Using S1 and S2 sequence probes, the number of integrated copies of S1 and S2 in N-cytoplasm mtDNA has been determined as well as the structure of the DNA regions flanking the integration sites.

MATERIALS AND METHODS

a) Strains

Recombinant clones pZmS42 and pZmS21 were derived from the S1 and S2 DNA elements of S-mtDNA, respectively (3). λ mn(S⁺)9 and λ mn(S⁺)10 are recombinant clones derived from N-mtDNA, containing sequences homologous to the S2 DNA element (R.D.T., manuscript in preparation). The cosmid, Homer I, was a gift from P.W.J. Rigby (Homer I was derived from pAT153 by the insertion of a 1.7 kb Bgl II fragment, having the cos sequence of bacteriophage λ Charon 4A, into the Bam HI site). The λ packaging strains BHB2688 and BHB2690 were a gift from B. Hohn.

b) Isolation of mtDNA

MtDNA was isolated from normal maize lines, WF9 or B37, essentially as previously described (5), except that the mitochondria were lysed with 1% sodium dodecyl sulphate and the DNA purified by CsCl equilibrium centrifugation, $\rho_{\text{ave}} = 1.7\text{g/cc}$. The CsCl gradients were fractionated, and an aliquot of each fraction was electrophoresed on a 1% (w/v) agarose gel in order to locate the mtDNA. The mtDNA was dialysed against 1mM Tris-HCl pH 8.0, 1 mM NaCl, 0.1 mM EDTA and stored frozen at -20°C .

c) Cloning of mtDNA

WF9-N mtDNA was partially digested with Hae III and sized on a 15 ml, 10.4 - 21% (w/v) sucrose gradient by centrifugation at 25,000 rpm, 4°C for 19 h in an MSE 6 x 16.5 rotor. The gradient was fractionated into four size classes: 1, > 50 kb; 2, 35-50 kb; 3, 22-35 kb and 4, 12-22 kb.

Homer I was linearised with Eco RI and the sticky ends filled in using the large (Klenow) fragment of E. coli DNA polymerase I (Boehringer, batch No. 1278113).

Following the polymerase reaction the Homer I had lost the capacity to self ligate. DNA in each size class (2, 3 or 4) of the partially digested mtDNA and the linear Homer I DNA were mixed in equimolar amounts and blunt end ligated using T4 DNA ligase (BRL). Approximately 1 μg mtDNA was ligated to 1 μg Homer I DNA with 2 Weiss units of T4 DNA ligase in a 25 μl reaction volume for 4 h at 20°C .

In vitro λ packaging was done by the method described by Blattner (6), using the packaging strains BHB2688 and BHB2690 (7). The efficiency of each set of packaging extracts was assayed using λ C1857 Sam 7 and was typically $1-2 \times 10^8$ plaques per μg DNA. The packaging reactions were diluted 3-4 fold with phage buffer (22 mM KH_2PO_4 , 49 mM Na_2HPO_4 , 85.6 mM NaCl, 1 mM MgSO_4 , 1 mM CaCl_2 and 0.001% gelatin) and mixed with 2 vol

E. coli HB101 which has been grown to $E_{650}^{1\text{cm}}$ 1.0 in L-broth, 0.4% maltose, pelleted and resuspended in $1/20$ vol 0.01 M Mg Cl₂. Cells were incubated for 15 min at 20°C and then plated on to L agar containing 50 µg/ml ampicillin and incubated at 37°C for 1-2 days.

Packaging efficiencies were 1220, 624 and 114 colonies per µg mtDNA for size classes 2, 3 and 4 respectively.

Cloning and propagation of clones was performed in category II containment as defined by G.M.A.G. (U.K.).

d) Maintenance of the recombinants

Individual colonies were picked into 0.2 ml L-broth containing 50 µg/ml ampicillin in 96-well microtitration plates (Flow Laboratories). The colonies were arranged into arrays of 8 x 10, grown to saturation by incubating for 2 days at 37°C. Maintenance and storage was as described by Gergen *et al.* (8). Replicas of the cultures were made on to L-agar-ampicillin prior to their transfer to Whatman 540 filter paper.

Clones were identified as cos ZmNmt(X)c(Y), where x is the gradient fraction number and Y is the clone number. The labelling system is abbreviated to (X)c(Y) e.g. 2c75.

e) Growth of cosmids and isolation of DNA

Cosmid DNA was isolated from 100 ml chloramphenicol-amplified cultures. Cleared lysates (9) were extracted with 75 mM NaCl, 50 mM EDTA pH 8.0 saturated phenol. The aqueous phase was further extracted with phenol-chloroform (1:1) and the nucleic acids precipitated by the addition of 2 vol ethanol. The nucleic acids were resuspended in 2 ml 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM EDTA; RNAase A and RNAase T1 were added to 100 µg/ml and 10 units/ml, and dialysed overnight at 4°C. Ammonium acetate was added to 0.2 M and the DNA precipitated by addition of 2 vol ethanol. Samples were vacuum dried and resuspended in 0.5 ml 1 mM Tris-HCl pH 8.0, 1 mM NaCl, 0.1 mM EDTA. Yields of cosmid DNA tended to vary considerably between 100 ng to 2.0 µg per ml of amplified culture. Contaminating E. coli was estimated to be generally less than 10% and did not prove to be a problem in these studies.

f) Enzymes

Restriction endonucleases Sac II and Sma I were purchased from New England Biolabs. Bam HI was purified on ultragel (LKB), DE-52 (Whatman) and Biorex (Biorad). T4 DNA ligase was purchased from BRL, E. coli DNA polymerase from Boehringer and Polynucleotide Kinase from P-L Biochemicals.

g) Restriction enzyme analysis

Restriction endonuclease digests were electrophoresed in the presence of 0.5 µg/ml ethidium bromide, in horizontal slab agarose gels at 2v/cm for 16-24 h. Bands were visualized and photographed under 256 nm illumination. DNA was transferred to nitrocellulose (Millipore), as described by Southern (10). The molecular weights of restriction fragments were determined relative to fragments of known molecular weight. Agarose gels, 0.25%, were used to determine molecular weights between 49 to 5 kb; 1% agarose gels allowed determination of size in the 8 to 0.5 kb range. It is possible that very small fragments (for example 200 bp or less) may have been missed.

h) Labelling of nucleic acids and hybridisation

DNA was labelled by nick translation using α -³²P-dATP (Amersham > 400 (Ci/mMol) as described elsewhere (11,12). RNA was 5'-labelled following partial alkaline hydrolysis with γ -³²P-ATP (Amersham, >2000 Ci/mMol) and polynucleotide kinase (13).

Unincorporated nucleotide triphosphates were removed by chromatography on a 1.2 ml Sephadex G100 column constructed in a 1 ml disposable syringe.

The procedure for hybridisation of Whatman 540 paper or nitro-cellulose were essentially similar (8,14). Filters were prehybridised for a minimum of 1 h in hybridisation buffer and the ³²P-labelled DNA or RNA was injected directly into the hybridisation bags.

RESULTS

Identification of cosmids carrying S1 and S2 sequences

The recombinants, pZmS42, carrying an S1 DNA sequence, and λ mn(S⁺)9 and λ mn(S⁺)10, carrying the S2 DNA sequences, were used as probes to identify clones containing homologous sequences, Table 1. The cosmid DNAs were initially screened to check their size (Fig. 1). Two recombinants 2c12 (Fig. 1, track 2) and 2c68 (Fig. 1, track 6) carried obvious deletions and were omitted from the study. Further checks for deletions were made by hybridising the nick translated cosmid DNA to Bam HI digests of the respective cosmid and mtDNA. For any cosmid carrying a unique mtDNA sequence all the Bam HI fragments should co-align with mt genomic Bam HI fragments except for the cosmid fragment containing the Homer I sequence and its mt flanking sequences.

The mtDNA sequences attached to the Homer I sequence should hybridise to a further two genomic Bam HI fragments, these being the fragments flanking the Bam HI fragments of the cosmid. However, all the recombinant

Table 1. Recombinants carrying sequences homologous to S1 and S2 identified from 80 selected clones.

	Probe		
	λ mn(S ⁺)9	λ mn(S ⁺)10	pZmS42
Positive Colonies	2c12	2c12	2c11
	2c44	2c13	2c27
	2c75	2c36	2c36
	2c77	2c44	2c73
		2c68	2c68
		2c73	
		2c75	
		2c77	

2c13 was subsequently demonstrated not to contain any homology to the probe λ mn(S⁺)10.

cosmids hybridised to more genomic Bam HI fragments than expected for a unique mtDNA sequence. Therefore sequences are present in these cosmids which must be repeated in the mt genome. The results are illustrated by hybridisation of 2c44 to a Bam HI digest of mtDNA (Fig. 2). Four fragments not found in a 2c44 Bam HI digest can be readily seen. The 6.5 kb and probably the 4.8 kb Bam HI fragments flank the Bam HI insert fragments of 2c44 (see Fig. 3). The 3.9 kb Bam HI fragment is partly homologous to the 2.1 kb Bam HI fragment (see Fig. 3). The 3.6 kb Bam HI fragment hybridises with all the S1 and S2 recombinant cosmids with the possible exception of 2c36. The sequence responsible for this cross-hybridisation in the S1 and S2 recombinant cosmids has not yet been identified, nor is the genomic localisation of this 3.6 kb Bam HI fragment known.

Figure 1. Analysis of the DNA preparations of 2, 2c12; 3, 2c13; 4, 2c36; 5, 2c44; 6, 2c68; 7, 2c73; 8, 2c75 and 9, 2c77 on a 0.5% agarose gel. Tracks 1 and 10: λ and Homer I, FI.

Figure 2. Bam HI digest of 2c44 (A) and B37-N mtDNA (B) probed with nicktranslated 2c44. X: Bam HI restriction fragment containing the Homer I sequence.

As most Bam HI fragments of related cosmids co-align, and also co-align with the Bam HI genomic fragments, deletions within these fragments can be excluded. Two deletion events have been detected, a 0.1 kb deletion from the 1.8 kb Bam HI fragment of 2c11 (compare tracks 1 and 4, Fig. 4A) and a 2.0 kb deletion from the 6.9 kb Bam HI fragment of 2c73 (compare tracks 1, 3 and 4 with track 2, Fig. 4C and D). This latter fragment contains DNA sequences homologous to the S1 DNA element found in S mtDNA.

Restriction mapping

The DNA isolated from the cosmids identified with λ mn(S⁺)9, λ mn(S⁺)10 and pZmS42 were analysed with the restriction enzymes Sac II, Sma I and Bam HI. Restriction maps were constructed using overlaps between the clones and Southern hybridization analysis. It was apparent from the Sac II and Sma I data (Table 2) that by aligning the Sac II sites defining the 20.5 kb restriction fragment common to 2c44, 2c75 and 2c77 clones (identified by λ mn(S⁺)9) and 2c36, 2c73, 2c27 and 2c11 clones (identified by pZmS42) that a large repeated sequence existed (Figure 3).

Copy number and size of the repeated sequence

The number of copies of the repeated sequence appears to be two, based

Table 2. Molecular weight (kb) of mtDNA fragments

	Recombinant						
	2c11	2c27	2c36	2c73	2c44	2c75	2c77
Sac II Fragments	20.5	20.5	32.5*	20.5*	26.0*	20.5	20.5
	13.7	18.8*	13.7	20.5	15.5	15.5*	18.8*
	10.5*	3.57		3.57	3.8	4.85	3.8
	3.57				3.05	3.8	
					1.47		
Sum of all fragments	47.37	42.87	45.3	44.57	49.82	44.7	43.1
Sma I Fragments	40.0*	36.0*	27.5		19.5*	27.0*	25.0*
	5.7	5.7	17.0*		8.1	6.5	6.5
					6.5	5.7	5.7
					5.7	3.65	3.65
					3.65		
					2.4		
					1.16		

* Fragment containing the Homer I sequence.

on several observations: (1) probing the gene bank with cosmid sequences carrying the entire repeat does not identify any further clones; (2) no further fragments homologous to the Sac II 3.57 kb and 3.8 kb unique-repeat junction fragments have been identified. The equivalent Bam HI fragments, 2.1 kb and 3.9 kb, are the only two homologous unique-repeat junction fragments identified by probing Bam HI digested mtDNA with λ mn(S⁺)10.

(3) The 4.85 kb Sac II fragment of 2c75 has been determined to be present in exactly the same stoichiometry as the 3.57 kb and 3.8 kb Sac II unique-repeat junction fragments (Fig. 6). This indicates that the entire repeat is contained within 2c75 and its ends are defined by the 3.57 and 4.85 kb Sac II fragments. The repeat length is therefore approximately 26 kb.

The relative orientation of the repeats cannot be determined at present as no DNA sequence linking the repeats has been isolated.

Localisation of the S1 and S2 DNA sequences

Bam HI digests of the cosmid DNAs were probed with λ mn(S⁺)10, pZmS42 and pZmS21, Fig. 4. λ mn(S⁺)10 hybridised not only to its own two component Bam HI fragments of 2.1 kb and 0.95 kb (Fig. 4B) but also to two other fragments: (1) a 4.4 kb fragment which is homologous to the Bam HI insert fragment in λ mn(S⁺)9 and (2) a 3.9 kb fragment which cross hybridises to the 2.1 kb λ mn(S⁺)10 fragment. The 3.9 and 2.1 kb fragments represent the unique-repeat junctions flanking the S1 and S2 DNA sequences (Fig. 3).

Hybridisation with the essentially S1 specific probe pZmS42 identified

Figure 3. Restriction endonuclease maps of regions of the mt genome spanned by cosmids containing sequences homologous to (A), the S1 DNA element and (B), the S2 DNA element. For example 2c36 indicates the cosmid, the length of the mt DNA insert and the region of the mt genome which it covers, see Table 2. [stippled box], identifies the Bam HI fragment carrying homology to the S1 specific probe, pZmS42 (Fig. 6 and text). [dotted box], identifies the fragments hybridizing to λ mn(S⁺)9 and 10, but excluding the homology due to the repeated sequence (see Fig. 6 and text). Restriction fragments of 2c36 not overlapped by 2c11 have been omitted. Similarly, restriction fragments of 2c44 not overlapped by 2c75 have been omitted. The block diagrams illustrate the position of the integrated S1 and S2 sequences relative to the repeated sequence, which are those sequences contained within the two vertical broken lines.

the expected 6.9 kb Bam HI fragment (3). The cosmid 2c73 apparently has suffered a deletion of 2.0 kb within the Bam HI fragment carrying the S1 sequence (Fig. 4, C and D, track 2). Faint hybridisation to the 4.4 and 0.95 S2 Bam HI fragments was also observed consistent with its small amount of homology with S2 (3).

Hybridisation of the recombinant pZmS21, an S1 probe with homology to S2 demonstrates that the homology is to the 4.4 kb S2 Bam HI fragment. No homology to the 2.1 kb and 0.95 S2 fragments was observed. The position of the S1 and S2 DNA sequences in their integrated form are therefore found to be adjacent to the repeats (Fig. 3).

Does the 26 kb repeated sequence encode the rRNA genes ?

In order to determine whether the repeated sequences flanking the S1

Figure 4. A: Bam HI digests of 1, 2c36; 2, 2c73; 3, 2c27; 4, 2c11; 5, 2c75; 6, 2c77 and 7, 2c44 run on a 1% agarose gel. Autoradiograms of Bam HI digests probed with: B, λ mn(S⁺)10; C, pZmS42 and D, pZmS21.

▼: fragments resulting from a partial digest of the DNA.

and S2 DNA sequences contained the rRNA genes, 5'-labelled mtrRNA was hybridised to Sac II restriction digests of mtDNA and the S1 and S2 cosmid DNAs (Fig. 5). The 5'-labelled mtrRNA hybridised strongly to 2c13, a mt rRNA recombinant, in contrast to all the cosmids containing mtDNA sequences from the 26 kb repeat and its flanking sequences. This indicates that the mt rRNA genes are not located either in or near the 26 kb repeated sequence. This was confirmed when labelled RNA was hybridised to the colony bank: all clones giving positive signals were unrelated to those associated with

Figure 5. A: Sac II digest of 1, 2c44; 2, 2c77; 3, B37-N mtDNA; 4, 2c75; 5, 2c73; 6, B37-N mtDNA; 7, 2c36 and 8, 2c13. Autoradiograph following hybridisation with 5'-labelled mtRNA.

the S1 and S2 DNA sequences (unpublished data).

Estimation of genome size

As S1 and S2 have unique flanking sequences it can be assumed that they only occur once in the genome. The S1 sequence was found in 4/79 and the S2 sequence in 3/79 clones (Table 1). Two cosmids, 2c12 and 2c68, were found to carry deletions. The probe λ mn(S⁺)10, which carries homology not only to S2 but also to the repeated sequence, identified 9/79 clones (Table 1). Therefore, it would appear that a unique sequence, e.g. S1 or S2, is represented approximately once in every 20 clones while the repeated sequence is found in approximately 1 in 10 clones. The cosmid Homer I has a minimum insert capacity of 32 kb and a maximum of 47 kb, giving minimum and maximum genome size estimates of 640 kb and 940 kb respectively. The average insert size of the cosmids in this study was 39.4 kb (Table 2), giving an estimated genome size of 790 kb.

In a densitometric scan of a total Sac II digest of mtDNA the 4.85, 3.8 and 3.57 kb fragment areas (Fig. 6), which derive from S1 and S2 sequence cosmids, were compared with the total 'peak' area. A value for genome size determined by this method gives 426 ± 105 kb.

Figure 6. Densitometer tracing of a Sac II digest of B37-N mtDNA. 3 of the 4 Sac II unique-repeat junction fragments are indicated.

DISCUSSION

Large duplications of sequence have not been reported in any other mt genome studied so far, though *Tetrahymena* mtDNA has terminal inverted repeats of approximately 2.7 kb which code for the 21S rRNA (15). As a DNA sequence linking the S1 adjacent repeat with the S2 adjacent repeat has not yet been isolated the relative organisation of the repeats cannot be determined.

In E.M. studies of maize mtDNA, circular DNA molecules have been detected (16). All four cytoplasmic types contain a 1.94 kb plasmid species as well as other type-specific low-molecular-weight DNAs (2). In mtDNA from N-cytoplasm the size classes of the circular molecules observed and their relative number proportions are 68 kb (48%), 50 kb (33%), 100 kb (14%) and 138 kb (4%) (16). These values would allow the S1-linkage group (approximately 60 kb) and the S2-linkage group (approximately 60 kb) to reside either on different circles of the same size (e.g. 68 kb) or on different sized circles (e.g. 68 kb and 100 kb) or they could reside on the same circle (the 138 kb only). However, restriction fragments associated with the S1-linkage group and the S2-linkage group are in the same stoichiometry and also in the same apparent stoichiometry with the majority of the remaining restriction fragments (see Fig. 6) which would indicate a simpler genome than that predicted from the E.M. data (16).

In mtDNA from N, S and T cytoplasms there is apparently no common

circle size (16), apart from the 1.94 kb plasmid species (2). The proportion of circles observed as a percentage of the total mtDNA is extremely low and it is possible that they could have arisen by intra-molecular recombination events, perhaps involving the repeated sequences. Such a mechanism has been postulated for the formation of the head-to-head dimers of lettuce and spinach chloroplast DNA (17).

The chloroplast DNA of some plant species has been shown to contain two or three repeated sequences which may be in either an inverted or tandem arrangement (18,19,20). In all cases the repeated sequences include the sequences coding for the rRNAs. However, it appears that the 26 kb repeat sequence flanking the S1 and S2 sequences does not contain the rRNA genes. These have now been located elsewhere on the physical map (unpublished data).

The size of mtDNA of higher plants has been estimated to range from 100 kb to 300 kb (17,21,22). This is substantially larger than other known mt genomes (23). The mt genome of maize has been estimated at 277 kb (24) which is smaller than the estimates of 426 kb and 760 kb obtained in this manuscript. With the possibility of a complex genome, perhaps consisting of circular molecules of different sizes and the presence of repeats, the true complexity will only be obtained on completion of a physical map.

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