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Inverted repeats in chloroplast DNA from higher plants*

(circular DNA/electron microscopy/denaturation mapping/circular dimers)

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ABSTRACT **The circular chloroplast DNAs from spinach, lettuce, and corn plants have been examined by electron microscopy and shown to contain a large sequence repeated one time in reverse polarity. The inverted sequence in spinach and lettuce chloroplast DNA has been found to be 24,400 base pairs long. The inverted sequence in the corn chloroplast DNA is 22,500 base pairs long. Denaturation mapping studies have shown that the structure of the inverted sequence is highly conserved in these three plants. Pea chloroplast DNA does not contain an inverted repeat. All of the circular dimers of pea chloroplast DNA are found to be in a head-to-tail conformation. Circular dimers of spinach and lettuce were also found to have head-to-tail conformation. However, approximately 70–80% of the circular dimers in preparations of lettuce and spinach chloroplast DNA were found to be in a head-to-head conformation. We propose that the head-to-head circular dimers are formed by a recombination event between two circular monomers in the inverted sequence.**

The chloroplast DNA (ctDNA) from higher plants exists as covalently closed circular DNA molecules (1–6). The ctDNA from the alga *Euglena gracilis* has also been found to be circular (7). The ctDNA molecules from different species of higher plants have significantly different sizes and range from a M_r of 85.4×10^6 for corn ctDNA (2) to a M_r of 97.2×10^6 for lettuce ctDNA (2, 3). *Euglena* ctDNA has a M_r of approximately 90×10^6 (2, 8). The excellent agreement between the molecular weights of ctDNAs from higher plants determined by kinetics complexity and electron microscopy suggests that the sequence of a circular ctDNA molecule represents the entire informational content of the ctDNA (ref. 3; unpublished results). This suggestion has been further confirmed by denaturation mapping studies on pea ctDNA which have shown that all of the pea ctDNA molecules exhibit the same denaturation pattern (6).

We have been studying the structural relationships among the ctDNAs from different species of higher plants in order to obtain information on the divergence of ctDNAs through evolution. In this communication the ctDNA molecules from four different higher plants (pea, spinach, lettuce, and corn) have been examined for the presence of sequences that are repeated in reverse. Such sequences renature spontaneously after denaturation (9) and have been observed in a large variety of DNAs ranging from bacterial plasmids (9) to eukaryotic chromosomal DNA (10). The data obtained have shown that the ctDNAs from lettuce, spinach, and corn plants contain a similar sequence, comprising approximately 16% of their monomer length, that is repeated one time in reverse. In pea ctDNA this sequence has not been found in inverted repeats. The structure of ctDNA circular dimers has been also investigated and the results suggest that they might be formed by a recombination mechanism.

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MATERIALS AND METHODS

DNA. Covalently closed circular ctDNA from corn (*Zea mays*), spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*), and pea (*Pisum sativum*) plants was prepared as previously described (3). The ctDNAs were treated with γ irradiation so that 50% of the DNA molecules contained approximately one single-strand break per molecule (nicked circular DNA) (2, 3, 6). ϕX174 bacteriophage (ϕX) DNA and monomers of open circular replicative form DNA of ϕX (ϕX RFII DNA) were provided by Robert C. Warner.

Formation of Inverted Repeats. Nicked circular ctDNA was denatured at 5–10 μg/ml by treatment with 0.1 M NaOH/0.02 M EDTA for 1–2 min on ice. The DNA solution was neutralized by the addition of 1.8 M Tris·HCl/0.2 M Tris base to a final Tris concentration of 0.2 M. The DNA was kept on ice for 10–15 min and mounted for electron microscopy.

Electron Microscopy. DNA was mounted under conditions that are nondenaturing for ctDNA by spreading it from a solution containing 50% formamide (Matheson, Coleman and Bell), 0.1 M Tris·HCl (pH 8.5), 0.01 M EDTA, and cytochrome *c* (Calbiochem) at 50 μg/ml onto a hypophase containing 20% formamide, 0.01 M Tris·HCl (pH 8.5), and 0.001 M EDTA at a solution temperature of 23°C. Partially denaturating conditions were achieved when the formamide concentrations of the spreading solution and the hypophase were increased to 78% and 48%, respectively. The temperature was maintained at 23°C. ctDNA was present in the spreading solution at 0.25 μg/ml, and ϕX viral DNA and ϕX RFII DNA monomers were each present at 0.05–0.1 μg/ml to serve as length standards in these experiments. The details of spreading, staining, and shadowing of grids, of electron microscopy, and of length calibrations have been described (2, 6). Length measurements were made with a Hewlett-Packard digitizing board and computer which were kindly made available by the Department of Biological Chemistry, Harvard Medical School. The contour lengths were calculated by using a fully smoothed program.

RESULTS

Inverted Repeat in Chloroplast DNAs. When preparations of nicked circular lettuce, spinach, and corn ctDNA molecules were denatured and examined in the electron microscope, DNA molecules that contained one duplex region were observed. A typical example of a partially duplex spinach ctDNA molecule is presented in Fig. 1*A*. About 40% of the partially duplex ctDNA molecules observed had a central duplex region, a small

Abbreviations: ctDNA, chloroplast DNA; ϕX, *Escherichia coli* bacteriophage ϕX174; ϕX RFII DNA, open circular replicative form DNA of ϕX; nicked circular DNA, circular DNA containing a single-stranded break; kb, kbase (1000 nucleotides or nucleotide pairs).

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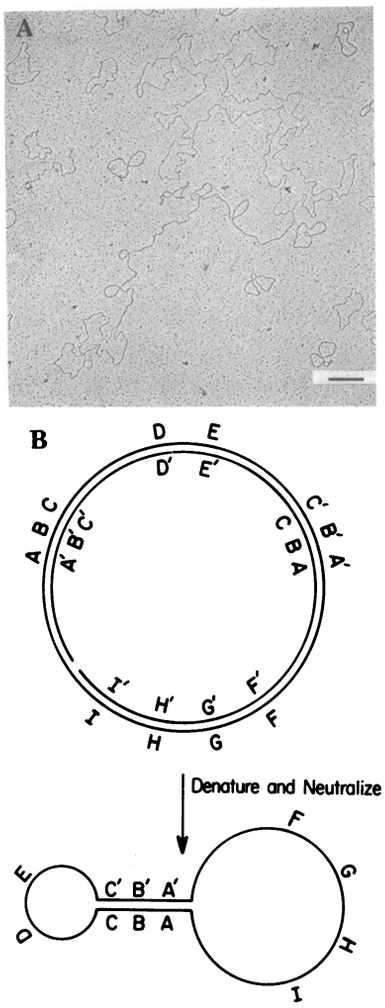

FIG. 1. Self-renatured circular ctDNA molecule. (*A*) Electron micrograph of a self-renatured spinach ctDNA molecule. The small circles are single-stranded and double-stranded ϕX DNA. The bar indicates 1.0 μm. (*B*) Illustration of the formation of a self-renatured molecule from a circular molecule containing an inverted repeat.

single-stranded loop on one end of the duplex region, and a large single-stranded loop on the other end of the duplex region. The remaining partially duplex ctDNA molecules contained a single-stranded loop at one end of the duplex region and had two single-stranded branches at the other end of the duplex region. As many as 70–90% of the denatured ctDNA molecules were found in the form of partially duplex ctDNA molecules (400 DNA molecules from each DNA preparation were examined). The remaining DNA molecules were single-stranded linear molecules of length less than that of ctDNA.

A summary of length measurements of partially duplex lettuce, spinach, and corn ctDNA molecules is presented in Table 1. The duplex segment of lettuce and spinach ctDNA is 24.4 kbase (kb)‡ long and amounted to approximately 16% of the native length of these two ctDNAs (2, 3). The corn duplex segment is 22.5 kb long which is smaller than the lettuce and spinach duplex segments but it still amounts to approximately 16% of the native length of corn ctDNA, because the molecular size of corn ctDNA is smaller than that of lettuce and spinach ctDNAs (2). The small single-stranded loop in partially duplex spinach ctDNA appears to be slightly smaller (1 kb) than that

‡ The length of ϕX RF DNA was taken to be 5375 ± 10 base pairs, as determined by sequence analysis (11) in order to make these calculations.

Table 1. Length measurements on self-renatured ctDNA molecules

	Small loop, ϕX units*	Duplex region, ϕX units	Large loop, ϕX units	*n*
Lettuce	3.64 ± 0.13	4.56 ± 0.08	16.21 ± 0.48	21
Spinach	3.44 ± 0.15	4.53 ± 0.15	16.0 ± 0.84	18
Corn	2.34 ± 0.20	4.18 ± 0.06	14.9 ± 0.62	18

* The data are presented as the ratio of a given length to the length of single-stranded or double-stranded ϕX DNA that were used as internal standards. ± indicates SD.

in lettuce ctDNA, whereas the large single-stranded loops of these two ctDNAs are quite similar in size. Both the small and large single-stranded loops in partially duplex corn ctDNA molecules are significantly smaller than the corresponding structures in lettuce and spinach ctDNA.

These results are consistant with corn, lettuce, and spinach ctDNA molecules that contain a sequence that is repeated once in reverse polarity and can participate in intramolecular renaturation. The structure and formation of a partially duplex ctDNA molecule from a circular ctDNA molecule containing a sequence repeated in reverse is illustrated in Fig. 1*B*.

Denatured pea ctDNA molecules did not form any self-renatured DNA molecules. Four different pea ctDNA preparations were studied as described above and no self-renatured molecules like those observed in corn, spinach, and lettuce ctDNA were found ($P < 0.05\%$). The pea ctDNA preparations used for these experiments were highly intact, and approximately 40% of the single-stranded ctDNA molecules were unit length single-stranded circles whereas most of the rest of the single-stranded DNA molecules were unit length linear molecules. Incubation of the denatured ctDNA molecules under less stringent renaturing conditions (12, 13) did not result in any intramolecular renaturation. Mixing experiments showed that preparations of pea ctDNA would not inhibit self-renaturation by denatured spinach and lettuce ctDNA molecules. The pea ctDNA molecules did not self-renature during the mixing ex-

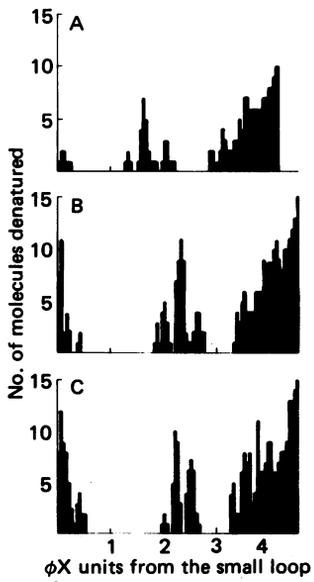

FIG. 2. Denaturation maps of the duplex segment of self-renatured ctDNA molecules. (*A*) Corn; (*B*) lettuce; (*C*) spinach. The denaturation maps are oriented so that the small loop of the self-renatured molecules is on the left side of the figure and the large loop is on the right side. All lengths were standardized by using ϕX DNA as the internal standard (6).

periments and were observed as linear and circular single-stranded DNA molecules the size of pea ctDNA (2).

Denaturation Mapping of the Inverted Repeat. When self-renatured ctDNA molecules were mounted for electron microscopy from a spreading solution containing 78% formamide onto a hypophase containing 48% formamide, the duplex region of these DNA molecules was found to be partially denatured. The extent of denaturation ranged from 12% for corn ctDNA to 17% for spinach ctDNA. Denaturation maps of the duplex region of self-renatured corn, lettuce, and spinach ctDNA molecules were constructed from partially denatured DNA molecules as previously described (6) and are presented in Fig. 2. The three denaturation maps show a striking degree of similarity. At the large-loop side they all have a highly denatured region that is 1.3 ϕX units long. Further in from the large loop, all three denaturation maps have a native region that is 0.55–0.65 ϕX unit long followed by three denatured regions covering a distance of 0.75–0.95 ϕX unit. To the small-loop side of the three denatured regions, spinach and lettuce ctDNA have a native region 1.4 ϕX units long followed by two small denatured regions at the small-loop end of the duplex segment. Corn ctDNA is somewhat different in this region. It has a smaller native region that is 0.95 ϕX unit long followed by a single denatured region at the small-loop end of the duplex segment. It is not clear whether the denatured region at the small-loop end of the corn duplex segment is the same as the corresponding denatured region in the spinach and lettuce duplex. However, the shortening of the native region in this area could fully account for the difference in length between the corn duplex segment and the duplex segments of lettuce and spinach ctDNAs. With the exception of the differences at the small-loop side of corn ctDNA, the sequences of the inverted repeats in these three ctDNAs appear to be highly related.

The Structure of Circular Dimers. Circular dimers constitute as much as 3–4% of the circular ctDNA molecules from higher plants (2). A circular dimer can conceivably consist of two monomers joined in either a tandem repeat (head-to-tail circular dimer) or in an inverted repeat (head-to-head circular dimer). These two arrangements can be distinguished after denaturation of a relaxed circular dimer, because head-to-tail circular dimers should yield dimer-length single-stranded circular and linear molecules whereas a head-to-head circular dimer should renature to form a monomer-length double-stranded linear molecule (14). This approach has been used to examine the structure of pea, lettuce, and spinach ctDNA circular dimers.

After denaturation and neutralization of pea ctDNA, 1% of the single-stranded molecules was either dimer-length linears or circles (16 circles and 23 linears were observed). No monomer-length duplex molecule was found ($P < 0.025\%$). These results are consistent with a head-to-tail conformation of most pea ctDNA circular dimers and confirm the previous results obtained by denaturation mapping of pea ctDNA circular dimers (6).

When relaxed circular lettuce and spinach ctDNA molecules were denatured and neutralized, two new types of dimer-length molecules were found. A typical electron micrograph of the first type of dimer is presented in Fig. 3A. The molecule has a small, equal-length, single-stranded loop on each end. Each loop is attached to a separate duplex segment of equal length and the two duplex segments are connected to each other by two equal-length, single-stranded segments. The duplex segments are equal in length to the inverted repeat discussed above. The small single-stranded loops are the size of the small spacer between the inverted sequences of the monomer, and each internal single-stranded segment of the dimer is the same length as the large spacer between inverted sequences of the monomer.

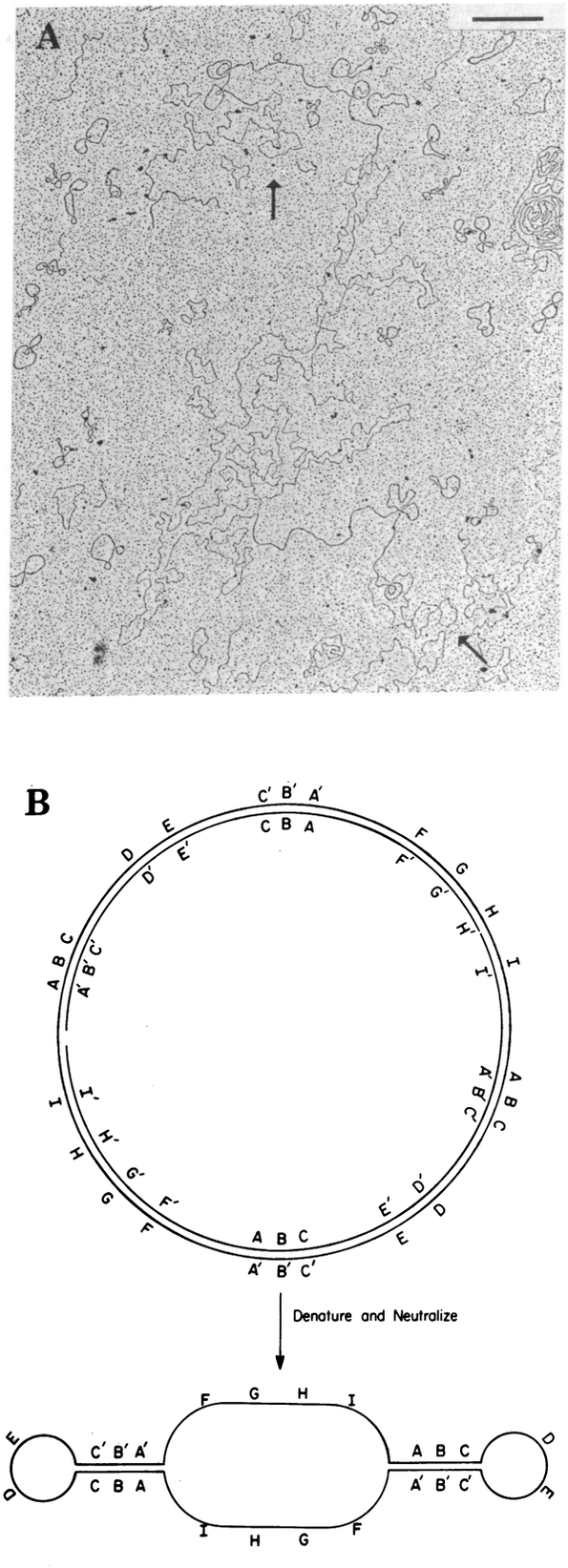

FIG. 3. Self-renatured head-to-tail circular dimer. (*A*) Electron micrograph of a self-renatured ctDNA head-to-tail circular dimer. The arrows point to the single-stranded loops at the ends of the molecule. The small circles are single-stranded and double-stranded ϕX DNA. The bar indicates 1 μm. (*B*) Illustration of the formation of a self-renatured molecule from a head-to-tail circular dimer containing two sets of inverted sequences.

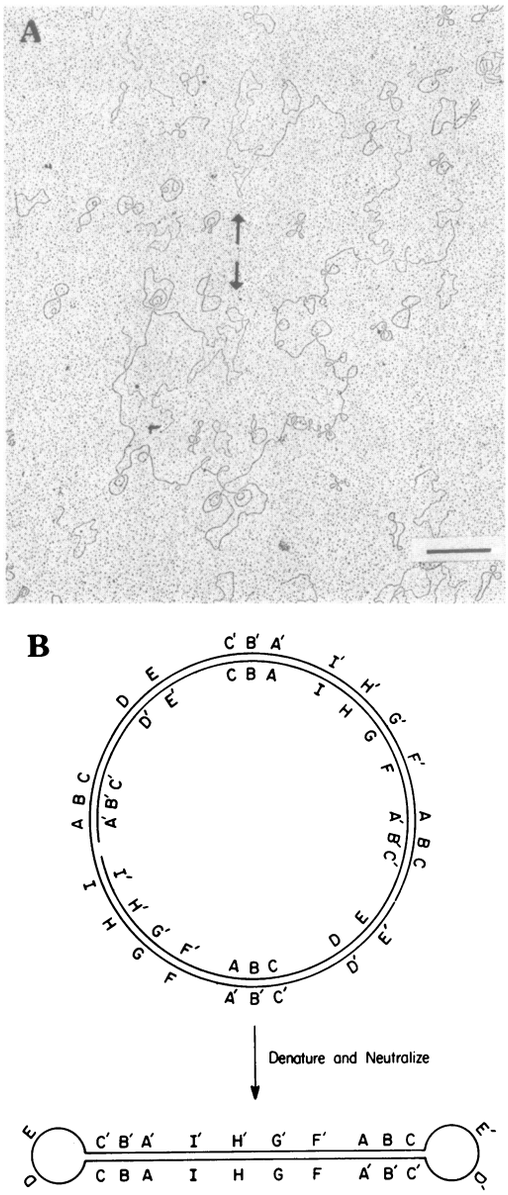

FIG. 4. Self-renatured head-to-head circular dimer. (*A*) Electron micrograph of a self-renatured ctDNA head-to-head circular dimer. The arrows point to the single-stranded loops at the ends of the molecule. The small circles are single-stranded and double-stranded ϕX DNA. The bar indicates 1 μm. (*B*) Illustration of the formation of a self-renatured molecule from a head-to-head circular dimer containing two sets of inverted sequences.

This molecule is consistent with its formation from a head-to-tail circular dimer[§] and its formation is illustrated in Fig. 3*B*. An electron micrograph of the second type of circular dimer is presented in Fig. 4*A*. The molecule consists of two equal-length, single-stranded loops joined by a long duplex segment. The length of the single-stranded loops is equal to the length of the small spacer between the inverted sequences of the monomer, and the length of the duplex segment is equal to the sum of the lengths of two of the inverted sequences and one large spacer. This molecule is consistent with its formation from a head-to-head circular dimer[¶] and its formation is illustrated in Fig. 4*B*. Approximately 1.5% of the self-renatured lettuce and spinach ctDNA molecules were circular dimers (2000 ctDNA molecules from each plant were examined) and the head-to-head dimers were approximately 4–5 times as prevalent as the head-to-tail dimers (24 of 30 dimers in spinach ctDNA and 24 of 29 dimers in lettuce ctDNA).

DISCUSSION

The results presented above are consistent with the idea that lettuce, spinach, and corn ctDNAs each contain a sequence amounting to approximately 16% of their genome length (2, 3) that is repeated once in reverse polarity. Denaturation mapping studies suggest that the repeated sequence in the ctDNAs is highly related. The greater similarity between the repeated sequences of lettuce and spinach ctDNAs than between either of these two ctDNAs and corn ctDNA is in line with the established evolutionary divergence of these plants (15). It is not obvious why these ctDNAs have such a large sequence repeated in reverse. However, this organization does have some advantages over two sequences repeated in tandem. An intramolecular recombination event between two tandemly repeated sequences could lead to the excision of the segment located between the two repeated sequences, whereas an intramolecular recombination event between two inverted sequences could, at worst, reverse the polarity of segment between the two sequences but would not result in the physical loss of any genetic material. Intramolecular recombination events between the two inverted sequences would also tend to prevent their divergence from each other. In any event, the inverted conformation is probably not strictly required for ctDNA function, because pea ctDNA does not have this sequence repeated in reverse. It will be interesting to see whether pea ctDNA has a similar sequence that is possibly repeated once in a tandem repeat.

A sequence that is 16% of the length of ctDNA is a large proportion of the ctDNA to be repeated and suggests that the genes coded for by this sequence are important for the function of the ctDNA. Studies on the structure of corn ctDNA (16, 17) show that one copy of the two sets of chloroplast specific rRNA genes (18) is located on each of the corn ctDNA inverted sequences. In view of the striking similarity among the denaturation maps of the corn, spinach, and lettuce ctDNA inverted sequences, it is probable that the inverted DNA sequence also codes for the chloroplast rRNA genes in spinach and lettuce ctDNAs. In any event, a minimum of 12–15% (18, 19) of each inverted sequence would be required to code for one set of chloroplast rRNA genes (not including spacers), which probably leaves room for other genes on the inverted sequences.

The studies presented here have also provided information on the structure of circular dimers of ctDNA. The finding that most, if not all, pea ctDNA circular dimers are in a head-to-tail configuration is consistent with previous work (6) and is similar to the results obtained with circular dimers of ϕX RF DNA (20) and mitochondrial DNA (14). The finding of circular dimers of lettuce and spinach ctDNAs that are in a head-to-head configuration is unusual. The formation of head-to-head circular dimers appears to be associated with the presence of the inverted repeat in ctDNA. This could most easily be explained if circular dimers of ctDNA were formed by a recombination

[§] The conformation in which the large spacers are present as the external single-stranded loops and the small spacers are present as the internal single-stranded segments is possible but was not observed. Statistical analysis of the renaturation process (9) indicates that the rate of formation of this conformation should be approximately 0.1 of the rate of formation of the conformation presented in Fig. 3.

[¶] The conformation in which the large spacers are present as external single-stranded loops and the small spacers are present in the internal duplex segment is possible but was not found. The observed conformation has the greatest amount of hydrogen bonding and its rate of formation should be 10 times the rate of formation of the conformation having the large spacers as the external single-stranded loops (9).

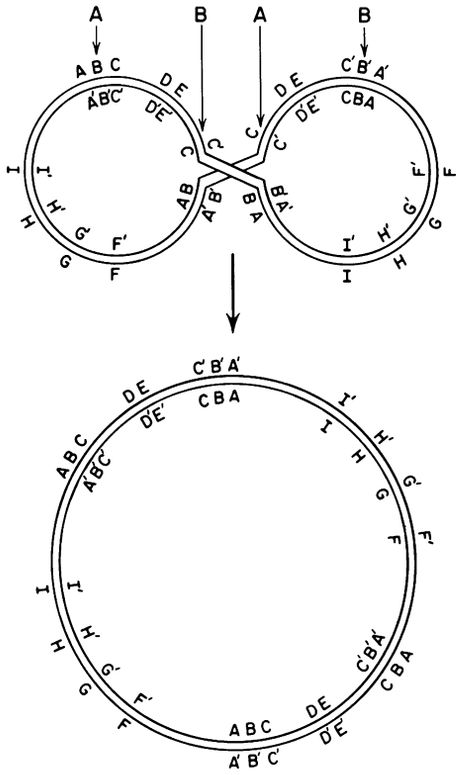

FIG. 5. Illustration of the formation of a head-to-head circular dimer by a recombination event between two circular monomers. Recombination between one pair of inverted sequences (designated by the arrows A and B) leads to the formation of a head-to-head circular dimer. Recombination between the other possible pairing of inverted sequences (designated by the arrows A and A or B and B) would lead to the formation of a head-to-tail circular dimer (not shown).

event between two circular monomers. A head-to-head circular dimer would be formed by a recombination event between two circular monomers at their inverted sequences, in which one monomer was inserted into the other monomer in reverse polarity as illustrated in Fig. 5. A yeast circular DNA molecule containing an inverted repeat has recently been shown to form head-to-head circular dimers (21). Recombination has been shown to be involved in the metabolism of circular dimers formed by several of the small single-stranded DNA-containing *Escherichia coli* bacteriophages (22–25) and several *E. coli* plasmids (26, 27). Our findings suggest that the many ctDNA molecules present in each chloroplast (28) can recombine with each other.

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