Volume 12 Number 15 1984 Nucleic Acids Research

Recombination sequences in plant mitochondrial genomes: diversity and homologies to known mitochondrial genes

David B.Stern+ and Jeffrey D.Palmer*

Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, CA 94305, and *Division of Biological Sciences, University of Michigan, Ann Arbor, MI 48109, USA

Received 2 April 1984; Revised and Accepted 10 July 1984

ABSTRACT

Several plant mitochondrial genomes contain repeated sequences that are postulated to be sites of homologous intragenomic recombination (1-3). In this report, we have used filter hybridizations to investigate sequence relationships between the cloned mitochondrial DNA (mtDNA) recombination repeats from turnip, spinach and maize and total mtDNA isolated from thirteen species of angiosperms. We find that strong sequence homologies exist between the spinach and turnip recombination repeats and essentially all other mitochondrial genomes tested, whereas a major maize recombination repeat does not hybridize to any other mtDNA. The sequences homologous to the turnip repeat do not appear to function in recombination in any other genome, whereas the spinach repeat hybridizes to reiterated sequences within the mitochondrial genomes of wheat and two species of pokeweed that do appear to be sites of recombination. Thus, although intragenomic recombination is a widespread phenomenon in plant mitochondria, it appears that different sequences either serve as substrates for this function in different species, or else surround a relatively short common recombination site which does not cross-hybridize under our experimental conditions.

Identified gene sequences from maize mtDNA were used in heterologous hybridizations to show that the repeated sequences implicated in recombination in turnip and spinach/pokeweed/wheat mitochondria include, or are closely linked to genes for subunit II of cytochrome c oxidase and 26S rRNA, respectively. Together with previous studies indicating that the 18S rRNA gene in wheat mtDNA is contained within a recombination repeat (3), these results imply an unexpectedly frequent association between recombination repeats and plant mitochondrial genes.

INTRODUCTION

Recent restriction mapping studies indicate that the entire sequence complexity of two mitochondrial genomes, from turnip (1) and maize (2) may be contained on a single circular molecule, or "master" chromosome. In addition, a single major repeated element in turnip mtDNA (1), and several major repeated elements in maize mtDNA (2; D. M. Lonsdale, pers. comm.), are postulated to be sites of intragenomic recombination, thereby generating smaller circular subgenomes. Evidence for analogous, repeated sequences also exists for mtDNAs from wheat (3) and spinach (D.B. Stern and J.D. Palmer, unpublished data),

although complete physical maps of these genomes are not yet available.

It is of interest to know whether intragenomic recombination is a general feature of plant mitochondrial genomes, and if so, whether recombination occurs within the same sequences in different species. Our approach has been to use filter hybridizations to investigate possible cross-homologies among the turnip, spinach, maize and wheat recombination repeats and also between them and a variety of other higher plant mtDNAs. We show here that a repeated sequence implicated in recombination in one species may not be involved in recombination in a second, despite its presence in both genomes. Furthermore, several apparently unrelated mtDNA sequences exist as recombination repeats in different species. We also find that these repeats display an unexpectedly frequent association with either structural or rRNA genes in most genomes.

MATERIALS AND METHODS

Watermelon (*Citrullus lanatus* cv. Florida Giant), zucchini (*Cucurbita pepo* cv. Zucchini dark green) and muskmelon (*Cucumis melo* cv. Hales's best jumbo) seeds were purchased from Ferry-Morse, Inc. Cucumber seeds (*Cucumis sativus* cv. Beit Alpha MR) were from FMC Corporation and maize seeds (*Zea mays* B37-N) were purchased from Pfizer Genetics. Mung bean (*Vigna radiata* cv. berken) and pea (*Pisum sativum* cv. Alaska) seeds were from W. Atlee Burpee. Spinach (*Spinacia oleracea*) leaves were purchased from a local grocery, and wheat (*Triticum aestivum*) seeds from a local feed store. Green leaves from turnip (*Brassica campestris*), two species of pokeweed [*Phytolacca americana* and a plant identified as *Phytolacca heterotepala* (4)] and *Atriplex halimus* were harvested from plants growing or grown on the grounds of the Carnegie Institution.

Mitochondria were purified from etiolated shoots or green leaves by the DNAse I procedure (5). MtDNA was purified from lysed (5) mitochondria by two cycles of CsCl/ethidium bromide ultracentrifugation (6).

Restriction endonuclease digestions, agarose gel electrophoresis, preparation of nitrocellulose filters and hybridizations with nick-translated (7) DNA were carried out as described (6). Transfer of gels to GeneScreen (New England Nuclear) was carried out exactly as for nitrocellulose, except that 1 x SSC (0.15 M NaCl/30 mM trisodium citrate) was used for blotting. After hybridization, filters were washed extensively in 2 x SSC/0.1% sodium dodecyl sulphate at 65°C prior to autoradiography.

Plasmid DNA was prepared by a modification of the alkaline lysis procedure of Birnboim and Doly (8). Spinach mtDNA restriction fragments were ligated into the Sal I site of pUC8 (9). The cloned 4.8 kb Sma I and electroeluted

(10) 1.16 kb Sst II maize mtDNA restriction fragments containing the genes for the mitochondrial 26S and 18S rRNAs, respectively, were described previously (11). pE3.2 consists of pUC8 and a 3.2 kb Eco RI fragment that contains an entire copy of the "2 kb" repeat sequence found in turnip mtDNA (1). pZmE1 (12) was a gift of Dr. C. J. Leaver, and consists of pBR322 and a 2.4 kb Eco RI fragment of maize mtDNA which contains cox2, the gene for subunit II of cytochrome c oxidase. p64E contains a 3.5 kb Bam HI fragment of the maize mtDNA cosmid clone 2c11 (13) cloned into pBR322.

RESULTS

Definition of recombination repeat

In this report we will use the term "recombination repeat" to denote a sequence which is present in two copies relative to the rest of the genome but which exists in four different genomic environments as a result of apparent recombination between repeats. The operational criteria defining such recombination repeats are the following (cf. refs. 1-3):

1. Digestion of total mtDNA with any restriction enzyme that does not cleave within the repeat generates four restriction fragments, each of which contains the entire repeat sequence (Fig. 1).
2. These four repeat-containing fragments each contain one copy of the repeat element flanked by four paired combinations of four unique sequences (Fig. 1). Thus, pairwise combinations of these fragments exist which conserve genome size, i.e. the two repeat-containing fragments present in one genomic orientation isomer together contain the same DNA sequences as the two repeat-containing fragments present in the other isomer.
3. Relative to any entirely unique fragment, the four repeat-containing fragments are all substoichiometric since each is present in only one of two genomic isomers. The relative amounts of the two pairs of fragments depend on the concentrations of the two orientation isomers.

Repeats possessing the above features are called "recombination" repeats because the two pairs of repeat-containing fragments which conserve genome size are interconvertible by a single reciprocal recombination event within the repeat. Since there is at present no direct evidence bearing on the frequency of the recombination that is postulated to occur between such repeats, we stress that in this paper we use recombination repeat only to denote these physical manifestations consistent with recombination, without implying any knowledge of its actual frequency.

Homology relationships between identified mtDNA recombination repeats

Filter hybridizations were performed to determine whether the same

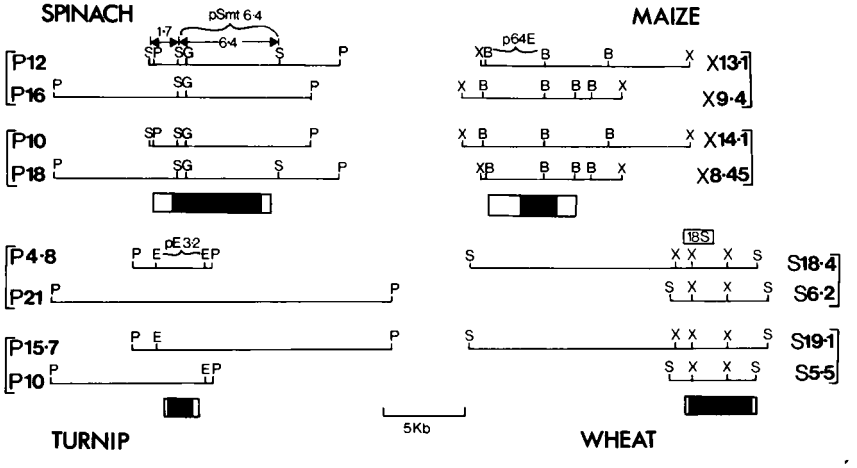

Figure 1. Maps of four, repeat-containing, substoichiometric restriction fragments from each of spinach, turnip, maize and wheat mtDNAs. Selected cleavage sites are shown for Pst I (P), Eco RI (E), Sal I (S), Bgl I (G), Xho I (X) and Bam HI (B). Turnip, maize and wheat maps are redrawn from refs. 1-3, and spinach maps are from our own unpublished data. The solid bar indicates the minimum extent of the repeat, and the open extensions of the bar its maximum extent. The restriction sites defining the minimum and maximum extents of the various repeats are not all shown; detailed mapping information is given in refs. 1-3 for turnip, maize and wheat. The fragments are bracketed so that the upper two restriction fragments in each set of four represent those found in one orientation isomer, while the lower two represent those found in the other.

sequences exist as recombination repeats in the maize, spinach and turnip mitochondrial genomes. Restriction endonucleases were selected which do not cleave within the repeats from each of these three genomes (see Fig. 1), so that four substoichiometric repeat-containing restriction fragments were resolved for each genome and could be detected by hybridization. The substoichiometric nature of the repeat-containing Pst I fragments of turnip and spinach mtDNAs is apparent in an ethidium bromide-stained gel (Fig. 2A). Because the maize mitochondrial genome is relatively large (ref. 2, Table 1), the four repeat-containing Xho I fragments are only visible by hybridization.

Figure 2 shows that pSmt6.4, a plasmid carrying a 6.4 kb Sal I fragment that contains approximately 85% of the 5 kb repeat element found in spinach mtDNA (Fig. 1), and pE3.2, which contains the entire turnip 2 kb repeat (Fig. 1), each hybridize to spinach, turnip and maize mtDNAs. On the other hand, p64E, which contains most of a 3 kb recombination repeat from the maize mitochondrial genome (2), does not hybridize to either spinach (Fig. 3D) or turnip (not shown) mtDNA. Each of these three clones, however, hybridizes to

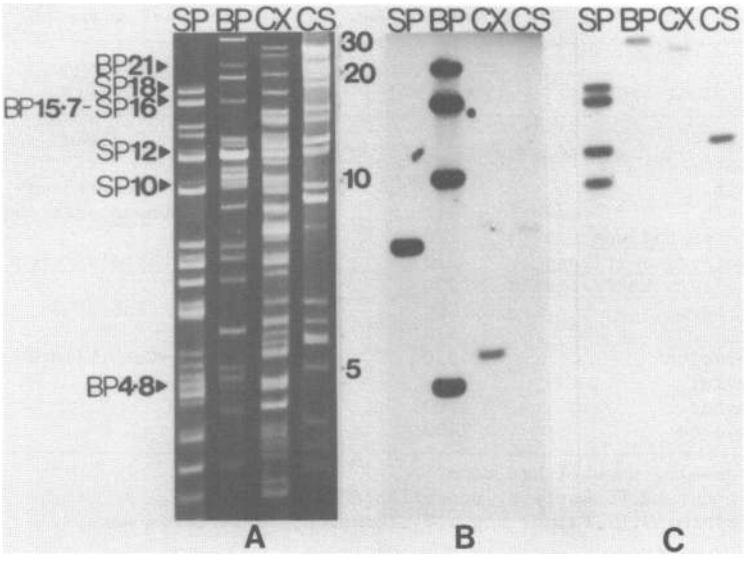

Figure 2. Cross-homologies of recombination repeats from turnip and spinach with mtDNAs from turnip, spinach and maize. Spinach mtDNA was digested with Pst I (SP), turnip mtDNA with Pst I (BP) and maize mtDNA with Xho I (CX) and Sac II (CS), electrophoresed in a 0.7% agarose gel and stained with ethidium bromide (A). The gel was transferred bidirectionally to GeneScreen and probed with nick-translated pE3.2 (B) and pSmt6.4 (C). Substoichiometric spinach mtDNA Pst I fragments are indicated by arrows and designated SP 18, SP 16, SP 12 and SP 10. Substoichiometric turnip mtDNA fragments of BP 21, BP 15.7 and BP 4.8 can be seen in lane 2. BP 10.1 (Fig. 1) is obscured by another genomic fragment (1). Size standards are given on the right in kb, and were deduced from Sal I, Eco RI and Hind III digests of phage lambda DNA and a Hae III digest of ØX 174 DNA. The bright band of 11.3 kb in lane BP is an extrachromosomal mitochondrial plasmid described previously (37). The relatively weak hybridization in lane CS relative to that in lane CX results from an incomplete Sac II digest of maize mtDNA.

the expected four fragments from its own genome [Fig. 2C, track SP; Fig. 2B, track BP; Fig. 3D, track Co(X)]. The spinach and turnip repeats must contain largely different sequences, since each hybridizes to restriction fragments in the other genome which do not contain the major recombination repeat, identifiable as four substoichiometric bands.

We note that pSmt6.4 does not contain the entire 5 kb repeat of spinach (Fig. 1). However, a cloned Sal I fragment of 1.7 kb, which contains the remainder of the repeat (Fig. 1), gave virtually identical hybridizations in all cases considered in this study (data not shown). Similarly, although p64E does not contain the entire 3 kb repeat of maize mtDNA (Fig. 1), hybridization patterns were virtually identical when a cloned 2.1 kb Bam HI fragment which

Table 1. Plant Species Used for mtDNA Repeat Analysis

Plant Species	Mt Genome Size (kb)	Taxonomic Designation- Subclass	Family	Ref.
Maize	570	Monocot	Poaceae	*
Wheat	440	"	"	**
Turnip	218	Dicot	Brassicaceae	1
Spinach	312	"	Chenopodiaceae	***
Atriplex halimus	270	"	"	***
Phytolacca americana	320	"	Phytolaccaceae	***
Phytolacca heterotepala	320	"	"	***
Mung bean	400	"	Fabaceae	***
Pea	430	"	"	***
Watermelon	330	"	Cucurbitaceae	28
Zucchini	840	"	"	28
Cucumber	1500	"	"	28
Muskmelon	2400	"	"	28

*D.M. Lonsdale, unpublished data.
**B. Lejeune and F. Quetier, unpublished data.
***D.B. Stern, J.D. Palmer and W.F. Thompson, unpublished data.

contains the remainder of the repeat was used to probe the gel shown in Fig. 3A (data not shown).

Conservation of repeated mtDNA sequences among angiosperms

Although there was no detectable homology between a clone (p64E) containing most of the maize mtDNA 3 kb repeat and either spinach or turnip mtDNA, the cloned repeated sequences from spinach and turnip had homology to all three genomes tested (Fig. 2). It was therefore of interest to investigate homologies between the spinach and turnip repeats with mtDNAs from other plant species, both to see if these sequences are universal among plant mitochondrial genomes and if they function as recombination repeats in any other species. A wheat mtDNA 4 kb repeat, for example, is expected to hybridize to mtDNAs of all other plant species, since it contains the genes for the 18S and 5S mitochondrial rRNAs (3). Similarly, much of the homology between the spinach and turnip mtDNA repeats and other plant mtDNAs can be attributed to the presence of part or all of known mitochondrial genes within, or closely linked to, the recombination sequences (see last section of RESULTS).

The most striking results were obtained with pSmt6.4, which contains most of the spinach mtDNA 5 kb repeat (Fig. 1). This probe hybridizes strongly to four fragments of Phytolacca heterotepala mtDNA digested with either Pvu II or Bst EII [Fig. 3B, tracks PH(P) and (T)]. Substoichiometric restriction fragments of P. heterotepala mtDNA, of the same size as those hybridizing to pSmt6.4, are visible in an ethidium bromide-stained gel as Pvu II bands of 30 kb, 26 kb and 12 kb [Fig. 3A, track PH(P)] and a Bst EII band of 6.9 kb [Track

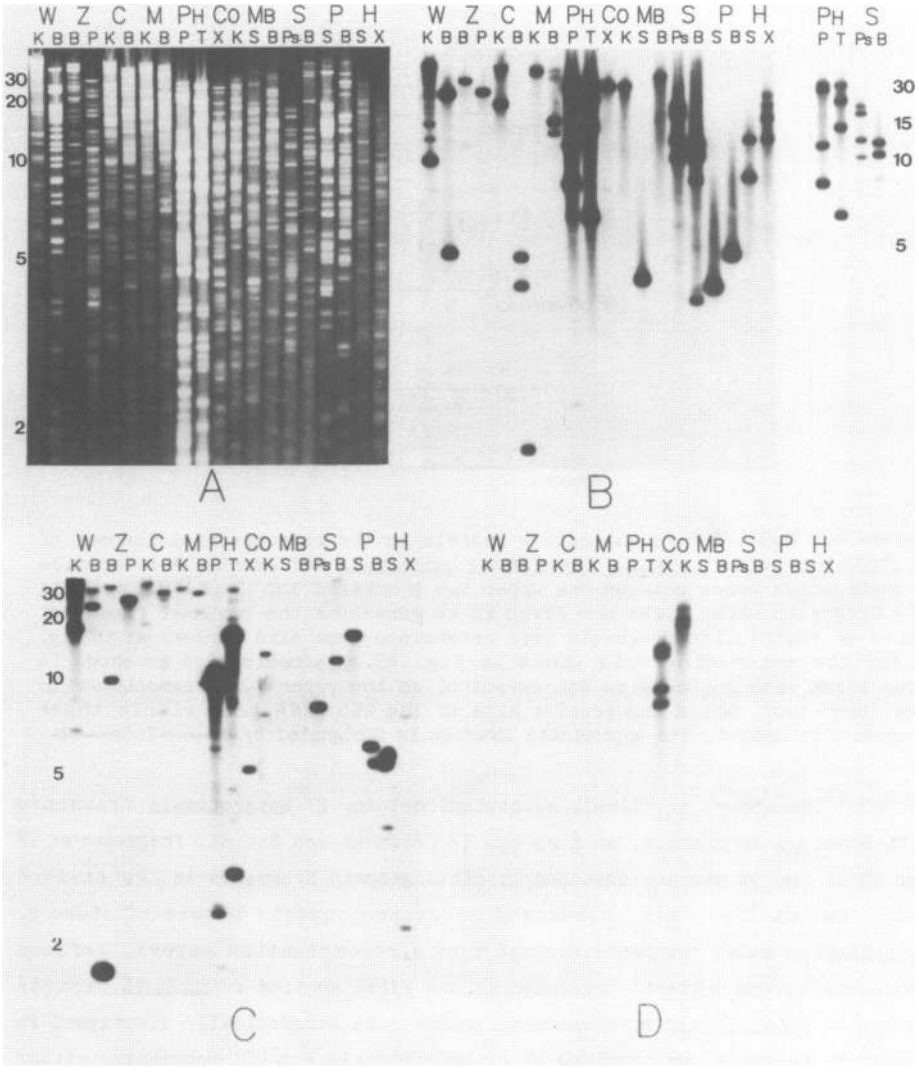

Figure 3. Cross-homologies between spinach, turnip and maize recombination repeats and mtDNAs from ten flowering plants. A) Ethidium bromide-stained 0.7% agarose gel of mtDNAs of watermelon (W), zucchini (Z), cucumber (C), muskmelon (M), Phytolacca heterotepala (PH), maize (CO), mung bean (MB), spinach (S), pea (P), and wheat (H) digested with various combinations of the following restriction enzymes: Kpn I (K), Bgl I (B), Pvu II (P), Bst EII (T), Xho I (X), Sal I (S), and Pst I (Ps). The gel shown in Figure 3A, or one similar to it, was transferred to nitrocellulose and probed with nick-translated pSmt6.4 (B), pE3.2 (C) or p64E (D). A shorter exposure of the P. heterotepala and spinach tracks of Fig. 3B is shown to the right of Fig. 3B. Size standards are in kb. The digestion of watermelon mtDNA by Kpn I is incomplete (track W(K).

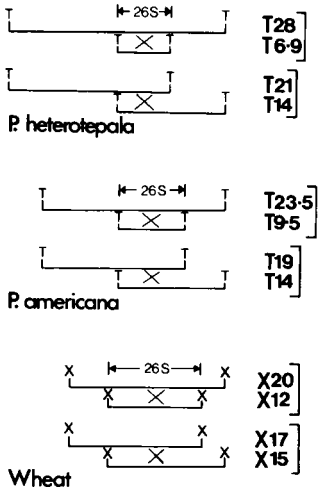

Figure 4. Model for recombination repeats in the mitochondrial genomes of Phytolacca heterotepala, Phytolacca americana and wheat. A reversible recombination event between the upper two bracketed Xho I (X) or Bst EII (T) fragments whose sizes are given in kb generates the two lower fragments. Sizes of restriction fragments were determined from size markers as in Fig. 2 and the autoradiographs shown in Fig. 4B. Recombination as shown is consistent with genome size conservation, as the upper two fragments sum to the lower two. While the precise site of the 26S rRNA gene within these fragments is unknown, its approximate location is designated by ◀——26S——▶.

PH(T)]. The other, putatively substoichiometric, P. heterotepala fragments with homology to pSmt6.4, an 8 kb Pvu II fragment and Bst EII fragments of 28 kb, 21 kb and 14 kb, are obscured by other genomic fragments in the stained gel. The multiplicity, sizes and substoichiometric nature of these P. heterotepala mtDNA fragments suggest that a recombination repeat, defined according to the criteria presented in the first section of RESULTS, exists in the P. heterotepala mitochondrial genome. As schematically diagrammed in Figure 4, pairwise combinations of P. heterotepala Bst EII fragments, either of 28 kb and 6.9 kb or 21 kb and 14 kb, are interconvertible by a single postulated recombination event. Further support for this recombination model, specifically for our inference that an element with homology to the spinach repeat is common to all four P. heterotepala fragments identified by pSmt6.4 probing, comes from the observation that the 1.7 kb Sal I fragment adjacent to pSmt6.4 (Fig. 1) hybridizes to the same four Pvu II and Bst EII fragments (data not shown).

Four wheat mtDNA Xho I restriction fragments also hybridize to pSmt6.4

[Fig. 3B, lane H(X)]. The sizes of these fragments are also consistent with a recombination model, as discussed in the next section. Although the spinach repeat probe hybridizes strongly to the other seven mtDNAs tested (Fig. 3B), in none of these cases do the hybridization patterns suggest the presence of a recombination repeat.

The turnip repeat probe, pE3.2, hybridizes to all the mtDNAs tested with the exception of mung bean mtDNA (Fig. 3C). This probe hybridizes to the four expected Pst I fragments from turnip mtDNA (Fig. 2B), but to only one or two restriction fragments from most of the other genomes (Fig. 3C). Multiple hybridizations are seen between pE3.2 and watermelon [track W(B)], P. heterotepala [tracks PH(P) and PH(T)] and wheat [track H(X)] mtDNAs. None of these hybridization patterns are consistent with a recombination phenotype, as was seen with pSmt6.4 (Fig. 3B), either in terms of the number and size of hybridizing bands or their molarity relationships [e.g. stained gel of W(B)]. Thus, we infer that the sequences contained in pE3.2 function in recombination only in the turnip mitochondrial genome.

A cloned 3.5 kb Bam HI fragment (p64E), which contains most of the maize 3 kb recombination repeat, shows the expected (2) hybridization to four maize mtDNA Xho I fragments [Fig. 3D, track Co(X)], but even at prolonged exposure shows little or no homology to nine other plant mtDNAs tested (Fig. 3D, Table 1).

Overall, three types of hybridization patterns were observed with the three repeat-containing probes. The spinach mtDNA probe, pSmt6.4, hybridized with all 10 other mtDNAs, including fragments in both P. heterotepala and wheat which are diagnostic of an apparent recombination repeat. The turnip probe, pE3.2, hybridized with all the mtDNAs with the exception of mung bean mtDNA, but does not appear to contain sequences involved in recombination in other plant species, although sequences homologous to it are repeated in several other genomes. The maize probe, p64E, contains sequences which are apparently unique to maize mtDNA, since under our hybridization conditions the probe hybridizes only to maize mtDNA.

Genes within mtDNA recombination repeats

We wished to ascertain whether any identified mitochondrial genes had homology to repeated sequences within plant mitochondrial genomes, in light of the observations that the wheat mtDNA 4 kb repeat contains the genes for two mitochondrial rRNAs (3) and that both the spinach 5 kb repeat and the turnip 2 kb repeat have homology to virtually all plant mtDNAs tested (Figs. 3B and 3C). Available plant mitochondrial gene sequences, all from maize,

included a 1.16 kb Sst II fragment internal to the 18S rRNA gene (11), a cloned 4.8 kb Sma I fragment (pS4.8) which contains virtually the entire 26S rRNA gene plus approximately 1.3 kb of flanking sequences (11,14), and pZmE1, a cloned 2.4 kb Eco RI fragment which contains the entire gene for subunit II of cytochrome c oxidase plus 0.8 kb of flanking sequences (12).

The hybridization patterns of 18S rDNA to the 13 angiosperm mtDNAs listed in Table 1 were relatively simple (data not shown): In most genomes only a single restriction fragment hybridized to 18S rDNA, a few genomes contained two or three hybridizing fragments, and only wheat showed the four bands characteristic of intramolecular recombination. Detailed evidence that the 18S and 5S rRNA genes are completely contained within a recombination repeat in wheat has recently been publshed (ref. 3; also see Fig. 1).

More complex hybridization patterns were obtained when pS4.8, a maize mtDNA clone containing 99% of the 26S rRNA gene, was used to probe restriction digests of 13 angiosperm mtDNAs (data not shown). Most notably, pS4.8 appeared to hybridize to the same spinach mtDNA fragments as did pSmt6.4, a clone containing sequences of the spinach repeat. To demonstrate conclusively that the spinach repeat and related repeats in several other species contain 26S rDNA sequences, pSmt6.4 and pS4.8 were hybridized to replica filter blots prepared from a gel containing digests of mtDNAs from the related species Phytolacca heterotepala, Phytolacca americana and Atriplex halimus, and mtDNAs from spinach, wheat and maize (Fig. 5). It is immediately apparent that the hybridization patterns are nearly identical: both pSmt6.4 and pS4.8 hybridize strongly to four Pst I fragments of spinach mtDNA [track S(P)], and four fragments of P. heterotepala and P. americana mtDNAs digested either with Pvu II or Bst EII [tracks PH and PA (V) and (B)]. Most of these Phytolacca mtDNA fragments can be visualized in an ethidium bromide-stained gel (Fig. 5A) and appear to be substoichiometric. These include the two largest Pvu II fragments of P. americana mtDNA, which appear as a single band on the autoradiograph. These data suggest that the spinach mtDNA recombination repeat encodes a significant portion of the 26S rRNA, and that the 26S rRNA gene is repeated within the mitochondrial genomes of P. heterotepala and P. americana in a manner structurally analogous to that in spinach. Figure 4 presents recombination models for P. heterotepala (discussed earlier) and P. americana mtDNAs consistent with these data.

Four Xho I fragments of wheat mtDNA hybridize to pS4.8, and very weakly to pSmt6.4 [Figs 5B and 5C, track W(X)]. The sizes of these restriction fragments are consistent with a recombination model (Fig. 4) in which the 20

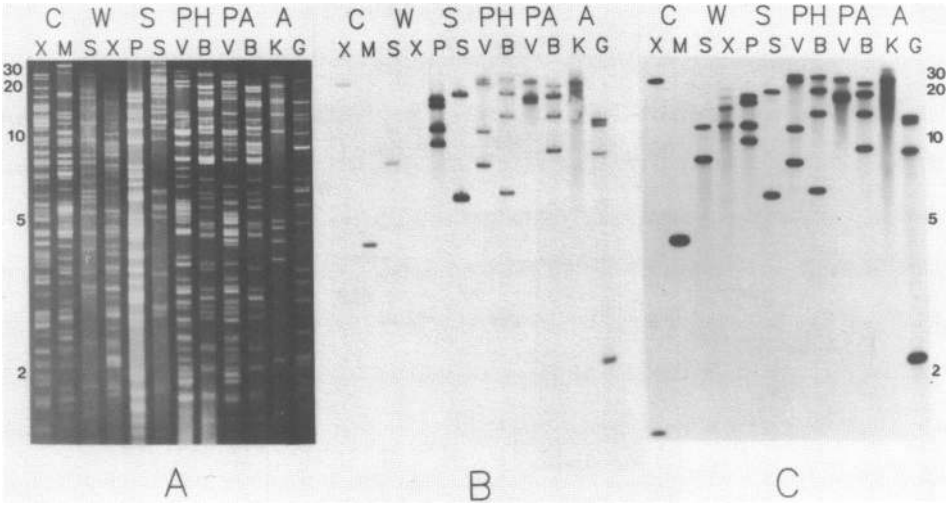

Figure 5. The 26S rRNA gene is largely coextensive with the spinach, wheat and Phytolacca mtDNA recombination repeats. MtDNAs isolated from maize (C), wheat (W), spinach (S), Phytolacca heterotepala (PH), Phytolacca americana (PA) and Atriplex halimus (A) were digested with combinations of Xho I (X), Sma I (M), Sal I (S), Pst I (P), Pvu II (V), Bst EII (B), Bgl I (G) and Kpn I (K) and electrophoresed in a 0.7% agarose gel (panel A). The gel was transferred bidirectionally to nitrocellulose, and the replicates probed with nick-translated pSmt6.4 (B) and pS4.8 (C). Size standards are in kb.

kb and 12 kb Xho I fragments are present in one genomic isomer, and the 17 kb and 15 kb Xho I fragments in another, thus conserving the 32 kb of sequence complexity hybridizing to pS4.8 or pSmt6.4. Alternatively, the 26S rRNA gene may contain an Xho I site and be present in two distinct genomic locations. That only two Sal I fragments of wheat and spinach mtDNAs hybridize to pSmt6.4 suggests that the spinach 5 kb repeat and this repeated sequence in wheat share a Sal I site. This supposition is supported by the observation that pSmt1.7, which contains the remainder of the spinach 5 kb repeat (Fig. 1), hybridizes to the same four wheat mtDNA Xho I fragments as pSmt 6.4, but to two Sal I fragments of different sizes than those with homology to pSmt6.4 (data not shown). Thus, in wheat it appears that the 18S (Fig. 1, ref. 3) and 26S rRNA genes (Fig. 4) are each part of independent recombination repeats.

As a final note, we emphasize that our conclusions regarding the presence of 26S gene-associated recombination repeats in wheat and both pokeweed species must necessarily remain tentative and preliminary, in the absence of any detailed analysis of cloned fragments containing the putative repeats. In fact, such analysis has recently been carried out in wheat and completely

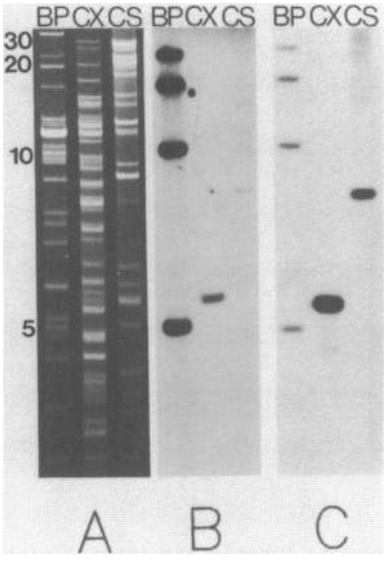

Figure 6. The cox2 gene is largely coextensive with the turnip recombination repeat. Turnip mtDNA was digested with Pst I (1), and maize mtDNA with Xho I (2) and Sac II (3), electrophoresed in a 0.7% agarose gel (A), transferred bidirectionally to GeneScreen and probed with nick-translated pE3.2 (B) which contains the turnip repeat, and pZmE1, a cloned maize mtDNA fragment which contains the cox2 gene (C). Size scale is in kb.

confirms our inference that the wheat 26S gene is contained within a recombination repeat as defined in this paper (L. Bonen, D. Spencer and M. Gray, pers. comm.; D. Falconet and F. Quetier, pers. comm.). Furthermore, we have obtained hybridization patterns similar to those seen in Figure 5C using a 3.3 kb Sma I - Sal I fragment completely internal (14) to the maize 26S rRNA gene (data not shown). This result further confirms our inference that the spinach and pokeweed repeats do indeed encode part or all of the 26S rRNA.

Four Bgl I fragments of Atriplex halimus mtDNA also hybridize to pSmt6.4 and pS4.8 [Figs. 5B and 5C, track A (B)]. The sizes of these fragments - 2.3 kb, 9 kb, 13 kb and 14 kb - are not reconcilable with a simple recombination model, since no pairwise combinations of these Bgl I fragments exist which conserve genome size. Additionally, restriction endonuclease digestions of A. halimus mtDNA with 14 endonucleases, including Bgl I, reveal no evidence of sequence heterogeneity [data not shown, except for Fig. 5A, tracks A(K) and A(B)], in contrast to spinach, a member of the same family and Phytolacca (pokeweed), a member of the same order. Therefore, unlike in spinach and pokeweed, the A. halimus repeat sequences encoding 26S rRNA do not appear to

be engaged in intragenomic recombination.

Although the hybridization patterns of pSmt6.4 and pS4.8 to these mtDNAs are nearly identical, several minor differences are apparent (Fig. 5). In addition to hybridizing to all the fragments identified by pSmt6.4, pS4.8 hybridizes to a 1.34 kb Xho I fragment of maize mtDNA, and also very weakly to 17.1 kb and 1.7 kb Sal I fragments of spinach mtDNA. The 1.34 kb Xho I fragment lies almost completely within pS4.8, but does not contain rDNA sequences (14). The weak hybridization to the 1.7 kb and 17.1 kb Sal I fragments of spinach mtDNA, which contain the remainder of the spinach repeat, indicates that the region of homology between pS4.8 and spinach mtDNA extends across the Sal I site located within the spinach repeat (Fig. 1). We do not yet know whether the 26S rRNA gene is completely contained within the spinach repeat. In addition to these minor qualitative differences in hybridization, quantitative differences are also apparent (Fig. 5).

A third gene-containing probe used to investigate possible repeated genes in higher plant mtDNAs was pZmE1, a maize mtDNA clone (12) containing cox2, the gene for subunit II of cytochrome C oxidase. pZme1 hybridizes to only a single restriction fragment in most of the 13 mtDNAs (Table 1) tested (data not shown). The most notable exception is turnip mtDNA. Reciprocal hybridizations of pE3.2, the cloned turnip repeat, and pZmE1 to maize and turnip mtDNAs demonstrate that the recombination repeat in turnip mtDNA contains part or all of the cox2 gene (Fig. 6). Both of the probes hybridize to the four repeat-containing Pst I fragments of turnip mtDNA, although with differential intensities.

DISCUSSION

The primary finding of this study is that different sequences exist as recombination repeats in different plant mtDNAs. At the level of filter hybridizations we cannot distinguish between two alternative implications of this observation: 1) that different sequences present within the different recombination repeats actually serve as recombination sites (assuming that recombination is a continuing and site-specific process), or 2) that a single common, but relatively short recombination site is embedded within the different repeats. Sequences homologous to some of these recombination repeats (the cox2 repeat in turnip, the 26S repeat in spinach, the 18S repeat in wheat) are present in essentially all angiosperms examined, but only exist as recombination repeats in one (cox2 in turnip; 18S in wheat) or a few (26S in spinach, two species of pokeweed, and wheat) species. Another recombination

repeat (from maize) is found in only a single species among those surveyed here. A second major conclusion is that part or all of three distinct mitochondrial genes of known function, the genes for the 18S and 26S rRNAs and for subunit II of cytochrome c oxidase, exist within the various characterized recombination repeats.

Sequence variation between plant mtDNA repeats

By analogy to both prokaryotic and eukaryotic systems, there is some reason to believe that DNA sequences which facilitate site-specific recombination will be conserved between species. An identical decanucleotide, for example, is found both at the end of the insertion element IS 1 and within the attachment site of phage lambda (15). Furthermore, Chi sites of generalised recombination (16) are found not only within the genomes of phage lambda and E. coli (17), but also near the T-DNA of the Agrobacterium tumefaciens Ti plasmid (18) and within mouse immunoglobulin genes (19). These sites comprise only short segments of DNA, however, and if recombination within plant mitochondrial genomes is site-specific at an equally short sequence present within all recombination repeats, the cross-homologies would probably have been undetectble by our filter hybridizations.

Alternatively, intragenomic recombination in plant mtDNA could be mediated by longer sequences. The site of recombination in the yeast 2 micron circle, for example, may be as long as 65 base pairs (20). Thus, some of the homology between the spinach mtDNA 5 kb repeat and the mtDNAs of Phytolacca heterotepala and P. americana may result from a conserved recombination site either included within or physically linked to the 26S rRNA gene. That this result is not found for Atriplex, which is in the same family (Chenopodiaceae) as spinach, is somewhat surprising. We speculate that the recombination function within the spinach 5 kb repeat either has been lost in an Atriplex-specific lineage following the divergence of spinach and Atriplex, or independently gained in spinach and pokeweed (a member of the same order, but different family, Phytolaccaceae).

Another aspect of sequence comparisons between plant mtDNA repeats is their differential hybridization to heterologous plant mtDNAs. In particular, the maize repeat showed virtually no homology to any other plant mtDNA tested, including wheat, in filter hybridizations (Fig. 3D). Both the spinach and turnip repeats do hybridize to all or nearly all the other mtDNAs, but only the spinach repeat appears to function in recombination in other plant species.

The lack of hybridization between the turnip mtDNA repeat and mung bean mtDNA (Fig. 3C) is surprising given that the turnip repeat probably contains part or all of the cox2 gene and also hybridizes strongly to all other mtDNAs

tested. One explanation might be that the cox2 gene is evolving unusually rapidly in a mung bean-specific lineage. Results consistent with this hypothesis have been obtained, since under relatively nonstringent conditions (30% formamide/5xSSC/42°C) pZmE1 does hybridize to mung bean mtDNA (data not shown). In animal mtDNA the cox2 gene is evolving significantly faster in primates than in other mammals (21,22).

Intragenomic recombination in plant mitochondria and chloroplasts

The phenomenon of isomeric interconversion of higher plant mtDNA molecules is reminiscent of an isomerization mechanism recently proposed for chloroplast DNA (23-25). Nonetheless, there are several significant differences between mitochondrial and chloroplast recombination repeats. First, recombination repeats in angiosperm ctDNAs are on average an order of magnitude larger in size (20-30 kb) than the recombination repeats thus far described in plant mtDNAs (2-5 kb), although no correlation has been established between the size of a repeat and the length of the sequence which may actually function in recombination. The universal inverted orientation of the ctDNA repeats contrasts with the situation in mitochondria, where large repeated DNA sequences have been found in both inverted [spinach (our unpublished data) and maize (D.M. Lonsdale, pers. comm.)] and direct [turnip, maize (refs. 1,2)] orientations. Lastly, the sequence content of the ctDNA inverted repeat has remained relatively constant and unrearranged during the evolution of the angiosperms (26,27), while in plant mitochondria largely or completely different sequences exist as recombination repeats. These differences between mtDNA and ctDNA repeat sequences reflect, in a general sense, the different ways in which the two organellar genomes are evolving in terms of structure. Plant chloroplast genomes are highly conserved in size and organization (26,27), whereas plant mitochondrial genomes are quite variable in size (28) and in organization (29).

Functional genes within plant mtDNA repeats

The wheat, spinach and turnip mitochondrial genomes contain repeated sequences that are implicated in intragenomic recombination. These repeat sequences, and/or closely linked unique sequences, also encode part or all of 18S rRNA, 26S rRNA and subunit II of cytochrome c oxidase, respectively. Furthermore, structurally analogous repeats which may function in recombination in wheat and two species of pokeweed also contain 26S rRNA gene sequences. Altered gene expression resulting from recombination in or near genes has been documented in both prokaryotes and eukaryotes. For example, inversion at the H2 locus of Salmonella switches expression of the two genes for flagellar antigens (30). Site-specific recombination is also required for the expression of a functional immunoglobulin kappa chain (31,32).

Whether the entire 26S rRNA gene is contained within the spinach, pokeweed and wheat repeats, or the entire cox2 gene within the turnip repeat, is not yet established. Only part of the large mitochondrial rRNA gene of the yeast Kloeckera africana is repeated and thus only a single functional copy of this gene exists per genome (33). In wheat mtDNA, however, the entire 18S rRNA gene and also the adjacent 5S rRNA gene are completely contained within the same recombination repeat and thus these genes are present in two copies (3). In mtDNA of the protist Tetrahymena pyriformis, the complete large rRNA gene is present twice (within inverted repeats) but the small rRNA gene is present in only one copy per genome (34). However, in the fungus Achlya ambisexualis both the small and large mitochondrial rRNA genes are completely contained within an inverted repeat (35).

With the exception of the 3 kb maize repeat, all examined plant mtDNA recombination repeats, and/or sequences closely linked to these repeats, contain functional gene sequences. In considering the size of the plant mitochondrial genome (200-2400 kb) and the number of polypeptides synthesized by isolated mitochondria (20-30) (36), it seems likely that plant mtDNA consists largely of non-coding sequences. Thus, although only a few mitochondrial recombination repeats have been characterized so far, there does appear to be a surprising propensity for these repeats to be closely linked to functional genes. The implications of this finding are presently unclear.

ACKNOWLEDGEMENTS

We thank Dr. W.F. Thompson for providing laboratory facilities used to perform these experiments, and Dr. C. J. Leaver for the gift of pZmE1. This work was supported by funds from the Carnegie Institution of Washington and NIH Training grant GM-07276-08 to D.B.S. This is C.I.W.-D.P.B. publication #803.

+To whom correspondence should be addressed

REFERENCES

1. Palmer, J.D. and Shields, C.R. (1984) Nature 307, 437-440.
2. Lonsdale, D.M., Hodge, T.P., Fauron, C.M.-R. and Flavell, R.B. (1983) in ICN-UCLA Symposia on Molecular and Cellular Biology, Goldberg, R.B. Ed., Volume 12, New Series, pp. 445-456, Alan R. Liss, New York.
3. Falconet, D., Lejeune, B., Quetier, F. and Gray, M.W. EMBO J. 3, 297-302.
4. Howell, J.T. (1960) Leafl. West. Bot. 9, 81-83.
5. Kolodner, R. and Tewari, K.K. (1972) Proc. Natl. Acad. Sci. USA 69, 1830-1834.
6. Palmer, J.D. (1982) Nucleic Acids Res. 10, 1593-1605.

7. Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975) Proc. Natl. Acad. Sci. USA 72, 1184-1188.
8. Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523.
9. Vieira, J. and Messing, J. (1982) Gene 19, 259-268.
10. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning, pp.164-166, Cold Spring Harbor Laboratory.
11. Stern, D.B., Dyer, T.A. and Lonsdale, D.M. (1982) Nucleic Acids Res. 10, 3333-3340.
12. Fox, T.D. and Leaver, C.J. (1981) Cell 26, 315-323.
13. Lonsdale, D.M., Thompson, R.D. and Hodge, T.P. (1981) Nucleic Acids Res. 9, 3657-3669.
14. Dale, R.M.K., Mendu, N., Ginsburg, H. and Kridl, J.C. (1984) Plasmid 11, 141-150.
15. Ohtsubo, H. and Ohtsubo, E. (1978) Proc. Natl. Acad. Sci. USA 75, 615-619.
16. Smith, G.R., Kunes, S.M., Schultz, D.W., Taylor, A. and Triman, K.L. (1981) Cell 24, 429-436.
17. Triman, K.L., Chattoraj, D.K. and Smith, G.R. (1982) J. Mol. Biol. 154, 393-398.
18. Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M. and Chilton, M.-D. (1982) Proc. Natl. Acad. Sci. USA 79, 6322-6326.
19. Kenter, A.L. and Birshtein, B.K. (1981) Nature 293, 402-404.
20. Broach, J.R., Guarascio, V.R. and Jayaram, M. (1982) Cell 29, 227-234.
21. Brown, G.G. and Simpson, M.V. (1982) Proc. Natl. Acad. Sci. USA 79, 3246-3250.
22. Cann, R.L., Brown, W.M. and Wilson, A.C. (1984) Genetics 106, 479-499.
23. Palmer, J.D. (1983) Nature 301, 92-93.
24. Palmer, J.D., Osorio, B., Watson, J.C., Edwards, H., Dodd, J. and Thompson, W.F. (1984) Proc. UCLA Symposia on the Biosynthesis of the Photosynthetic Apparatus, Alan R. Liss, New York, in press.
25. Bohnert, H.J. and Loffelhardt, W. (1982) FEBS Lett. 150, 403-406.
26. Palmer, J.D. and Thompson, W.F. (1982) Cell 29, 537-550.
27. Kolodner, R. and Tewari, K.K. (1979) Proc. Natl. Acad. Sci. USA 76, 41-45.
28. Ward, B.L., Anderson, R.S. and Bendich, A.J. (1981) Cell 25, 793-803.
29. Sederoff, R.R., Levings, C.S. III, Timothy, D.H. and Hu, W.W.L. (1981) Proc. Natl. Acad. Sci. USA 78, 5953-5957.
30. Zieg, J., Silverman, M., Hilmen, M. and Simon, M. (1977) Science 196, 170-172.
31. Sakano, H., Hüppi, K., Heinrich, G. and Tonegawa, S. (1979) Nature 280, 288-294.
32. Seidman, J.G., Max, E.E. and Leder, P. (1979) Nature 280, 370-375.
33. Clark-Walker, G.D., McArthur, C.R. and Sriprakash, K.S. (1981) J. Mol. Biol. 147, 399-415.
34. Goldbach, R.W., Borst, P., Bollen-de-Boer, J.E. and Van Bruggen, E.F.J. (1978) Biochim. Biophys. Acta 521, 169-186.
35. Hudspeth, M.E.S., Shumard, D.S., Bradford, C.J.R. and Grossman, L.I. (1983) Proc. Natl. Acad. Sci. USA 80, 142-146.
36. Leaver, C.J. and Gray, M.W. (1982) Ann. Rev. Plant Physiol. 33, 373-402.
37. Palmer, J.D., Shields, C.R., Cohen, D.B. and Orton, T.J. (1983) Nature 301, 725-728.