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Inverted Repetitious Sequences in the Macronuclear DNA of Hypotrichous Ciliates*

(electron microscopy/site-specific endonuclease/DNA structure)

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ABSTRACT **The low-molecular-weight macronuclear DNA of the hypotrichous ciliates *Oxytricha*, *Euplotes*, and *Paraurostyla* contains inverted repetitious sequences. Up to 89% of the denatured macronuclear DNA molecules form single-stranded circles due to intramolecular renaturation of complementary sequences at or near the ends of the same polynucleotide chain. Other ciliated protozoans, such as *Tetrahymena*, with high-molecular-weight macronuclear DNA and an alternative mode of macronuclear development, appear to lack these self-complementary sequences.**

The denatured macronuclear molecules of hypotrichs are held in the circular conformation by a hydrogen-bonded duplex region, which is probably less than 50 base pairs in length, since the duplex regions are not visible by electron microscopy and since the circles in 0.12 M phosphate buffer are not retained during hydroxylapatite chromatography at 60°. The existence of extremely small circles, with contour lengths shorter than the smallest pieces of native DNA, suggests that inverted repetitions containing nicks (broken phosphodiester bonds in duplex DNA) or gaps (interruptions with missing nucleotides) are present at internal positions as well as at the ends of native molecules. Estimates from length measurements of native and denatured *Oxytricha* macronuclear DNA indicate an average of 1.7 nicks per duplex molecule. Thus, in order to account for the high frequency of circle formation, a restriction-type enzyme(s) must exist which inserts single-strand, site-specific nicks or gaps at internal positions in the macronuclear DNA of *Oxytricha*.

Most ciliated protozoa have a macro- and a micronucleus. After conjugation, a new macronucleus is derived from a single, diploid micronucleus by a temporally well-defined developmental program. In hypotrichous ciliates macronuclear development is characterized by a biphasic cycle of extensive DNA synthesis (1) which involves the formation of polytene chromosomes that are cut into band-length pieces (2). The transection of the chromosomes is followed by degradation of most of the DNA in each band (3). Bostock and Prescott (4) have shown that only some micronuclear DNA sequences are retained during the formation of the macronucleus.

The following information is available about the DNA that remains in the mature macronucleus of hypotrichs. (*i*) The DNA is in small pieces having the physical size equivalent to one to a few genes (5). The low-molecular-weight nature of the DNA may have some bearing upon the unusual cytological structures, termed replication bands, where DNA synthesis characteristically occurs in hypotrichous ciliates (6). (*ii*) The renaturation kinetics of denatured macronuclear DNA are approximately second-order, indicating the presence of a single DNA component with a complexity about 13 times greater than *Escherichia coli* DNA (M. Lauth, J. Heumann, B. Spear, and D. M. Prescott, manuscript in preparation). (*iii*) The macronuclear DNA pieces possess a polarity, i.e., one end is different from the other, because a large region at one end of each molecule melts at a slightly lower temperature than the rest of the molecule, and because RNA polymerase binds exclusively at only one end (7).

The experiments reported here demonstrate that single-strand nicks or gaps and inverted repetitious sequences are other important structural features of macronuclear DNA.

Abbreviations: SSC, standard saline–citrate, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5; RF, replicative form of phage ϕX174 DNA.

* This paper is No. IV in the series, "DNA of Ciliated Protozoa." The preceding paper is ref. 7.

MATERIALS AND METHODS

Preparation of Oxytricha Macronuclear DNA. The hypotrich *Oxytricha* was cultivated aseptically in 0.1% Cerophyl (Cerophyl Laboratories, Kansas City, Mo.), with axenically grown *Tetrahymena* as the food organism (4). *Oxytricha* DNA was labeled by feeding *Tetrahymena* that had been cultured in defined medium (8) containing [^{3}H]thymidine (New England Nuclear, 25 μCi/ml, 20 Ci/mmol). The resultant specific activity of the purified DNA was 2500 cpm/μg.

For DNA isolation, the hypotrichs were collected on a 10 μm nylon mesh; macronuclei were prepared by the technique of Prescott *et al.* (5) and stored at −20°.

Thawed macronuclear preparations were lysed at 60° for 1–2 hr in a solution of 0.5% Sarkosyl, 0.15 M NaCl, 0.1 M EDTA (pH 8.0) containing 0.5 mg/ml of Pronase CB (Calbiochem, predigested for 90 min at 37° to eliminate DNase activity). To separate the DNA from protein and RNA, 4.86 ml of the nuclear lysate was added to 6.23 g of solid CsCl (Schwarz/Mann) and centrifuged to equilibrium at 33,000 rpm for 62 hr in the Spinco no. 65 fixed-angle rotor at 20°. Following centrifugation, four-drop fractions were collected from the bottom of the tube, 0.5 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) was added to each fraction, and the DNA band was located by its A_{260}. Pooled fractions were dialyzed against SSC and stored at −20°.

Macronuclear DNA preparations were shown to be free of *Tetrahymena* DNA contamination by analytical equilibrium density gradient centrifugation. The buoyant density of *Oxytricha* macronuclear DNA is 1.701 g/cm^3 and that of *Tetrahymena* DNA is 1.690 g/cm^3 (4).

The macronuclear DNAs of *Euplotes* and *Paraurostyla* were prepared similarly by isolating macronuclei, followed by nuclear lysis and CsCl density gradient centrifugation.

Preparation of Bacteriophage ϕX174 DNA. ϕ*Xam3*, a lysis-defective amber mutant of ϕX174, and *Escherichia coli* (HF

4704) were kindly provided by Dr. Dan S. Ray. ϕX replicative form (RF) DNA was prepared by blocking viral single-stranded DNA synthesis with chloramphenicol. The procedure was as described previously (9) except that RNA digestions and the Sephadex G-100 chromatography step were eliminated. Single-strand breaks were then introduced into the covalently closed molecules (form I) by photolysis ("photonicking") for 45 min in the presence of ethidium bromide as described by Smith and Vinograd (10). The nicked molecules were separated from ϕX RF I by ethidium bromide–CsCl density gradient centrifugation, the dye was removed by dialysis against Dowex 50, and the DNA in SSC was used as a double-stranded DNA size standard for electron microscopy.

The same general protocol was used to prepare ϕX single-stranded circular DNA, but chloramphenicol was omitted. Infected cells were vigorously aerated for 2 hr while [^{14}C]-thymidine was added in aliquots of 5 μCi at 1, 30, and 60 min post-infection. Collection and lysis of infected *E. coli* were as described above except that sucrose gradients were centrifuged for 12 hr at 24,000 rpm in the Spinco SW 27 rotor at 5°. The radioactivities of aliquots were measured, and fractions containing mature viral DNA were pooled and dialyzed against SSC. The DNA was further purified by neutral CsCl density gradient centrifugation for 48 hr at 40,000 rpm in the Spinco SW rotor at 20° (ρ_0 = 1.723 g/cm^3). This constituted the single-stranded DNA standard for electron microscopy.

Conditions for the Formation of Single-Stranded Circles in Macronuclear DNA. Single-stranded circles were prepared as follows: macronuclear DNA (0.5–1.0 μg) was denatured in 0.5 ml of 0.1 M NaOH, 0.02 M EDTA at 22–24° for 10 min. Next, 50 μl of 1.8 M Tris·HCl, 0.2 M Tris, and 0.5 ml of formamide (99% pure, Matheson, Coleman and Bell) were added so that the final annealing pH was 8.0. Renaturation was allowed to continue for 10 min at 25°, after which cytochrome *c* was added and the sample was immediately spread for electron microscopy. Alternatively, the renatured sample was dialyzed against 0.07 M Tris·HCl, pH 7.6, for exonuclease III digestion or against SSC for electron microscope visualization by the aqueous Kleinschmidt technique.

Electron Microscopy. DNA samples were mounted by either a modified Kleinschmidt protein-monolayer technique (11) or by the formamide procedure (12). For formamide mounting, the spreading solution generally contained 0.5 μg/ml of DNA and 0.1 mg/ml of cytochrome *c* in 50% formamide, 0.01 M EDTA, 0.1 M Tris, pH 8.5. The DNA-containing solution was spread on the surface of distilled water.

Formvar-coated copper grids, stabilized with carbon on the reverse side, were used. The DNA was picked up on the Formvar surface of the grid, stained with uranyl acetate, shadowed with platinum–palladium, and photographed at a magnification of 6640× or 9880× with a Philips EM 300.

Molecules were enlarged on a Nikon profile projector, traced, and contour lengths were determined with a Keuffel and Esser map measurer. As an internal standard, either photo-nicked ϕX RF DNA or single-stranded ϕX circular DNA was cospread with the sample.

For some experiments, DNA samples were treated in a 5% (v/v) formaldehyde solution for 15 min at 60° before spreading to prevent the formation of hydrogen-bonded duplexes.

Exonuclease III Incubation Conditions. Exonuclease III, [deoxyribonucleate (double-stranded) 5′-nucleotidohydrolase, EC 3.1.4.27] an enzyme that releases mononucleotides from the 3′ end of a DNA double helix (13), was used to determine if the single-stranded circles, which rapidly form after denaturation of macronuclear DNA, were sealed by a region of duplex DNA. The exonuclease III reactions were carried out as described by Wolfson and Dressler (14). The enzyme was generously provided by Dr. Tom Kornberg.

Single-stranded macronuclear DNA circles were dialyzed overnight to remove the formamide (four changes against 0.07 M Tris·HCl, pH 7.6). The solution was then made 7 mM in $MgCl_2$ and 10 mM in 2-mercaptoethanol. Two units of exonuclease III (13) were added to 0.25 μg of single-stranded circular DNA and the mixture was incubated at 45° for 20 min. Incubation was at 45° to increase the specificity of the enzyme for the 3′ end of double-stranded DNA (13). To insure completion of the reaction, an additional unit of enzyme was added and allowed to act for 20 min, at which time the reaction tube was transferred to 0°. The material was then processed for electron microscopy.

Control experiments, in which single-stranded ϕX DNA circles were incubated with exonuclease III under the same conditions, were performed to exclude the possibility of conversion of circular to linear molecules by an endonuclease activity. A preparation containing 87% (160/183) phage circles remained essentially unaltered after exonuclease III incubation (85%, 182/214), indicating the absence of any endonuclease contaminant.

Hydroxylapatite Chromatography. Hydroxylapatite (DNA grade) was obtained from Bio-Rad (Richmond, Calif.). Small columns (1 ml bed volume) equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) were maintained at 60°. [^{3}H]-Thymidine labeled, single-stranded rings were prepared by the standard formamide method as described above and dialyzed against 0.12 M sodium phosphate buffer. The sample (0.7 μg of DNA) was then applied at 60° to the upper layers of hydroxylapatite in the columns. The columns were washed with five 1 ml aliquots of 0.12 M sodium phosphate buffer at 60° to remove all unbound material. With these conditions, less than 5% of single-stranded DNA binds to hydroxylapatite, whereas 99.9% of double-stranded DNA is bound. To remove the bound material, the columns were washed with five 1 ml aliquots of 0.4 M sodium phosphate buffer at 60°.

RESULTS

Inverted repetitions in macronuclear DNA

When macronuclear DNA of hypotrichous ciliates is alkaline or heat-denatured and allowed to renature for 10 min at 25° in 50% formamide (pH 8.0), up to 89% (Table 1) of the single-stranded molecules assume a circular conformation (Fig. 1). These single-stranded circles are variable in length and generally show no visible projections or tails (Fig. 1b). In contrast, native *E. coli* DNA, sheared by syringe passage to approximately the same size and denatured and annealed under identical conditions, occurs only as single-stranded linear pieces.

The single-stranded circles must result from intramolecular base sequence complementarity, since the renaturation conditions are four orders of magnitude below the $C_0t_{1/2}$ value (15) for the bimolecular reaction of *Oxytricha* macronuclear DNA (M. Lauth, J. Heumann, B. Spear, and D. M. Prescott, manuscript in preparation). Therefore, inverted repetitious sequences must be present at or near the ends of each DNA strand.

Many of the circular single-stranded molecules are so small

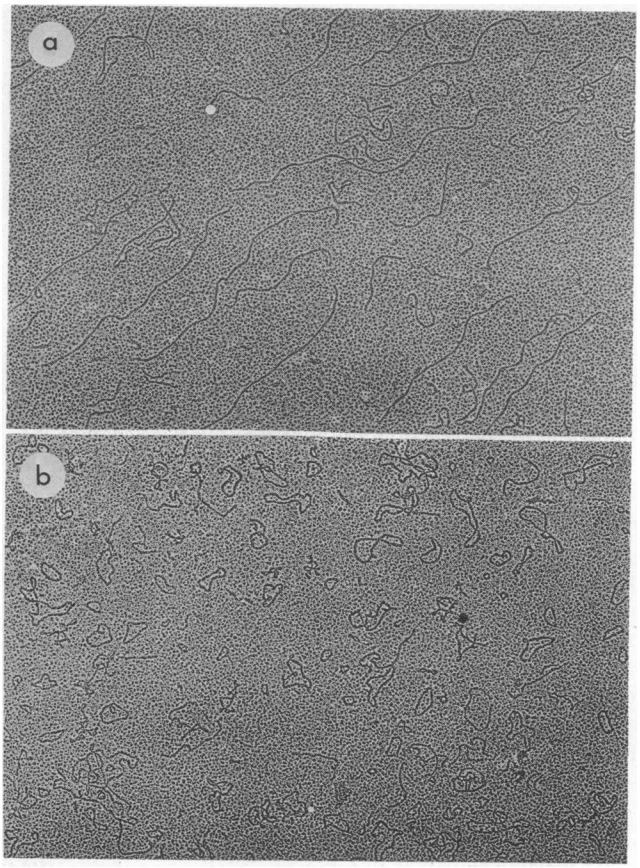

FIG. 1. Electron micrographs of *Oxytricha* macronuclear DNA (a) before and (b) after alkaline denaturation and renaturation. The conditions for denaturation and partial renaturation are described in *Materials and Methods*. Most of the denatured DNA is in the form of single-stranded circular molecules. Magnification ×16,750.

that they can represent only a part of the smallest native molecule (Fig. 1). In addition, the mean contour length for single-stranded circles is less than the mean contour length for the pieces of native macronuclear DNA. The measurements are compared in Fig. 2. For this experiment, either photonicked, duplex ϕX RF DNA or single-stranded ϕX circles,

TABLE 1. *Frequency of single-stranded circles in alkaline-denatured macronuclear DNA of hypotrichs*

Forms of single-stranded DNA	*Euplotes* %	*Oxytricha*† (I) %	*Oxytricha*† (II) %	*Paraurostyla* %
Circles	88.7	67.8	60.5	47.2
Linears	5.2	16.6	21.3	42.1
Lariats	0.5	2.6	5.0	1.3
Unknowns*	5.6	13.0	13.2	9.3
Total number of molecules	1136	1394	1322	610

* Unknowns result from collapsed molecules or aggregates. Many of these may be circular, since the frequency of unknowns is significantly decreased after exonuclease III treatment, which results in mainly linear molecules.

† The roman numerals refer to two different preparations which were dialyzed overnight against SSC or Tris buffer at 4° before spreading.

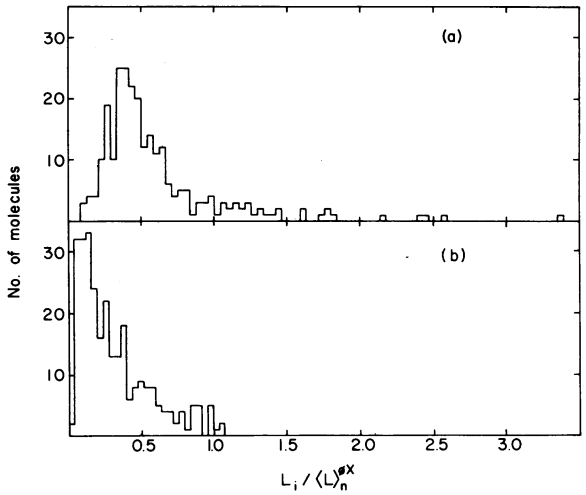

FIG. 2. Length measurements of (a) native and (b) denatured *Oxytricha* macronuclear DNA relative to ϕX174 DNA. L_i represents the contour lengths of native or denatured macronuclear DNA molecules which were normalized to the number-average contour length, $\langle L \rangle_n^{\phi X}$, of either double- or single-stranded ϕX DNA. The calculated number-average molecular weight of native macronuclear DNA is 2.08×10^6 (252 molecules), and that of denatured DNA is 0.56×10^6 (282 molecules), based on a value of 1.7×10^6 for the molecular weight of the single-stranded phage DNA (16). To facilitate the identification of single-stranded ϕX circles, denatured macronuclear DNA was spread onto a hypophase maintained at 35° so that most of the *Oxytricha* molecules were linear.

cospread with the corresponding double- or single-stranded sample, were used as standards. If there were no internal nicks in macronuclear DNA, then the distributions of contour lengths of native and denatured molecules should superimpose. Fig. 2 demonstrates that the single-stranded molecules are smaller than native macronuclear DNA; therefore, the polynucleotide chains of native DNA must contain interruptions, i.e., single-stranded nicks or gaps must be present in the native DNA. In addition, the majority of the single-stranded pieces form circles (89%), indicating that self-complementary base sequences exist internally as well as at or near the ends of macronuclear DNA. This implies than an enzyme(s) recognizes a specific sequence or a set of sequences in macronuclear DNA and inserts a single-strand nick or gap. The placement of the "nick," possibly within a palindromic sequence, is such that the ends of the single-stranded pieces are complementary, permitting the molecules to cyclize.

The number-average molecular weight of *Oxytricha* macronuclear DNA is 2.08×10^6 and that of denatured DNA is 0.56×10^6 (Fig. 2). If $M_s = M_d/(n + 2)$, where M_s and M_d are the number-average molecular weight of single- and double-stranded DNA, respectively, then there are 1.7 site-specific "nicks," n, per native macronuclear duplex. This may, in fact, be an underestimate, since the modal value for the length of denatured DNA approaches the limit of resolution of the spreading technique so that shorter DNA fragments would be missed. Thus, the number of nicks per macronuclear DNA duplex is probably greater than 1.7.

Single-stranded molecules other than the simple circular forms were also present (Fig. 3). Lariats, defined as single-stranded circles with single-stranded tails, constituted up to 5% of the total (Table 1); other forms were present at lower frequencies. The self-complementary regions of duplex struc-

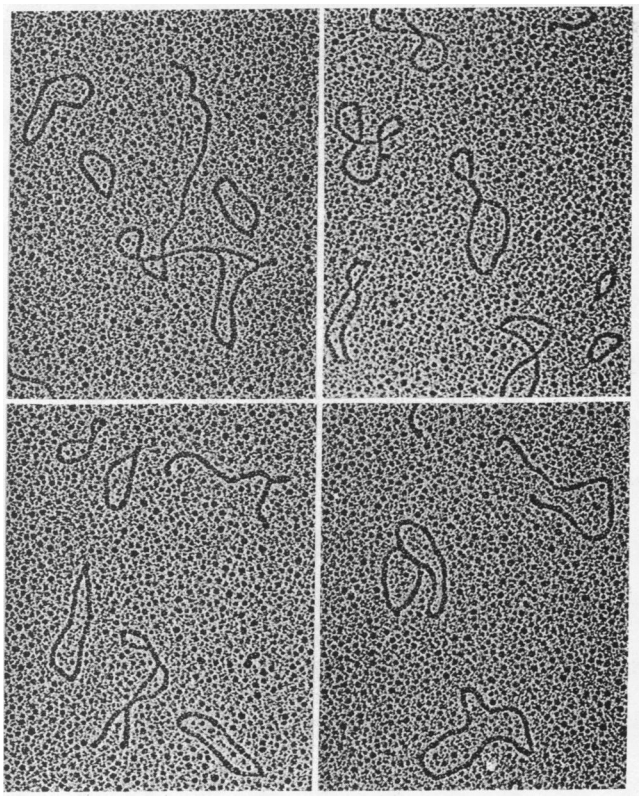

FIG. 3. Selected examples of denatured *Oxytricha* macronuclear DNA. The conditions for denaturation and partial renaturation are described in *Materials and Methods*. The regions of duplex structure are attributed to inverted repetitious sequences. Magnification ×34,500.

ture visible in these forms might possibly result from the absence of a nick at an inverted repeated sequence.

Evidence that the circles are single-stranded

(*i*) As previously mentioned, the C_0t value for circle formation is four C_0t decades below that at which macronuclear DNA renatures as a bimolecular reaction; thus, the circles are not duplex structures. (*ii*) Double-stranded and single-stranded DNA can be distinguished by electron microscopy. When single-stranded macronuclear rings are cospread with double-stranded ϕX RF DNA by the aqueous Kleinschmidt technique, the ϕX RF DNA remains as extended circular molecules, whereas the renatured macronuclear DNA completely or partially collapses. (*iii*) Circles in 0.12 M sodium phosphate buffer do not bind to hydroxylapatite at 60°, indicating not only that the circles are single-stranded, but also that the duplex region which holds the DNA in a circular conformation is probably less than 50 base pairs (17).

Evidence that circles are held together by a hydrogen-bonded duplex region

In order to test for ring closure by a hydrogen-bonded duplex region, a preparation of single-stranded circles was incubated with exonuclease III, which removes mononucleotides from the 3′ end of duplex DNA but not from the 3′ end of single-stranded DNA (13). In two experiments using excess exonuclease III, 67% and 69% of the circles were converted to linear single-stranded molecules. Accordingly, at least 68% of the circles are sealed by hydrogen bonding between the ends of a single DNA strand, whereas 32% of the circles resisted exonuclease III activity. Exonuclease III resistance is presumed to result from a nonbase-paired 3′ end in about one-third of the single-stranded circles.

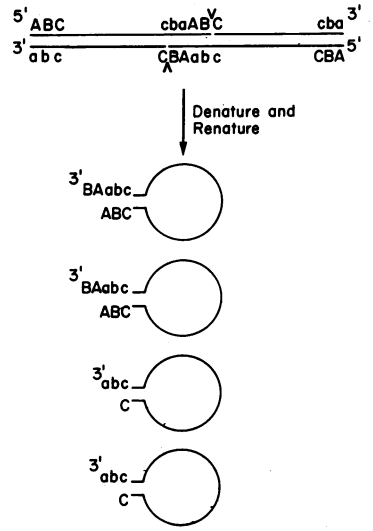

FIG. 4. Schematic representation of a possible arrangement of inverted repetitious sequences in macronuclear DNA. The hypothetical molecule contains one complete palindrome and two "half-palindromes" at the ends. After denaturation and partial renaturation the products of intrastrand re-annealing have non-base-paired 3′ ends as shown. The symbol ∧ identifies the location of single-strand nicks in the native molecule; the letters ABC represent DNA sequences, and abc, their complementary sequences.

In order to determine whether the exonuclease-III-resistant molecules were also closed by a duplex region, macronuclear DNA was denatured and renaturation was prevented by incubation with 5% formaldehyde at 60° for 15 min before spreading for electron microscopy, or, alternatively, circles were spread from a solution containing 80% formamide. Both procedures resulted in almost total conversion to linear molecules.

Are inverted repetitions present in other ciliates?

The hypotrichous ciliates studied, *Oxytricha*, *Euplotes*, and *Paraurostyla*, all have low-molecular-weight macronuclear DNA, and all have a polytene chromosome stage during macronuclear development in exconjugants. The macronuclear DNAs from these organisms contain inverted repeated sequences as shown by the ability to form single-stranded circles. The lower frequency of circle formation in *Paraurostyla* and *Oxytricha* (Table 1), compared with *Euplotes* macronuclear DNA, may reflect the action of nonspecific nuclease activity during DNA extraction.

The macronuclear DNA of the holotrichous ciliate *Tetrahymena pyriformis* (strain HSM) sediments in a sucrose gradient as two heterogeneous fractions at 56 S and 33 S (18). When DNA from each fraction was tested for the ability to form circles by the standard procedure, only long linear molecules were found.

DISCUSSION

The three species of hypotrichous ciliates examined appear to have inverted repetitious DNA sequences at the ends of macronuclear DNA molecules and at internal sites. The presence of internal nicks, or perhaps gaps, at sites that permit circle formation suggests that a specific restriction-type enzyme recognizes a particular DNA base sequence and

creates a "nick." This observation thus constitutes evidence for a restriction-type enzyme in a eukaryotic organism.

The simplest DNA sequence to account for the high frequency of circle formation is a palindrome, a symmetrical sequence that reads identically in the 3′ to 5′ direction of both DNA strands. One possible arrangement of these sequences in macronuclear DNA is shown in Fig. 4. The hypothetical molecule consists of two "half-palindromes" at the ends and an internal palindrome containing staggered nicks. After denaturation of this molecule, single-stranded circles with non-base-paired 3′ ends rapidly re-form (Fig. 4). If the *trans* orientation of the staggered nicks were reversed from that shown in the diagram, then the single-stranded circles resulting from denaturation and renaturation would all have a non-base-paired 5′ end. If only a single nick were present at the center of the internal palindrome, then single-stranded circles perfectly base-paired at the ends would re-form from one strand following denaturation and renaturation, and the other strand would yield either a single-stranded figure-8 molecule or a single-stranded circle.

The model in Fig. 4 predicts that nucleotide sequences at the ends of denatured macronuclear DNA pieces should undergo an intermolecular hybridization with appropriate annealing conditions. As yet, intermolecular complementarity has proven difficult to detect because of the heterogeneity in size of macronuclear DNA.

Exonuclease III digestion, formaldehyde treatment, and spreading from a solution containing 80% formamide have demonstrated that the single-stranded rings are held together by a hydrogen-bonded duplex region. However, 32% of the circles are exonuclease-III-resistant, and thus probably have an extended 3′ single-stranded end as illustrated in Fig. 4. Duplex structures unevenly paired at the ends could be generated by (*i*) enzymes nicking at different sites within a palindrome or (*ii*) by repeated sequences of the type,

3′....cbacbacbaABCABCABC....5′
5′....CBACBACBAabcabcabc....3′

within a palindrome. In both cases, duplex regions with single-stranded tails at the ends could result.

The duplex region that holds the denatured molecules in the circular conformation is most likely 12 to 50 base pairs in length. The standard 50% formamide renaturation conditions used for circle formation are equivalent to annealing at 75° in 0.1 M Tris (19). Hershey *et al.* (20) report that lambda DNA molecules with cohesive ends are linear at 75° even in 0.6 M NaCl. The cohesive ends of lambda DNA are 12 nucleotides long (21). Therefore, since stable duplex regions are formed with denatured macronuclear DNA, the length of the duplex must be greater than 12 base pairs. With regard to the upper limit, the duplex region is probably less than 50 base pairs, since no consistent irregularities in the contour of single-stranded macronuclear DNA circles are visible by electron microscopy and since circles do not bind to hydroxylapatite at 60° in 0.12 M sodium phosphate. Apparently, the self-annealing duplex regions in macronuclear DNA are considerably shorter than inverted repetitions in the chromosomal DNA of other eukaryotic organisms, which are reported to range from 300 to 1200 nucleotides (22). Also, these sequences are apparently smaller than the presumed A·T-rich sequences that cause the observed differential melting at the ends of macronuclear DNA (7).

The function of the inverted repetitions in macronuclear DNA is not known. However, the termini of macronuclear DNA molecules are probably created during the transection of polytene chromosomes or during the subsequent DNA degradation stage of macronuclear development, while the internal DNA nicks or gaps must be introduced every cell cycle in vegetative cells. This suggests that inverted repetitious sequences are recognized during the cell cycle and in processing the DNA during macronuclear development. Both events may share a common enzymatic basis.

Certain palindromes do have established biological functions. Smaller palindromes (4 to 8 base pairs) provide recognition sites for restriction enzymes; larger palindromes may be control regions for transcription [see reviews by Boyer (23) and Lewin (24)]. Suggestions have been made that inverted repetitions are involved in DNA replication (25, 26) and recombination (27). Which of these or other possibilities applies to hypotrich DNA remains to be determined.

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