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The Presence of Covalently Linked Ribonucleotides in the Closed Circular Deoxyribonucleic Acid from Higher Plants*

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RICHARD KOLODNER,‡ ROBERT C. WARNER, AND KRISHNA K. TEWARI

From The Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92664

Single-stranded scissions are induced in the covalently closed circular chloroplast (ct–) DNAs from peas, spinach, and lettuce plants by treatment with alkali or by incubation with a mixture of ribonucleases A and T1. These scissions are due to the presence of covalently linked ribonucleotides in these closed circular DNAs. By comparing the scission rates of these ctDNAs to the scission rate of RNA, it has been estimated that pea and spinach ctDNAs contain a maximum of 18 ± 2 ribonucleotides/molecule, while lettuce ctDNA contains a maximum of 12 ± 2 ribonucleotides/molecule. Further studies with pea ctDNA by electron microscopic methods have shown that pea ctDNA contains 19 alkali-labile sites at specific locations. A map of the relative positions of the alkali-labile sites has been constructed. These alkali-labile sites are presumably due to the insertion of individual ribonucleotides.

Covalently linked ribonucleotides have been shown to be present in the DNA of a number of organisms. The covalently closed circular mitochondrial DNA from animal cells is generally known to be alkali labile (1), and the definite presence of ribonucleotides has been shown in the closed circular mitochondrial DNA from Hela cells, mouse L cells, and rat ascites hepatoma cells (2–4). The DNA from the animal virus, herpes simplex type 1, is known to be fragmented in alkali (5–8), and has recently been shown to contain ribonucleotides (9). The DNAs from *Escherichia coli* bacteriophages T2, T4 (10), and T5 (11) have also been shown to contain covalently linked ribonucleotides. A stretch of 24 ribonucleotides is found in *E. coli* col plasmid E1 DNA, when the DNA replicates in the presence of chloramphenicol, but not when the DNA replicates under normal conditions (12, 13). Presumably, this RNA serves as a primer for col E1 DNA replication (14, 15), and is then excised, except in the presence of chloramphenicol. RNA primers for DNA replication have also been found in a number of DNA-replicating systems (16–21). However, ribonucleotides have not been found to be covalently linked to the mature DNA in any of these replicating systems.

The chloroplasts from higher plants contain covalently closed circular DNA molecules having molecular weights in the range of 85×10^6 to 96×10^6 (22–25).[1, 2] We have investigated the effect of alkali and a mixture of RNases A and T1 on the structure of the closed circular chloroplast DNAs from pea, lettuce, and spinach plants. The results have shown that these DNAs contain covalently linked ribonucleotides. The kinetics of the alkali nicking of these ctDNAs has shown that each ctDNA[3] contains only one population of molecules. By comparing the rates of alkali nicking of these ctDNAs with the rate of alkali hydrolysis of RNA under identical conditions, we have estimated the number of ribonucleotides that are present in each of these ctDNAs. The pea and spinach ctDNAs contain a maximum of 18 ± 2 ribonucleotides, while lettuce ctDNA contains a maximum of 12 ± 2 ribonucleotides. We have further analyzed the structure of pea ctDNA by electron microscopic methods. These studies with alkali-fragmented DNA and reannealed alkali-fragmented DNA have shown that pea ctDNA contains 19 alkali-labile sites at specific locations. A map containing the relative location of each site has been constructed. These results suggest that individual ribonucleotides are located at the alkali-labile sites in the pea ctDNA.

EXPERIMENTAL PROCEDURE

Enzymes—RNase-free alkaline phosphatase was purchased from P-L Biochemicals. RNase-free DNase I and pancreatic RNase were obtained from Sigma Chemical Co. RNase T1, T4-induced lysozyme, and grade R pronase were from Calbiochem. The two RNases were heat treated at 100° for 20 min to inactive deoxyribonucleases. The pronase was self-digested at 37° for 4 hours to inactivate nucleases.

Nucleic Acid Preparation—Covalently closed circular ctDNA was isolated from pea, lettuce, and spinach plants as described (25). The DNA was dialyzed into buffer containing 0.1 M NaCl 0.05 M Tris 0.01 M EDTA, pH 8, divided into portions, and stored at –40°. The amount of

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‡ Predoctoral Trainee of National Institute of General Medical Sciences Grant GM-02063.

Present address, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115.

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[2] R. Kolodner, K. K. Tewari, and R. C. Warner, submitted for publication.

[3] The abbreviations used are: ctDNA, chloroplast DNA; RF, replicative form; form I DNA, native covalently closed circular DNA; form IV DNA, denatured covalently closed circular DNA.

closed circular DNA in a preparation was checked by sedimentation on 3 M CsCl 0.01 M EDTA, pH 8, in a Spinco model E analytical ultracentrifuge.[2] All of the preparations used in these experiments contained greater than 96% of the DNA as covalently closed circular molecules.

[^{32}P]RNA was isolated from *Escherichia coli* B that had been grown to late log phase in 25 ml of low phosphate medium containing 0.5 mCi/ml of $^{32}P_i$. The cells were harvested by centrifugation at 4° for 10 min at 10,000 × *g*, and were washed two times in 0.05 M Tris 0.01 M EDTA, pH 8 (Buffer A). The final *E. coli* pellet was suspended in 1 ml of Buffer A containing 100 μg/ml of T4 lysozyme, and was incubated at 37° for 10 min, 9 ml of H_2O were added, and the cells were disrupted by rapid Vortex mixing for 30 s. RNase-free DNase I (final concentration, 100 μg/ml) and $MgCl_2$ (final concentration, 10 mM) were added, and the incubation at 37° was continued for an additional 1 hour. The solution was extracted three times with phenol that had been saturated with 0.1 M Tris. The aqueous phase was extracted twice with diethyl ether, and was precipitated with 2 volumes of 95% ethanol at −40°. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min, and was washed four times with 70% ethanol at 4°. The final precipitate was dissolved in 1 ml of 0.2 × Buffer A, and was stored at −40°. The specific activity of the [^{32}P]RNA was 25 × 10^6 cpm/μg.

The RF DNA of *E. coli* bacteriophages φX 174 and G4 (25) was prepared as described (26).

Analytical Ultracentrifugation—The analytical ultracentrifugation experiments were performed in a Spinco model E analytical ultracentrifuge as previously described[2] (26). The alkaline sedimentation experiments were carried out in 3 M CsCl/0.2 M NaOH/0.01 M EDTA. The neutral sedimentation solvent was 3 M CsCl/0.01 M EDTA, pH 8. All experiments were performed at 20°.

RNA Hydrolysis—The rate of alkaline hydrolysis of the *E. coli* [^{32}P]RNA was determined by the method of Grossman *et al.* (4). Two micrograms of [^{32}P]RNA were incubated in 3 M CsCl/0.2 M NaOH/0.01 M EDTA (the alkaline sedimentation solvent) at 20°. At various times, 100 μl samples were removed and were neutralized with 200 μl of 0.2 M Tris-HCl; 100 μl of 0.25 M HCl were added (final pH, 2), and the sample was incubated for 1 hour at 37°. Then, 100 μl of 0.3 M KOH (final pH, 8.25) and 1.2 units of alkaline phosphatase were added, and the incubation at 37° was continued. After 1 hour, 100 μl of a solution containing 10 mM potassium phosphate (pH 6) and 10 mM sodium pyrophosphate, 200 μl of bovine serum albumin (1 mg/ml), 100 μl of 1 M HCl, and 0.5 ml of a 40% (v/v) suspension of Norit A (Matheson, Coleman and Bell) were added and mixed. The Norit was removed by centrifugation at 10,000 × *g* for 5 min, and three 20-μl portions of the supernatant were counted.

The $^{32}P_i$ released at a given time, which represents the number of phosphodiester bonds broken, was expressed as the fraction of the total radioactivity. The total available radioactivity was determined as described above, except that the Norit was omitted from the assay. The radioactivity was corrected for the 2% of the radioactivity that was released by alkaline phosphatase in the absence of alkaline hydrolysis. Greater than 97% of the total radioactivity was released by alkaline phosphatase after alkaline hydrolysis for 24 hours.

Electron Microscopy—The spreading techniques, heteroduplex formation procedures, and details of electron microscopy have been described elsewhere (27, 28). In these experiments, the lengths of the DNA molecules have been expressed as the lengths relative to double-stranded and single-stranded φX DNAs (φX units), which were used as internal standards.

RESULTS

Alkali Lability of ctDNA—Covalently closed circular pea ctDNA was centrifuged through 3 M CsCl/0.2 M NaOH/0.01 M EDTA, and the centrifugation cell was scanned at 6-min intervals. The DNA zone that was present in each scan is given in Fig. 1. This figure illustrates the decrease in the size of zones with time. The observed decrease in the size of the zones could not be accounted for by the effect of radial dilution. In order to quantitate the decrease in the amount of DNA present at any given time, the mass of each zone on our ultracentrifuge scans was measured and was corrected to the mass that would be present at a fixed radial position in the cell using the formula $R_1^2M_1 = R_2^2M_2$. R_1 and R_2 are two radial positions in the centrifuge cell, and M_1 and M_2 are the masses of the zones that represent the same amount of DNA at the two different radial positions. In Fig. 2 is plotted the log of the per cent of the DNA remaining against time for spinach ctDNA. The half-life of spinach form I ctDNA was 10 ± 1 min. Similar alkaline sedimentation experiments were performed with both φX 174 RF I and G4 RF I monomers and dimers. The results with G4 RF I DNA are also presented in Fig. 2. There was no detectable loss of either of these DNAs during alkaline sedimentation. Similarly, there was no loss of DNA when closed circular ctDNA was centrifuged through neutral sedimentation solvent. Thus, the disappearance of closed circular spinach ctDNA from the form IV zone during sedimentation through the alkaline sedimentation solvent is due to a specific effect of alkali on the DNA. The loss of spinach ctDNA from the form IV peak is due to alkali-induced single strand breaks which convert the rapidly sedimenting form IV configuration (264 S) to the more slowly sedimenting denatured single-stranded form of spinach ctDNA (57.7 S).

The closed circular ctDNAs from pea and lettuce plants were also analyzed by the above methods. Under identical conditions, pea form IV ctDNA disappeared with a half-life of 10 ± 1 min, while lettuce form IV ctDNA disappeared with a half-life of 15 ± 1 min. The half-life of each DNA was found to be independent of the preparation of DNA (two different preparations of each DNA were tested). In addition, the half-lives of lettuce, pea, and spinach ctDNA were independent of the batch of sedimentation solvent (two batches were tested) and the half-lives were independent of the speed of centrifugation (the experiments were performed at 16,000 rpm and 24,000 rpm). Furthermore, the half-lives of these DNAs were independent of the amount of DNA that was layered in these experiments (0.1 to 1.0 μg was used).

The kinetics of degradation of pea, lettuce, and spinach form I ctDNAs was first order, and showed only one component. In the above experiments, the amount of DNA present at the zero time was taken as the amount of DNA present in the first scan. In order to rule out the presence of a rapidly degrading component, the amount of closed circular DNA present at the time of layering was measured by sedimenting the same volume of sample through neutral solvent in a parallel cell in the same rotor. The mass of the zone was corrected to the standard radial position as described above, and was also corrected for the hyperchromic shift of denatured DNA. The

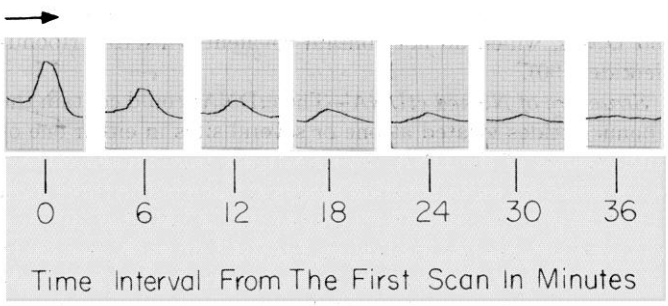

FIG. 1. Alkali breakdown of pea ctDNA. Pea form I ctDNA was centrifuged in the alkaline sedimentation solvent at 16,000 rpm, as described under "Experimental Procedure." The form IV peak from scans taken at 6-min intervals during this experiment is shown to illustrate the loss of DNA from the form IV zone. No other zones were present during this experiment, or in other experiments of this type. The zero time peak presented here was scanned at 4 min after layering, and the 36-min peak represents 4% of the applied DNA. The experiment presented here represents a period of 4.2 half-lives. Sedimentation is from *left* to *right*.

results of such an experiment are also presented in Fig. 2. This treatment produced kinetic data that were identical to those that were obtained when the amount of DNA present in the first scan was used as the zero time. Furthermore, the half-lives of each of the ctDNAs were unaltered. It has been possible to observe the degradation of each ctDNA for a minimum of four half-lives and, in all cases, the kinetic data were first order, showing only one component.

Ribonucleases A and T1 Nick ctDNA—The alkali sensitivity of ctDNA could be due to the presence of covalently inserted ribonucleotides. This was tested by incubating ctDNA with RNases A and T1 under conditions where these enzymes will digest RNA in an RNA-DNA duplex as well as double-stranded RNA (29). The pea form I ctDNA was incubated with a mixture of RNase A and RNase T1 for increasing lengths of time. The samples were pronase treated, and were analyzed by sedimentation in neutral 3 M CsCl. The covalently closed circular pea ctDNA (89.1 S) was successively converted to the open circular form (58.3 S). The kinetic data of this conversion are illustrated in Fig. 3. A 3-hour incubation with RNase A and (T1) quantitatively converted pea, lettuce, and spinach form I ctDNA to the open circular form, but did not produce any unit length linear molecules (≈50 S) or smaller molecules. G4 RF I monomers and dimers were also incubated with RNases A and T1 under identical conditions. These molecules were not nicked in these experiments (Fig. 3).

Number of Ribonucleotides in ctDNA—The preceding experiments indicated the presence of ribonucleotides in the form I ctDNA. To determine the number of ribonucleotides present in the ctDNA, we have compared the rate of alkaline hydrolysis of ctDNA to the rate of alkaline hydrolysis of *E. coli* [^{32}P]RNA under identical conditions. The kinetic data of hydrolysis of the [^{32}P]RNA were first order, and the RNA had a half-life of 180 min (Fig. 4). This half-life represents the rate of breakage of a single RNA-RNA phosphodiester bond. The rate of nicking pea and spinach ctDNA was 18 times as fast as the rate of breaking a single RNA-RNA phosphodiester bond. Therefore, pea and spinach form I ctDNA nick at rates that would be expected if they each contained a maximum of 18 ± 2 ribonucleotides. Similarly, the lettuce form I ctDNA was nicked 12 times as fast as an RNA phosphodiester bond, which corresponds to the presence of 12 ± 2 ribonucleotides in the lettuce ctDNA.

The ctDNAs we have examined are nicked at rates that are 100 to 150 times faster than the rates of nicking of viral and *E. coli* DNAs, which do not contain covalently inserted ribonucleotides (30).

Structure of Nicked ctDNA—The ctDNAs could contain the ribonucleotides located at one or several sites in either one or both strands of the DNA. To investigate this, nicked ctDNA was studied by sedimentation analysis. Each of the three form I ctDNAs was incubated for 3 hours in 0.2 M NaOH at 20°, and was then sedimented in alkaline 3 M CsCl. In each case, 20 to 40% of the DNA sedimented at the position of intact single strands, while the rest of the DNA sedimented more slowly as a broad zone. This indicated that the alkaline-labile sites could be located at multiple positions in both strands. When the form I ctDNAs were incubated for 3 hours with RNases A and T1, a similar sedimentation pattern resulted.

Using centrifugation techniques, it would require large quantities of ctDNA to determine if RNase treatment or alkaline hydrolysis of form I ctDNA produces specific size classes of fragments. Therefore, this problem was studied by

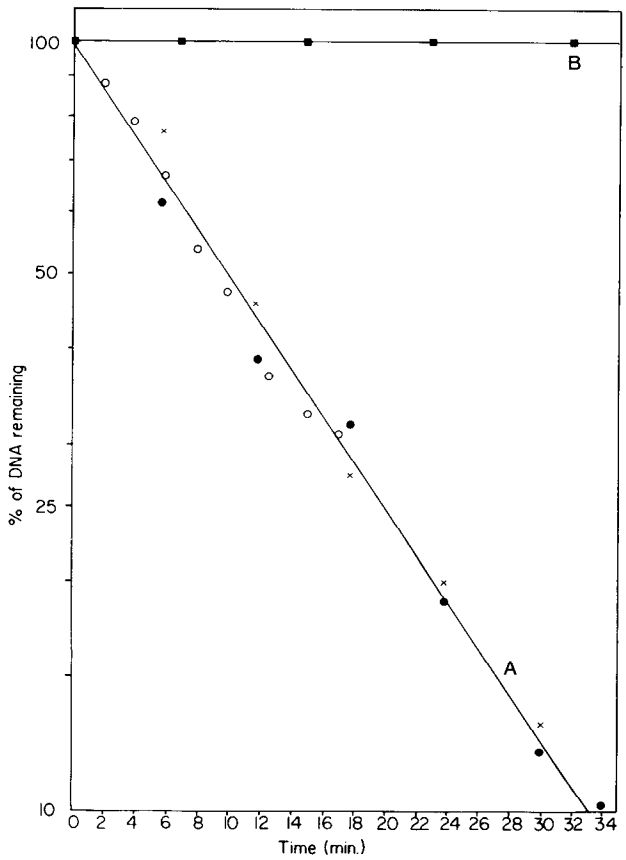

FIG. 2. Kinetics of the alkali nicking of ctDNA. Spinach ctDNA form I was sedimented in the alkaline sedimentation solvent at 16,000 rpm, and the size of the zones present at various times was determined as described under "Results." The log of the per cent of the DNA remaining at various times is plotted. O——O, DNA preparation 1 and solvent preparation 1, using the first scan at the zero time point. ×——×, DNA preparation 2 and solvent preparation 1, using the first scan as the zero point. ●——●, DNA preparation 1 and solvent preparation 2, using the amount of DNA present at the time of layering as the zero time point. Also presented (■——■) is a similar experiment performed with *Escherichia coli* bacteriophage G4 RF I DNA, in which both the monomers and dimers were analyzed.

electron microscopy. Pea form I ctDNA was incubated with 0.2 M NaOH at 20° for 16 hours (≈96 half-lives), as described in Fig. 5, and was mounted for electron microscopy by the formamide technique (28) to visualize single-stranded DNA. The length distribution of the fragment sizes produced by this treatment is presented in Fig. 5. A large number of fragment size classes were observed. The largest fragment was 12.4 ϕX units long. The length of intact pea ctDNA is 26.7 ϕX units (22). These results indicated that the alkali-labile sites in pea ctDNA were located in both strands of the DNA, since we did not observe unit length single-stranded circular molecules. It is quite possible that the alkali-labile sites are located at specific positions, because the length distribution of the fragments was not random.

Map of the Nicks in Pea ctDNA—The previous experiments had indicated that pea ctDNA might contain covalently inserted ribonucleotides located at specific sites. In order to investigate this further, the pea form I ctDNA was incubated in 0.2 M NaOH for 3 hours (≈ 18 half-lives), which will nick more than 99% of the molecules, but will not digest all of the alkali-labile sites. These fragments were then partially reannealed to produce molecules that generally had single-stranded tails and internal duplex sections. The formation of such a

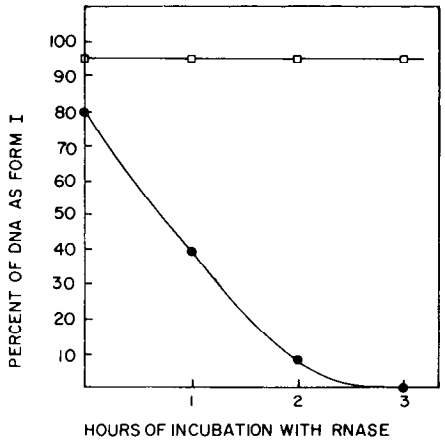

FIG. 3. Kinetics of ribonuclease nicking of ctDNA. One microgram of form I DNA was incubated at 37° for various times with 50 μg/ml of RNase A and 50 units/ml of T1 RNase in 0.01 M NaCl 0.005 M Tris 0.001 M EDTA. After each time period, the DNA was further digested with 100 μg/ml of pronase for one-half hour, and the sample was sedimented in neutral sedimentation solvent in the model E analytical ultracentrifuge at 24,000 rpm. The amount of the DNA present in the form I and form II zones was determined and standardized as described under "Results." The per cent of the DNA remaining as form I is plotted against incubation time for pea ctDNA (A) and G4 RF I monomers and dimers (B). The zero time point represents the nonspecific nicking that took place during a 3-hour incubation without RNase and with a one-half-hour incubation with pronase.

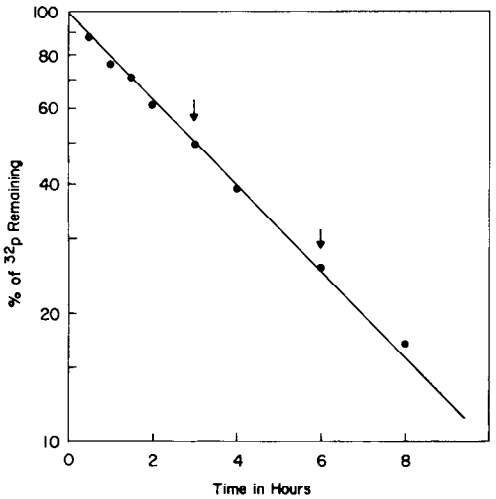

FIG. 4. Hydrolysis of [^{32}P]RNA in 3 M CsCl/0.2 M NaOH/0.01 M EDTA, at 20°. The log of the proportion of $^{32}P_i$ resistant to release by alkaline phosphatase at various times is presented. The details of this experiment are given under "Experimental Procedure" and by Grossman *et al.* (4). *Arrows* indicate the times at which 50% and 25% of the ^{32}P was resistant to digestion by alkaline phosphatase.

molecule is illustrated in Fig. 6. A molecule that is formed from two fragments will map the positions of four breaks relative to each other. A molecule constructed from three or more fragments can map the positions of more than four breaks. Two examples of such molecules are presented in Fig. 7. This procedure will generate a number of overlapping molecules from which a map can be constructed containing the positions of the nicks relative to each other. This map should be circular with a monomer repeat length of 26.7 φX units (the length of pea ctDNA), if the alkali-labile sites are at specific sites.

A map of the relative positions of the alkali-labile sites in pea ctDNA is presented in Fig. 8. Nineteen single strand breaks were located at distinct sites. All of the molecules were

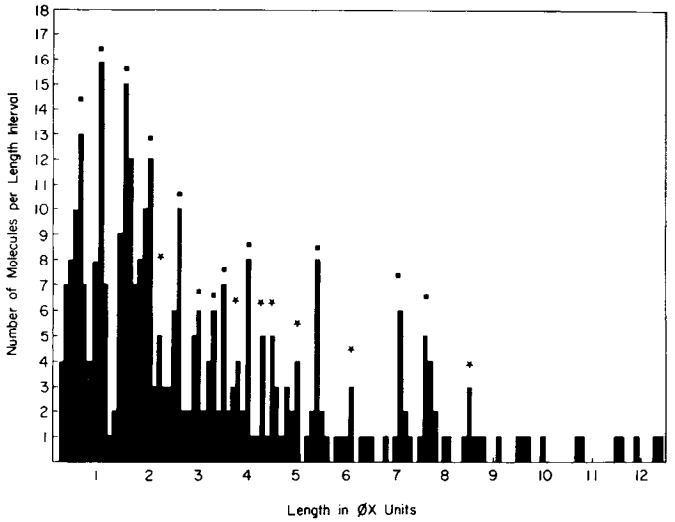

FIG. 5. Length distribution of fragments produced by the alkali hydrolysis of pea form I ctDNA. Pea form I ctDNA (10 μg/ml) was incubated at 20° in 0.2 M NaOH 0.04 M EDTA for 16 hours (~96 half-lives) and was neutralized with 1.8 M Tris-HCl 0.2 M Tris. This DNA was spread with single-stranded φX 174 DNA by the formamide technique; the spreading solution contained 50% formamide, and the hypophase contained 20% formamide as described (27, 28). Fields were selected and photographed randomly, and all of the molecules on a negative were measured. ■, size classes that match the fragment lengths predicted by the map that is presented in Fig. 9 and Table II. *, size classes that match fragment lengths resulting from incomplete digestion of pea form I ctDNA, as predicted by the map presented in Fig. 9 and Table II.

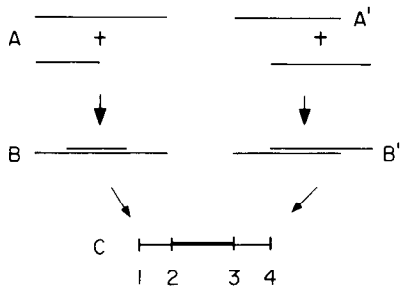

FIG. 6. Formation of a partially duplex molecule by the reannealing (*B*, *B'*) of two single-stranded fragments (*A*, *A'*). It can be seen that two molecules that appear identical in the electron microscope (*C*) could be produced by the reannealing of two different sets of two fragments. Regardless of its formation, such a molecule will map the position of four single strand breaks relative to each other (*C*, 1-4).

consistent with this map. It should be noted that at Sites 6 and 16, two single strand breaks were mapped on opposite strands at the same position. When two nicks mapping on opposite strands at the same site were found on a hybrid molecule (Fig. 7*B*; Fig. 8 *DS*), one end of the molecule appeared to be fully duplex. The repeat length of the map was 26.7 φX units. The large molecules fitted this repeat length with a standard deviation of 0.26 φX units (Fig. 8 *right*). This standard deviation is less than expected in measuring single-stranded molecules of this size (28).

Strand Location of the Alkali-labile Sites of Pea ctDNA—If the map presented in Fig. 8 accurately represents the locations of the alkaline-labile sites, it should be possible to make unambiguous strand assigments for each of the nicks. The two nicks that define a single strand tail of a reannealed molecule are located on opposite strands (see Fig. 6). Using this criteria, it was possible to make a list of the nicks that were located on opposite strands from each other. The nicks at positions 1, 5, 8,

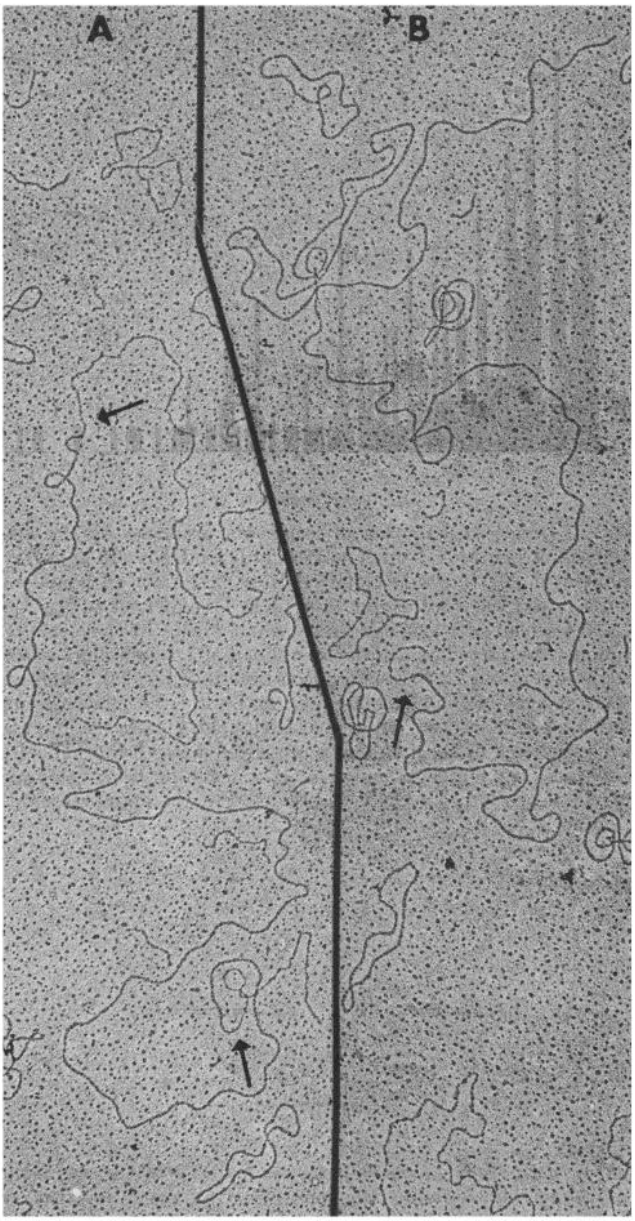

FIG. 7. Pea ctDNA molecules formed by reannealing alkaline hydrolysis fragments, as illustrated in Fig. 6. Pea form I ctDNA was hydrolyzed as described in Fig. 5 and 3 hours, reannealed for 1.5 hours as described (27, 28), and spread with single-stranded and double-stranded ϕX 174 DNA. *Arrows* mark the junctions between the single-stranded and duplex regions in the molecules. Molecule *B* has one fully duplex end. Both single-stranded and double-stranded circular ϕX 174 DNA molecules are present in these pictures. Magnification, × 20,000.

11, 12, 14, and 15 (Fig. 9, *Strand A*) were located on one strand, while the nicks at positions 2, 3, 4, 7, 9, 10, 13, and 17 (Fig. 9, *Strand B*) were located on the other strand. This method located nicks on both strands at positions 6 and 16, which agrees with the previous finding that there was one nick located on each strand at these two positions. A map of the relative positions of the alkali-labile sites in pea ctDNA is presented in Fig. 9 and Table I.

In Table II, we have presented the fragment sizes predicted from this map and the fragment sizes that were found in an extensive alkaline digest of pea form I ctDNA. The data of Table II show an excellent agreement between the predicted fragment sizes and the fragment sizes that were observed (Fig. 5, ■). There were several size classes (Fig. 5, *) which were not predicted from the map. However, these size classes correspond to the size predicted by the sum of two adjacent fragments in Fig. 9. These data are also presented in Table II. We have also observed several individual fragments (Fig. 5) which did not fit into any size class. These longer fragments could also arise from incomplete alkali digestion of pea form I ctDNA.

DISCUSSION

We have shown that covalently closed circular pea, lettuce, and spinach ctDNAs are nicked by exposure to alkali or by a mixture of RNases A and T1. These results are consistent with the idea that these covalently closed circular DNAs contain covalently incorporated ribonucleotides. Such ribonucleotides have been found in animal mitochondrial DNAs (2–4), herpes simplex virus DNA (9), and in the DNA from *E. coli* bacteriophages T2, T4 (10), and T5 (11).

The measured kinetic data of the nicking of pea, lettuce, and spinach form I ctDNAs at alkaline pH were first order and monophasic. There was no evidence for the existence of two classes of alkali-labile form I ctDNA molecules. In the case of animal mitochondrial DNA (2, 4), 35 to 45% of the form I DNA was found to nick 3 to 4 times faster than the rest of the form I DNA. The kinetics of form I ctDNA nicking was followed at 2-, 4-, or 6-min intervals for a period of at least four half-lives. During this period, 95% of the ctDNA was found to degrade as a single component. These results are in contrast to those observed with animal mitochondrial DNA.

We have estimated the number of ribonucleotides present in form I ctDNAs by comparing the rates of nicking of these form I ctDNAs to the rate of hydrolysis of a single RNA-RNA phosphodiester bond under identical experimental conditions. The spinach and pea ctDNAs were found to contain 18 ± 2 covalently incorporated ribonucleotides/molecule, while lettuce ctDNA was found to contain 12 ± 2 ribonucleotides/molecule. This difference between lettuce ctDNA and spinach and pea ctDNA is highly reproducible, but its significance is not understood. The number of ribonucleotides that we have estimated to be present in these ctDNAs is the maximum possible number that could exist, since the method assumes that all of the alkali-labile sites are ribonucleotides. The possible existence of other kinds of alkali-labile sites in DNA is discussed below.

The structure of pea ctDNA was extensively studied by examining alkali-digested and reannealed alkali-digested pea form I ctDNA in the electron microscope. The results showed that closed circular pea ctDNA contains 19 alkali-labile sites located at 17 specific positions. A map of these sites is presented in Fig. 9 and Table I. The excellent agreement between the number of alkali-labile sites measured electron microscopically and the number of ribonucleotides present determined from the alkaline hydrolysis rates suggests that all of the alkali-labile sites in pea ctDNA are due to covalently inserted ribonucleotides. It also suggests that there is one ribonucleotide/site. The similarity between the sedimentation profiles of alkali-nicked ctDNA and ribonuclease-nicked ctDNA also supports these observations. Grossman *et al.* (4) have suggested the existence of other kinds of alkali-labile sites besides ribonucleotides. The only other suggested mechanism (32) by which alkali-labile sites could be produced in DNA would not produce breaks at specific sites. We are presently

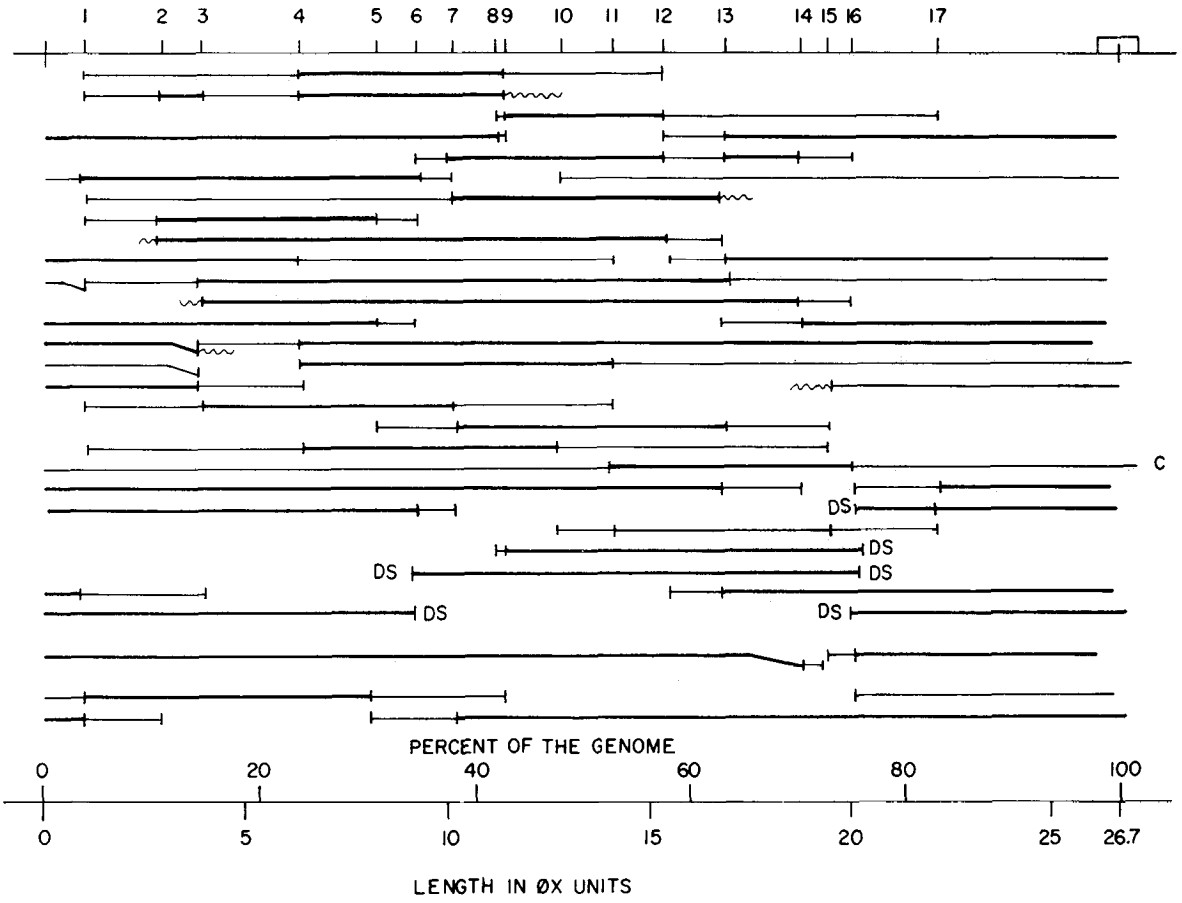

FIG. 8. Map of overlapping fragments of pea ctDNA produced from molecules generated by the procedures outlined in Figs. 6 and 7 and the text. All of the molecules containing duplex regions were photographed and the individual molecules were represented on graph paper. The positions of the single strand breaks in each molecule were aligned with the positions of the single strand breaks located in other molecules. This procedure is analogous to the partial digestion procedure that is used to order restriction endonuclease fragments (31). The map that is presented here is circular and was arbitrarily linearized for display purposes. *DS*, molecules with fully duplex tails; ~, single-stranded tail that ran off the edge of the negative; ——, single-stranded region; ■, duplex region. *Right side* of the figure shows the deviation of the long molecules from the unit length of pea ctDNA.

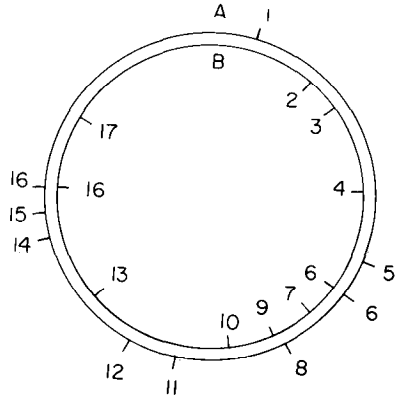

FIG. 9. Map of the positions of the alkali-labile sites in pea ctDNA. Strand assignments for each site are those described in the text. Positions of each site, relative to Site 1, are presented in Table I.

investigating the nature of the ribonucleotides that are located at these sites.

Alkali-labile sites are located on both strands at positions 6 and 16 of the map of alkali-labile sites in pea ctDNA. We have observed that when either of these pairs of nicks is present on the same hybrid molecule (Fig. 7*B*) one end appears to be fully duplex. It would not be possible to see a single-stranded tail that is 100 nucleotides long. Therefore, it is possible that the two alkali-labile sites at either position are as far apart as 100 nucleotides. Ribonuclease digestion of pea ctDNA produced only open circular molecules and not linear molecules, which supports the idea that the two alkali-labile sites at these positions are separated from each other. It will require further experimentation to resolve this point.

The significance of the individually inserted ribonucleotides in form I ctDNA is not understood at this time. The ribonucleotides could arise from a nonstringent DNA polymerase, but in that case, they would not be located at specific positions in the ctDNA molecule. It is possible that the ribonucleotides are remnants of RNA primers which are now generally believed to initiate DNA replication. In pea ctDNA, replication is initiated at two sites located on opposite strands of the DNA molecule. Replication is bidirectional, and at least 50% of the ctDNA is synthesized bidirectionally[1] (33). If the ribonucleotides resulted from incomplete excision of the primers that are used for the initiation of pea ctDNA replication, we would expect to observe only two alkali-labile sites. If they were remnants of primers for "Okazaki fragments," we would expect to observe on the order of 40 to 50 alkali-labile sites. In addition, they would be located more uniformly than the alkali-labile sites we have observed. It should be pointed out that in all systems in which RNA primers for DNA replication have been studied,

TABLE I

Relative positions of alkali-labile sites in pea ctDNA

Site	Per cent of genome	Kilobase pairs[a]	Number of determinations
1	0 S.D. ± 0.22	0 S.D. ± 0.30	11
2	6.92 S.D. ± 0.21	9.33 S.D. ± 0.28	4
3	10.67 S.D. ± 0.26	14.37 S.D. ± 0.35	9
4	19.96 S.D. ± 0.18	26.86 S.D. ± 0.24	7
5	27.12 S.D. ± 0.20	36.50 S.D. ± 0.27	5
6	30.71 S.D. ± 0.22	41.34 S.D. ± 0.30	7
7	34.13 S.D. ± 0.26	45.95 S.D. ± 0.35	7
8	38.20 S.D. ± 0	51.42 S.D. ± 0	4
9	38.95 S.D. ± 0	52.43 S.D. ± 0	5
10	43.94 S.D. ± 0.22	59.15 S.D. ± 0.30	3
11	48.99 S.D. ± 0.16	65.94 S.D. ± 0.21	5
12	53.99 S.D. ± 0.26	72.66 S.D. ± 0.35	7
13	59.44 S.D. ± 0.25	80.00 S.D. ± 0.34	10
14	66.52 S.D. ± 0.20	89.53 S.D. ± 0.27	5
15	69.10 S.D. ± 0.21	93.01 S.D. ± 0.28	6
16	71.57 S.D. ± 0.19	96.34 S.D. ± 0.43	10
17	79.31 S.D. ± 0.19	106.75 S.D. ± 0.26	4

[a] Calculated using 6.62×10^5 as the average molecular weight of a kilobase pair and 89.1×10^6 as the molecular weight of pea ctDNA.[3]

TABLE II

Fragment sizes produced by alkali digestion of pea ctDNA

Strand	Fragment[a]	Size[b] observed in the map of Fig. 9	Size[c] observed in the alkali digest of Fig. 5
A	1-5	7.24 S.D. ± 0.11	7.1 S.D. ± 0.13
	5-6	0.96 S.D. ± 0.11	1.0 S.D. ± 0.05
	6-8	2.0 S.D. ± 0.05	2.0 S.D. ± 0.07
	8-11	2.88 S.D. ± 0.05	2.95 S.D. ± 0.08
	11-12	1.34 S.D. ± 0.11	1.5 S.D. ± 0.06
	12-14	3.35 S.D. ± 0.12	3.5 S.D. ± 0.09
	14-15	0.69 S.D. ± 0.11	0.6 S.D. ± 0.04
	15-16	0.66 S.D. ± 0.14	0.6 S.D. ± 0.04
	16-1	7.59 S.D. ± 0.15	7.6 S.D. ± 0.14
	8-12	4.22 S.D. ± 0.07	4.3 S.D. ± 0.1
	11-12	4.69 S.D. ± 0.10	4.8 S.D. ± 0.11
	5-11	5.84 S.D. ± 0.12	6.1 S.D. ± 0.12
	12-16	4.70 S.D. ± 0.17	4.5 S.D. ± 0.1
B	2-3	1.0 S.D. ± 0.12	1.0 S.D. ± 0.05
	3-4	2.48 S.D. ± 0.12	2.6 S.D. ± 0.08
	4-6	2.87 S.D. ± 0.1	2.95 S.D. ± 0.08
	6-7	0.92 S.D. ± 0.12	1.0 S.D. ± 0.05
	7-9	1.3 S.D. ± 0.07	1.5 S.D. ± 0.06
	9-10	1.33 S.D. ± 0.08	1.5 S.D. ± 0.06
	10-13	4.13 S.D. ± 0.12	4.0 S.D. ± 0.1
	13-16	3.24 S.D. ± 0.15	3.25 S.D. ± 0.08
	16-17	2.06 S.D. ± 0.12	2.0 S.D. ± 0.07
	17-2	7.4 S.D. ± 0.15	7.6 S.D. ± 0.14
	6-9	2.2 S.D. ± 0.06	2.2 S.D. ± 0.07
	4-7	3.79 S.D. ± 0.11	3.8 S.D. ± 0.1
	17-3	8.38 S.D. ± 0.13	8.5 S.D. ± 0.14
	13-17	5.3 S.D. ± 0.12	5.4 S.D. ± 0.11

[a] Each fragment is defined as the shortest piece of DNA that would result from alkali cleavage of the two sites given (see Figs. 8 and 9).

[b] Calculated with the data of Table I, using 26.7 ϕX units as the length of pea ctDNA (22, 23).

[c] Taken as the sizes of the peak fractions in Fig. 5. The standard deviations were calculated according to Davis *et al.* (28).

the primer is completely excised in the mature DNA except under abnormal conditions. We consider it possible that these ribonucleotides have some function in ctDNA. These sites could be involved in transcription, recombination, or some other process that requires specific recognition sites. It will require further experimentation to test these possibilities.

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