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Isolation and characterization of a highly repetitious inverted terminal repeat sequence from *Oxytricha* macronuclear DNA

(necks/nuclease S1/T4 DNA polymerase)

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ABSTRACT **The low-complexity "gene-sized" linear DNA duplexes of the *Oxytricha* macronucleus sport short inverted terminal repeats; thus, each single strand is capable of forming a circle held together by a duplex "neck" [Wesley, R. D. (1975) *Proc. Natl. Acad. Sci. USA* 72, 678–682]. We have isolated necks from total, circularized, single-stranded macronuclear DNA by treatment with nuclease S1. Necks represent at least 2.2% of the total DNA, are homogeneous in size (23 base pairs), melt at 55° in 0.18 M Na^+, and reassociate extremely rapidly at 22° ($C_0t_{1/2}$ = 1.1 × 10^{-5} mol·liter^{-1}·sec) to form hybrid necks of the same thermal stability. From these and other results, we conclude that all necks on all the many thousands of different single-stranded circles are the same. The neck sequence is therefore highly repetitious—found in multiple copies (as inverted terminal repeats at flush duplex ends and probably also internally) on each natural "gene-sized" macronuclear DNA molecule—implying the possible participation of this sequence both in the general vegetative metabolism of macronuclear DNA and in the pre-vegetative process whereby macronuclear DNA is excised from the total *Oxytricha* genome.**

The macronucleus of *Oxytricha* (a hypotrichous ciliated protozoan) develops from a mitotic sister of the micronucleus after occasional conjugation. During this development, as much as 97% of the DNA sequence complexity is lost, the macronuclear DNA surviving as a heterogeneous-sized collection of about 17,000 different linear, "gene-sized," duplex molecules (number average, 3200 base pairs) (ref. 1; see ref. 2 for a recent review and other references). This situation must reflect a precise mechanism operating to separate those DNA sequences to be retained from those to be destroyed. This excision presumably occurs during the transection of the polytene chromosomes that develop in the macronuclear precursor. Little is known about this excision process, and it was the aim of the present work concerning the termini of the macronuclear DNA duplexes to provide some insight into the mechanism of macronuclear sequence excision from polytene chromosomes.

After brief annealing, the single strands of the macronuclear DNA molecules form intramolecular circles held together by short duplexes, or "necks." The necks are not visible by electron microscopy and were deduced to be between 12 and 50 base pairs long (1). Note that this type of single-stranded structure implies the existence of "inverted terminal repeats" in the native macronuclear DNA molecules.

Inverted repeated sequences have been described in the DNAs of a wide variety of prokaryotes, eukaryotes, and viruses. The subject is too large and heterogeneous to be appropriately reviewed here, and it seems probable that different inverted repeats have various different functions. However, it is interesting to note that the following "movable" DNA segments are terminated by inverted repeat sequences: the G segment of the coli-phage Mu DNA (3), many coli transposons [reviewed by Kleckner (4)], the L and S segments of herpes virus DNA (5), and the inserts found in rDNA genes of *Drosophila* (6). The data of Perlman *et al.* (7) appear to indicate general "insertion" of inverted repeat sequences throughout the *Xenopus* genome. Because of the striking analogy between such movable DNA elements and the discrete gene-sized duplexes of *Oxytricha* macronuclear DNA, it was of considerable interest to characterize further its inverted terminal repeats.

Because there are about 17,000 different native macronuclear DNA molecules (8), it is possible that there are a very large number of different inverted terminal repeat sequences—i.e., each different single-stranded circle might have its own unique neck. At the other extreme, it is possible that the same neck sequence is present on all the thousands of different single-stranded circles. To distinguish between these two extremes, we have isolated the neck structures from total macronuclear DNA single-stranded circles and studied some of their physical properties. Neck isolation was achieved by use of the single-strand-specific nuclease S1, which destroys most of the DNA of the circles, leaving behind the duplex necks.

MATERIALS AND METHODS

Enzymes. Bacteriophage T4 DNA polymerase (3900 units/mg) was a generous gift from D. Mace, N. K. Sinha, and B. Alberts. *Aspergillus oryzae* nuclease S1 was kindly donated by L. Gold.

DNAs. *Oxytricha* macronuclear DNA, kindly provided by R. Boswell, was purified as described (8). Synthetic 5′-^{32}P-labeled deoxyoligonucleotides, used as molecular weight standards (5B-6 is 17 bases long; 6-7, 20 bases; 5b-6-7, 26 bases), were a gift from M. H. Caruthers (9).

***In Vitro* Labeling of Native Macronuclear DNA 3′ Material.** Native DNA (7.55 μg) was placed in 54.5 μl of 0.125 mM dCTP/80 mM Tris·HCl, pH 8.1/6.7 mM $MgCl_2$/5.7 mM 2-mercaptoethanol/bovine serum albumin at 0.33 mg/ml. An aliquot (5 μl) was made 50 mM in Na_3EDTA and subsequently tested for single-strand circle-forming potential by electron microscopy. The digestion of 3′ termini was initiated by adding 2.5 units of T4 DNA polymerase. After 60 min at 37°, another 5-μl aliquot was transferred to EDTA to test circle-forming ability. The reaction mixture was then made 0.250 mM in dCTP, 0.125 mM in dATP, 0.125 mM in dGTP, and 0.020 mM in [α-^{32}P]TTP (1.72 × 10^4 cpm/pmol; New England Nuclear); another 2.5 units of polymerase was added, and the reaction was allowed to proceed another 10 min at 37°. Aliquots were transferred to glass fiber filters and analyzed for trichloroacetic acid-insoluble radioactivity. The reaction was terminated by addition of 10 mM Na_3EDTA, and 5 μl was again removed for

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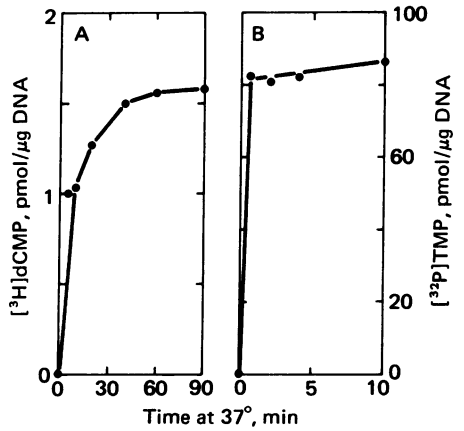


FIG. 1. Action of T4 DNA polymerase on native macronuclear DNA. (*A*) Incorporation of [^{3}H]dCMP into 3′ termini in the presence of one deoxynucleotide triphosphate. The reaction conditions are as described in *Materials and Methods*, except that dCTP was ^{3}H-labeled (785 cpm/pmol). (*B*) Incorporation of [^{32}P]TMP during repair in the presence of all four triphosphates.

assay of circle-forming potential. The remaining DNA was precipitated with ethanol and was all recovered in buffer A (20 mM NaCl/10 mM Tris·HCl, pH 8.1/0.1 mM Na_3EDTA).

RESULTS

It should be possible to produce necks from single-stranded circles formed from denatured macronuclear DNA by digesting the circles with the single-strand specific nuclease S1. In fact, in preliminary experiments (not shown) we were able to demonstrate that such a procedure does yield an S1-resistant DNA fraction that migrates as a small fragment in gel electrophoresis. We assume this material represents the necks, an assumption we will examine in the *Discussion*. Lightly labeled necks have been reproducibly isolated from long-term *in vivo* ^{32}P-labeled macronuclear DNAs (see Fig. 2); from the yields (mean, 2.2% in six experiments) and a crude estimate of recoveries at various steps (at most, 50%), we estimate that up to 4% of the total macronuclear DNA nucleotides participate in necks.

In order to obtain convenient quantities of radioactive S1-resistant neck band material of high specific activity, we used an *in vitro* labeling procedure suggested by Englund's work (10) with bacteriophage T4 DNA polymerase. The procedure relies on both the exonucleolytic (3′ → 5′) and the synthetic (5′ → 3′) activities of the polymerase. The exonuclease is quite active on 3′-OH termini at 37° but can be conveniently controlled by the addition of any one deoxynucleotide triphosphate. For instance, in the presence of dCTP the exonuclease proceeds uninhibited until it removes a dCMP from duplex DNA, at which point the polyermase activity very rapidly repolymerizes a new dCMP into place. Of course, the dCMP is soon removed again by the exonuclease and nearly always replaced by the polymerase; occasionally, the exonuclease removes the dCMP and the penultimate nucleotide before the polymerase can replace the dCMP, and digestion proceeds back in to the next dCMP, where the polymerase again impedes net digestion. Because dCMP addition is much more rapid than its removal, essentially all the strands that participate are 3′ terminated with dCMP (10). Fig. 1*A* shows such a digestion of native macronuclear DNA in the presence of [^{3}H]dCTP; the results indicate that most 3′ termini of macronuclear DNA do participate because, after 60 min of digestion, 1.56 pmol of dCMP is incorporated per μg of DNA, compared to 1.8 pmol/μg expected from the number average size of macronuclear DNA single strands, 0.56×10^6 g/mol (1). It should be noted here that, from the latter number, it was calculated that the DNA has about 1.7 strand interruptions per number average duplex (1); therefore, the 3′ termini of most of these strand interruptions, as well as the two external 3′ termini per duplex, must have participated in the present exonuclease digestion. However, the approach to equilibrium labeling in the present case was quite slow, in contrast to Englund's results (10). Whereas the exonuclease acts quite rapidly at 3′-OH termini, a low level activity is also reported on 3′-*P* termini (11), and the results of Fig. 1*A* may mean that macronuclear DNA strands are 3′-*P*-terminated.

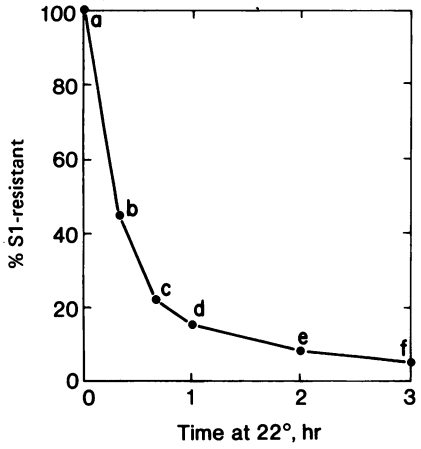
Table 1. Effects of T4 DNA polymerase on single-strand circle-forming potential of native macronuclear DNA

State of DNA	Percent Circles	Linears	Others	Total molecules scored
Exp. 1:				
Native	41	34	25	1081
Digested 60 min	8	68	24	791
Repaired	39	37	24	713
Exp. 2:				
Native	43	39	18	510
Digested 20 min	6	88	6	140
Repaired	40	49	11	376

* Note that, although only 41–43% of the single strands formed circles, in earlier experiments these values have ranged up to 68% (for *Oxytricha*) and up to 89% with the macronuclear DNA of the related protozoan *Euplotes* (1). Several factors may be responsible for such variability. First, many forms not scored as circles may in fact be tangled circles or other neck-mediated forms (1). Second, even low levels of random nicking have a magnified effect on circle-forming ability, because each random nick both destroys one circle-forming unit and simultaneously creates two strands incapable of forming circles. Finally, the neck structures are relatively unstable, and uncontrolled parameters of spreading may affect the results.

Once exonuclease digestion has proceeded to the desired point, the damage can be easily reversed by merely supplying the polymerase with all four nucleotide triphosphates and allowing normal DNA synthesis to repair the gaps created by the exonuclease. Fig. 1*B* shows the results of an experiment in which the native macronuclear DNA was digested for 60 min in the presence of dCTP, and repair was carried out in the presence of [α-^{32}P]TTP. Repair was very rapid. The extent of synthesis was 83 pmol of TMP per μg, or 47 TMPs at the 3′ end of each number average single strand. Assuming the base composition in these regions is that of total macronuclear DNA (G+C = 42%; ref. 8), we calculate that about 160 repairable nucleotides per each number average strand were removed during the 60-min digestion.

We might expect the circle-forming ability of the strands of this 60-min digested DNA to be decreased because the length of the inverted repeat sequences was estimated to be less than 50 nucleotides, and the 3′ repeat must be at or near the end of the single strand—that is, "terminal" (1). Furthermore, after polymerase repair, the circle-forming ability of the single strands should return, provided no nicking damage was sustained. To test these expectations, DNA aliquots were removed during the experiment described in Fig. 1*B* and the circle-forming abilities of their single strands were determined by electron microscopy, as described (1). The results (Table 1, Exp. 1) show that the single-stranded circle-forming potential of the native DNA is destroyed by the exonuclease digestion and is regained after polymerase repair. Note that the circles formed from the strands of the repaired DNA must be held together with necks, one side of which are newly synthesized. Of the circles formed from the repaired DNA in the first experiment



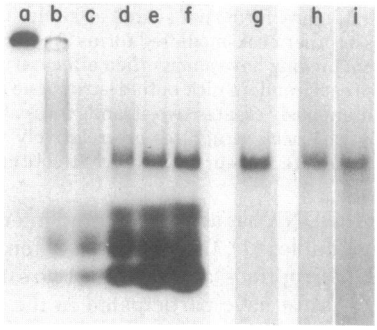


FIG. 2. Nuclease S1 digestion of *in vitro* labeled single-stranded circles. Native DNA labeled in its 3′ portions with [^{32}P]TMP (Fig. 1*B*) was denatured (100°, 10 min, buffer A) and briefly reannealed (22°, 10 min, 0.18 M Na^+ $C_0t = 2.7 \times 10^{-2}$ mol·liter^{-1}·sec). We calculate that an average-sized circle requires on the order of 1 min ($t_{1/e}$ = 67 sec) to circularize, based on the treatment of Schmid *et al.* (12). The resulting single-stranded circles were then digested with nuclease S1 (190 units/ml, 1 unit as defined in ref. 13) at 22° in 0.18 M NaCl/0.03 M sodium acetate, pH 4.6/0.68 mM $ZnCl_2$/0.2 mg of heat-denatured calf thymus DNA per ml. Aliquots were removed during the reaction for both gel electrophoresis and trichloroacetic acid precipitation. At the end of the reaction the remaining DNA was ethanol-precipitated and redissolved. (*Upper*) Trichloroacetic acid-insoluble materials remaining in total reaction; 100% = 8.2×10^6 cpm. (*Lower*) Gel electrophoresis. Various samples were subjected to electrophoresis through 10% polyacrylamide slabs containing 5 mM $MgCl_2$ (14). Labeled material was visualized by autoradiography; the arrows mark the position of the dye band (xylene cyanole ff). Lanes a–f: samples removed from the digestion reaction as shown in the graph; aliquots loaded in each well were of different sizes to obtain even exposure of the autoradiogram, and intensities are not comparable between wells. Lane g: an aliquot of the 3-hr digest after ethanol-soluble materials were removed. Lanes h and i: comparison of S1-resistant material from *in vitro* labeled and *in vivo* labeled macronuclear DNA single-stranded circles, respectively.

(Fig. 1*B*), 80% (1-8/39) are presumably held together by [^{32}P]TMP-labeled necks.

To prepare labeled necks, we denatured the ^{32}P-labeled repaired DNA, briefly reannealed it to allow single-strand circle (neck) formation, and treated with nuclease S1. The digestion kinetics are shown in Fig. 2. As expected, a fraction of labeled DNA did in fact resist S1 digestion, and it ran as a single band in gel electrophoresis. The material that remained S1-resistant after 3 hr of digestion was purified by ethanol precipitation (Fig. 2, lane g) and characterized further. All the experiments to follow were performed with this preparation. Note that such *in vitro* labeled material is indistinguishable in size from necks prepared from *in vivo* labeled DNA (Fig. 2, lanes h and i). From this result we can conclude that the 5′ termini at the duplex ends

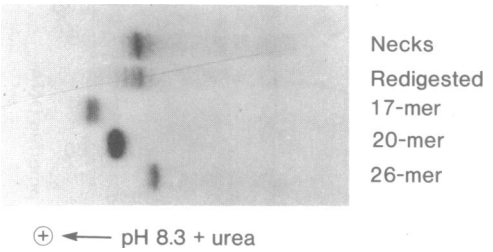


FIG. 3. Size determination of neck single strands. ^{32}P-Labeled necks (Fig. 2, lane g) and such necks re-treated with S1 nuclease (as in Fig. 4) were denatured and subjected to electrophoresis through 20% polyacrylamide/7 M urea (15), in parallel with three standards, as indicated. A plot of mobility of the standard vs. logarithm of length was linear, and the neck band had an apparent size of 23.4 bases by interpolation (not shown). The redigested material appears to have suffered "nibbling" (13), showing a series of bands differing by one nucleotide in length (15). One of these bands appears to run with the 20-mer standard; counting up to the main band suggests that the main band is 23 bases long.

of native macromolecular DNA molecules must not extend beyond the opposing 3′ termini—i.e., native macronuclear DNA does not have 5′ single-stranded "tails" (see below).

As a first step in characterizing the neck band material, the apparent size of the *in vitro* labeled preparation was determined by gel electrophoresis in parallel with chemically synthesized standards of known length (Fig. 3). The 20% polyacrylamide/7 M urea gel system used is capable of resolving short polynucleotides differing by single nucleotides in length (15). The necks were nearly homogeneous, with an apparent length of 23 nucleotides.

Because of the size homogeneity of the necks, it seemed possible that they might be sequence-related or even sequence homogeneous. As a test of this possibility, we undertook a study of sequence complexity of neck material by measuring its reassociation kinetics. In anticipation of that experiment, the thermal stability of necks was measured by monitoring neck denaturing by S1 challenge. The t_m was quite low (55° in 0.18 M Na^+), and the transition was wide (Fig. 4). Both are predictable consequences of the small size of the necks (see review, ref. 17). Because, unlike high molecular weight DNA, neck strands may completely separate at the t_m, one might expect the t_m to be dependent on neck concentration. In fact, when necks were melted at a 22-fold higher concentration, the t_m was 60° (not shown).

To measure the kinetics of reassociation of neck single strands, heat-denatured necks were reannealed at 22° in 0.18 M Na^+ and the return of S1-resistance was monitored. The data, shown in Fig. 5 could be readily fitted to a curve describing the second-order reassociation of a single component. The reassociation was strikingly rapid ($C_0t_{1/2} = 1.1 \times 10^{-5}$ mol·liter^{-1}·sec), on the order of 10^4 times faster than *Escherichia coli* DNA under standard conditions (19).

Reassociated neck material was tested for thermal stability in parallel with native necks (Fig. 4); no difference in t_m or transition breadth was detected.

DISCUSSION

We have isolated S1-resistant material from briefly reannealed macronuclear DNA single strands. The material is homogeneous in size, running in electrophoresis as a single band; its apparent length is 23 nucleotides.

Does this band material represent the inverted terminal repeat sequences of native DNA and the necks of the single-stranded circles? Several properties of this material coincide

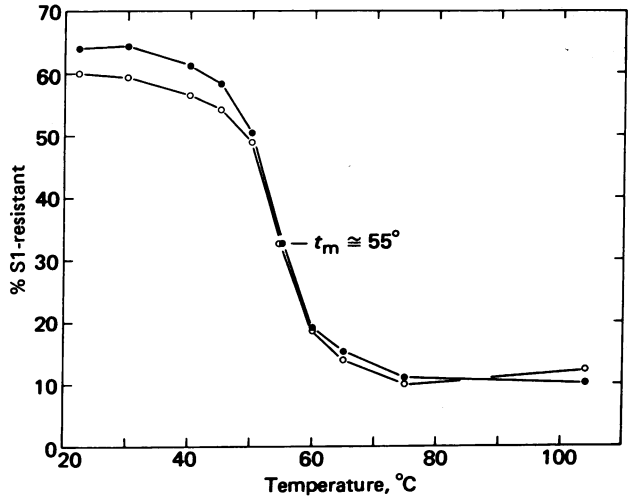


FIG. 4. Thermal melting of native and renatured necks in 0.18 M Na^+. Aliquots of ^{32}P-labeled necks (Fig. 2, lane g) contained 11.3 nM nucleotides, calculated from the radioactivity and the known neck nucleotide composition (29% T, unpublished data), in 180 mM NaCl/10 mM Tris·HCl, pH 7.5/0.5 mM Na_3EDTA. Some aliquots (open symbols) were previously denatured at 95° for 20 min and then renatured at 22.5° for 120 hr ($C_0t = 4.9 \times 10^{-3}$ mol·liter^{-1}·sec); others were held at 4° (solid symbols). Aliquots were then treated for 20 min at the temperature indicated and challenged with nuclease S1 for 3 hr as described in Fig. 2, but at 12° and 380 units/ml. Digestion products were separated from undigested necks by hydroxylapatite chromatography. Radioactivity was detected by scintillation counting in a Triton X-100/toluene cocktail; recovery of counts in the two fractions was complete. Note truncated abscissa; 100% = 1100 cpm. From the kinetics of S1 digestion of denatured and undenatured necks and from gel analysis of the products (not shown), we conclude that (*i*) about 30% of the radioactive material in the neck preparation that is not resistant to S1 in the experiment shown consists of heterogeneous-sized denatured macronuclear DNA that escaped digestion by S1 during the preparative digestion (Fig. 2); (*ii*) the S1-resistant material found in the fully denatured neck samples consists primarily of denatured material that has nonetheless escaped S1 digestion and is not duplex DNA. Difficulties in obtaining complete digestions with S1 have been noted (16).

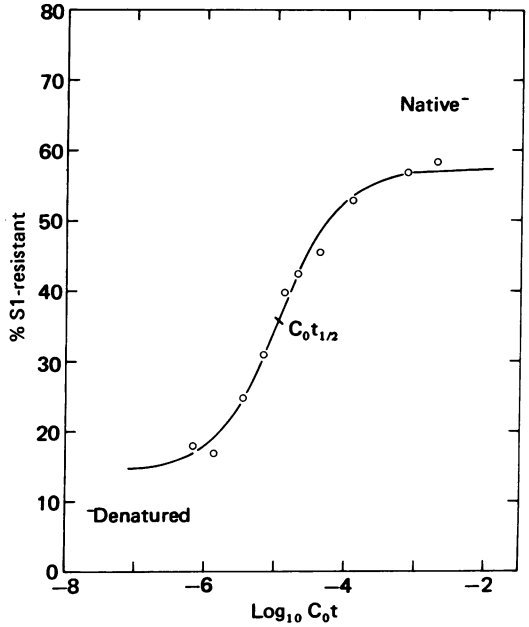


FIG. 5. Reassociation kinetics of neck single strands in 0.18 M Na^+. Aliquots of labeled necks (see Fig. 4) were denatured for 20 min in 100°, renatured at 22.5°, and challenged at various times by S1 digestion; the first time point was 1 min and the last was 46 hr. The data were fitted by a linear regression program (18) to a single second-order component curve (root mean square deviation = 0.014). $C_0t_{1/2} = 1.11 \times 10^{-5}$ mol·liter^{-1}·sec. Also indicated are the S1 resistances of undenatured DNA and denatured DNA not held at 22.5°.

with the known properties of necks. First, necks are duplex DNA whose two strands are attached by large regions of single-stranded material. The band material has the expected nuclease S1 resistance (Fig. 2); the thermal instability of the material (Fig. 4) is readily understood in terms of its being short DNA duplex fragments. Second, necks were judged to be 12–50 nucleotides long (1); the apparent size of the band material (23 nucleotides long) is within that range. Third, from electron microscopy and exonuclease III digestions of circles (1), the necks of single-stranded circles must be quite close to the molecular ends of the strands (i.e., "terminal"). Judging from T4 DNA polymerase exonuclease and repair experiments (Fig. 1*B*), the S1-resistant material must be derived from DNA within about 160 nucleotides of the 3′ termini of the single strands. Finally, nucleotide sequencing has shown that the band material shares the same sequence with the inverted terminal repeat that forms the necks (see below). On the strength of the above, we think that the S1-resistant band material is indeed the duplex necks of the single stranded circles.

The observation that the neck preparation is quite homogeneous in size (Fig. 3) suggests that all necks may be identical. Neck single strands reassociate extremely rapidly as a single component (Fig. 5), which is certainly consistent with possible neck sequence homogeneity. Estimation of sequence complexity from C_0t analysis can be only approximate in the best situations; in the present case, additional problems arise (short chain length, low complexity). It is thus safe to conclude only that neck sequence heterogeneity, if it exists, is not large.

The observation that renatured necks have the same thermal stability as native necks (Fig. 4) is also consistent with the idea that all necks are the same. It seems likely that mismatching in the hybrid necks would be readily detected in this experiment. Nonetheless, it remains possible that the necks could be comprised of a small number of unrelated families, represented roughly equally and all of the same length.

Recently it has been possible to show by DNA sequencing techniques that at least 40% of all the natural 5′ ends of macronuclear DNA carry the same 26-nucleotide sequence and that at least 90% of the 5′ ends are dCMP terminated; at least 50% of the necks carry this same sequence (2, 20).§ These results, taken together with the results presented in this paper, make us confident that all necks are the same, even though no one experiment can be so strongly interpreted. Thus, the neck sequence is highly repetitious, being present in each macronucleus on the order of 10^7 times (for each of 17,000 different duplexes; each kind is present about 10^3 times per macronucleus; see ref. 8).

Furthermore, each macronuclear DNA duplex must be bounded by a pair of neck copies, making an inverted terminal repeat. That is to say, all the macronuclear DNA molecules must be of the form

$$\begin{array}{l} ABC.....cba \\ abc.....CBA \end{array}$$

§ Although the inverted terminal repeat length is at least 26 nucleotides, and thus the necks would also be expected to be that long, the necks as isolated are clearly less than 26 nucleotides long (Fig. 3). This discrepancy is probably attributable to the fact that one end of the full-length neck consists of several adjacent A-T pairs, which are presumably unstable and removed by the S1 digestion (2).

Note that the neck copies are drawn directly at the duplex ends, and the ends are flush. These conclusions are supported by the following information. First, as mentioned above, most if not all 5′ termini of macronuclear DNA carry the same sequence and at least the 5′ portions of all the inverted repeats (ABC. . .) extend to the 5′-terminal nucleotide (2, 20). Second, no difference in size was detected between necks derived from untreated (*in vivo* labeled) native macronuclear DNA and from *in vitro* repaired DNA (Fig. 2). If the 3′ termini of untreated DNA were not flush with the 5′ termini, but stopped short, leaving the 5′ termini protruding (e.g., 3′ *bc*, with *a* missing), then necks that formed involving these 3′ termini would be

$$\begin{matrix} ABC \\ bc \end{matrix}$$

which upon S1 digestion would be reduced to shorter "BC" necks. In contrast, the *in vitro* labeled native DNA would have been different, with the missing *a* eventually being put in place by the repair action of the T4 DNA polymerase, leading finally to labeled

$$\begin{matrix} ABC \\ abc \end{matrix}$$

necks. Thus, that *in vivo* and *in vitro* labeled necks are the same size suggests that untreated macronuclear DNA external 3′ termini are at least flush with the 5′ termini. It is possible, however, that the 3′ termini might extend beyond the duplex, because the T4 DNA polymerase exonuclease is quite active against such 3′ protruding DNA (11) and such material could not be replaced by repair because no template exists for that region. However, exonuclease III treatment of circles causes the linearization of at least two-thirds of the single-stranded circles (1). Because this enzyme only digests 3′ termini that are base-paired to an opposing strand (21), at least two-thirds of the 3′ termini of macronuclear DNA must not extend beyond the *cba* sequence. In short, a neck copy is most likely at each end of each macronuclear DNA duplex.

These end-punctuations may serve to mark the location of the macronuclear sequences within the much larger set of sequences of the total *Oxytricha* genome. It should be recalled that the macronuclear DNA pieces are excised from that genome as it is found in the macronuclear precursor within the exconjugant cell (see Introduction). It is tempting to speculate that the inverted terminal repeats might participate directly in the excision process; for example, the neck sequence could serve as the focus of site-specific recombination mediated by neck-specific enzymes. Site-specific recombination events are presumably responsible for "mobility" of the various inverted repeat-terminated DNAs mentioned in the Introduction.

Although the data discussed above strongly indicate that every macronuclear DNA duplex carries a copy of the neck sequence at each terminus, some types of evidence suggest that most molecules also contain internal (nonterminal) neck sequence copies as well. First, if each molecule contains only the two terminal neck copies, the maximal expected yield of total DNA that could be isolated as neck material would be 1.4% (46/3200); however, we found that at least 2.2% and more likely up to 4% of the total nucleotides can form necks (see *Results*), indicating considerably more than the two neck copies per number average size duplex. The reader is directed elsewhere (2) for a further discussion of evidence concerning the issue of internal neck copies, their arrangement as contiguous pairs (as palindromes), and their possible involvement in the vegetative metabolism of macronuclear DNA.

To recapitulate, we have isolated the necks from macronuclear DNA single-stranded circles and have been able to conclude that they must all be identical, representing a highly repetitious sequence present about 10^7 times per macronucleus. The copies are certainly found as inverted repeat pairs on the termini of each macronuclear DNA duplex, suggesting the possible role of the neck sequence in the excision of the macronuclear DNA from the much more complex total *Oxytricha* genome. Furthermore, some types of evidence suggest that further neck sequence copies also exist internally in the macronuclear DNA duplexes, with a role in the general vegetative metabolism of macronuclear DNA.

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