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DNA primase and the replication of the telomeres in *Oxytricha nova*

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ABSTRACT

An enzymatic activity in crude extracts of macronuclei from the hypotrichous ciliate *Oxytricha nova* catalyzes the synthesis of RNA consisting of $(C_4A_4)_n$ using an oligodeoxynucleotide template of the telomeric sequence $(dG_4T_4)_n$. Single-stranded $(dG_4T_4)_n$ is an effective template if it has a random sequence at its 5' end. The enzyme will not use a $(dG_4T_4)_n$ template of any length (up to 64 bases) if it lacks a random sequence at the 5' end. With a random, single-stranded sequence at the 5' end, the $(dG_4T_4)_n$ oligodeoxynucleotide must be at least 36 bases long to work as a template. A 16-base, single-stranded region of $(dG_4T_4)_2$ is an effective template when joined to a 20-base double-stranded region of $(dG_4T_4)_n/(dA_4dC_4)_n$, a structural arrangement that is the same as the native telomere of *Oxytricha* macronuclear DNA. The RNA-synthesizing activity is unaffected by 1.0 mg/ml of α-amanitin. Macronuclear extracts have an α-amanitin-insensitive, RNA-polymerizing activity that can use a random 55mer oligodeoxynucleotide as a template. This enzyme activity may be the same one that uses $(dG_4T_4)_n$ templates to make $(C_4A_4)_n$ RNA. The $(C_4A_4)_n$ RNA made in the reaction can prime DNA synthesis by the *E. coli* DNA polymerase I Klenow fragment. Therefore, the RNA polymerase activity fulfills the requirements of the telomere DNA primase that we postulated for replication of telomeres in hypotrichs (Zahler and Prescott, 1988, Nucleic Acids Research **16**, 6953-6972).

INTRODUCTION

Two properties of the telomeres of eukaryotic chromosomes are well recognized. First, natural chromosome ends do not participate in fusion of chromosomal segments in translocational events. This contrasts with the readiness with which ends produced by chromosome breakage fuse with one another (1). Second, the ends of linear DNA molecules in chromosomes present a particular problem in replication, known as the 5' primer problem (2,3). The solutions to both problems probably reside in the

special properties of the molecular structure of the telomeric portion of the DNA molecule. The DNA of telomeres in yeast, ciliated protozoa, trypanosomes, slime molds, a plant, and humans are similar, possessing tandem repeats of a sequence that conforms to the formula $C_{1-8}(T/A)_{1-4}$ (4-6). In hypotrichous ciliates of the genera *Oxytricha* and *Stylonichia* the telomere sequence is

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                    5' CCCCAAAACCCCAAAACCCC.....
3' GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG.....
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(7). A complex of two proteins binds tightly and specifically to this telomeric sequence (8-11). Such telomere binding proteins probably have roles in preventing chromosome fusion at telomeres and in determining the length of the 3' telomeric extension (10).

Hypotrichous ciliates provide a particularly favorable opportunity to study telomere structure and function because all the DNA in the macronucleus occurs in small molecules with a number average size of 2200 bp (in *O. nova*). A single macronucleus contains over 2 x 10^7 DNA molecules, and therefore over 4 x 10^7 telomeres. About 3% of the total DNA in the macronucleus consists of telomeric sequences (12).

We recently described a telomere terminal transferase activity in macronuclear extracts of *O. nova* (13). In that paper we proposed a model for replication of the short linear DNA molecules and their telomeres in *O. nova* based on the telomere terminal transferase activity. In that model we postulated the presence in macronuclei of a DNA primase activity that could use the 16-base, single-stranded 3' $(dG_4T_4)_2$ extension of the telomere as a template for synthesis of a $(C_4A_4)_n$ RNA primer for initiation of DNA replication. In this paper we describe this primase activity in macronuclear extracts of *O. nova*.

METHODS

Cell Growth and Macronuclei Isolation

Growth of *Oxytricha nova* strain H0 and isolation of macronuclei were done as previously described (13). The TMS storage buffer used for these macronuclei was slightly modified from Zahler and Prescott (13) in that it contained no $CaCl_2$.

Synthetic Oligomers

Synthetic oligodeoxynucleotides were made as previously described (13) with the exception of middle 1/3A, #90, #95, CJD-20, CJD-36, CJD-36U, T_4G_4-64mer and Actin PCR2, which were gifts from D. Duhl, C. Thomas, C. Dunn, M.K. Raghuraman and A. Greslin. Oligodeoxynucleotides with possible

secondary structure were heated to boiling and allowed to cool to room temperature overnight.

Primase Reactions

Primase reactions were done using slight modifications of the telomere terminal transferase assays described by Greider and Blackburn (14,15) and Zahler and Prescott (13). Reaction mixtures were made on ice and done in a total volume of 40 µl. Each reaction contained 20 µl of thawed macronuclear extract, 10 µl of a 4X assay buffer (200 mM Tris-Cl pH 7.5, 40 mM $MgCl_2$, 56 units/ml RNase inhibitor from human placenta purchased from Sigma), and 2 µl of 1.0 mg/ml α-amanitin (Sigma). 1.0 µl of $\alpha^{32}P$ CTP (3000 Ci/mMol, 10 mCi/ml New England Nuclear) was the label in each reaction. Cold rNTPs or dNTPs were added as 1.0 µl of a 5 mM solution. Oligodeoxynucleotides were added as 1.0 µl of a 25 µM solution. DEPC (diethylpyrocarbonate) treated deionized H_2O was used to bring final reaction volumes to 40 µl.

Reactions were carried out at 14°C for 30 to 45 minutes. 14°C was determined to be the optimum temperature, and all reactions were largely complete after 15 minutes. Reactions were stopped by addition of 95 µl of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and 5 µl of 0.5 M EDTA. They were extracted with phenol and then with chloroform. 2 µl of 25 mg/ml yeast RNA, 20 µl of 5 M NH_4-acetate, and 0.5 ml of 100% ethanol were added to the samples, and they were stored at -20°C for one hour. Samples were spun down for 15 minutes in an eppendorf microcentrifuge at 4°C. Pellets were rinsed in 70% ethanol and then dried in a Speed-Vac. Dried pellets were dissolved in sequencing loading buffer and run on 16% acrylamide (19:1) sequencing gels as previously described (13).

Hybridization of Primase Reaction Product to Southern Blots

Plasmid pAZ1 was constructed by inserting the EcoR1 fragment of pMAC-4, which possesses the macronuclear actin gene of *O. nova* (described in 16), into the EcoR1 site of a pUC9 vector that has had its Ssp1 site removed by cutting pUC9 with Ssp1, treating with T4 DNA polymerase to create blunt ends, and religating the vector. pAZ1 was digested with the restriction enzymes indicated in figure 2b and run on a 1% agarose gel in TBE buffer along with undigested macronuclear DNA from *O. nova* and λ-BstEII size marker (New England Biolabs). Note that the restriction enzyme Ssp1 (lanes 4 and 5) only cleaved the DNA with about 50% efficiency. This gel was then blotted to Zetabind filter paper (17).

Probe for the southern blot was made by doing a primase reaction

with oligodeoxynucleotide #90 as described in the primase reactions section of Methods, but as a five-fold larger reaction. After a 30-minute incubation at 14°C, 0.6 ml of Nensorb reagent A was added to the reaction mixture. The reaction mixture was fractionated on a Nensorb™20 cartridge (New England Nuclear) according to the manufacturers instructions. Nucleic acid samples (RNA reaction product along with oligodeoxynucleotide template and any DNA and RNA present in the macronuclear extract) were recovered in 250 µl of 50% methanol and dried in a Speed-Vac for 2 hours. The recovered product was digested with RQ1 DNase (RNase-free DNase I from bovine pancreas at 1 unit/µl, Promega) prior to use a probe for the southern blot to remove any contaminating DNA.

The probe was hybridized to the filter in 5X SSPE (17), 1% SDS, 5X Denhart's solution, and 100 µg/ml sheared herring DNA at 42°C for 2 days. The blot was washed at a final stringency of 2X SSPE at 40°C. Blot was exposed to X-ray film with an intensifying screen.

Klenow Reactions

To make $(C_4A_4)_n$ RNA primers, primase reactions were performed as described above, but as five-fold larger reaction mixtures. After a 30-minute incubation at 14°C, 0.6 ml of Nensorb reagent A was added to each reaction mixture. The reaction mixture was fractionated on a Nensorb™20 cartridge (New England Nuclear) according to the manufacturers instructions. Nucleic acid samples were recovered in 250 µl of 50% methanol and dried in a Speed-Vac for 2 hours. These samples were then dissolved in 10 µl of DEPC treated deionized H_2O.

Klenow reactions were done in 10 µl total volume. Each had 1 µl of primase reaction product, 1 µl of 10X reaction buffer (0.5 M Tris-Cl pH 7.5, 0.1 M $MgSO_4$, 10 mM DTT), 1 µl of each indicated dNTP at 330 µM, and 1 µl of Klenow enzyme (5 units/µl, US Biochemical). Reactions were carried out at 25°C for 30 minutes. Reactions were stopped by addition of 95 µl of TE buffer and 5 µl of 0.5 M EDTA. 2 µl of 25 mg/ml yeast RNA, 10 µl of 5 M NH_4-acetate, and 400 µl of 100% ethanol were added, and samples were kept at -20°C for one hour. Ethanol precipitates were spun down and prepared for electrophoresis on sequencing gels as described above.

Those samples that were treated with DNase I had 1 µl of RQ1 DNase (RNase-free DNase I from bovine pancreas at 1 unit/µl, Promega) added to the Klenow reaction after 30 minutes and were incubated at 25°C for an additional 10 minutes before being ethanol precipitated as described above.

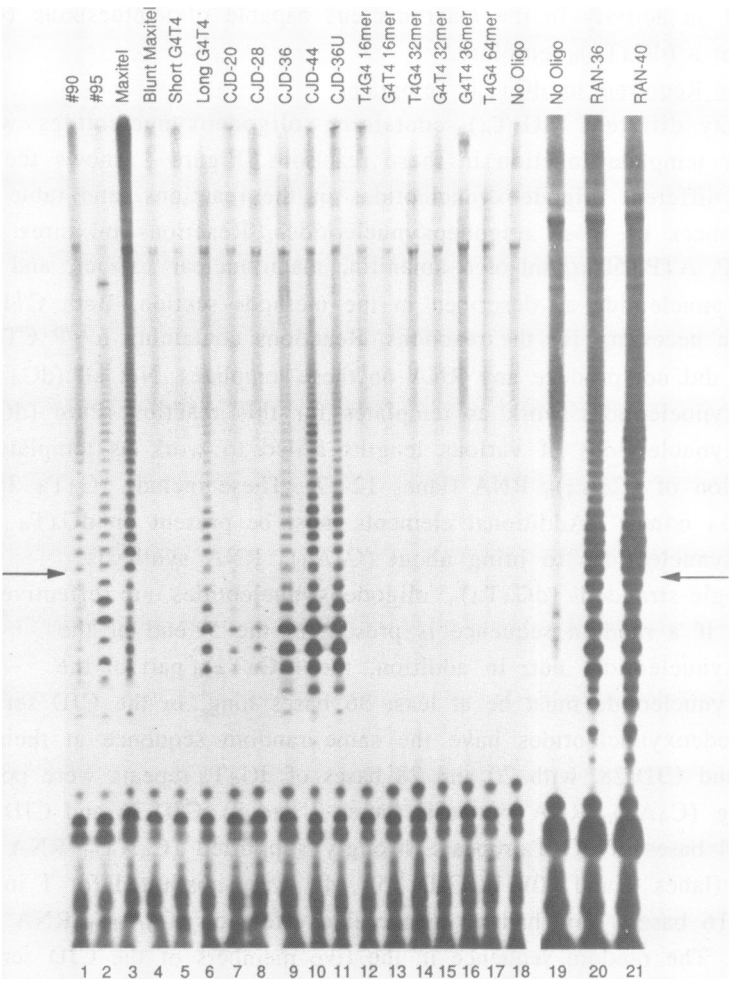


Figure 1. The primase reaction with different telomeric oligodeoxynucleotides. Macronuclear extracts were incubated with α^{32}P CTP, 125 μm ATP, 50 μg/ml of α-amanitin, and different oligodeoxynucleotides containing dG_4T_4 repeats. The oligodeoxynucleotide sequences and the lanes that they correspond to are indicated in table 1. The arrow is a 5' end-labeled 16mer marker consisting of $pdC_4dA_4dC_4dA_4$.

RESULTS

Using the basic protocol for telomere terminal transferase activity from Greider and Blackburn (14) as modified by Zahler and Prescott (13),

we found an activity in the macronucleus capable of synthesizing $(C_4A_4)_n$ RNA from a $(dG_4T_4)_n$ template.

Sequences Required in dG_4T_4 Templates

Many different $(dG_4T_4)_n$-containing oligodeoxynucleotides were tested for template function in these reactions. Figure 1 shows the results of using different oligodeoxynucleotides in the reactions, and table 1 shows the sequences of these oligodeoxynucleotides. Reactions mixtures contained $\alpha^{32}P$ CTP, ATP, 50 μg/ml of α-amanitin, macronuclear extract, and oligodeoxynucleotide as described in the methods section. Both CTP and ATP were necessary for the reactions. Reactions containing $\alpha^{32}P$ CTP, UTP and GTP did not produce any RNA on these templates. Not all $(dG_4T_4)_n$ oligodeoxynucleotides work as templates for this reaction. Pure $(dG_4T_4)_n$ oligodeoxynucleotides of various lengths failed to work as templates for transcription of $(C_4A_4)_n$ RNA (lanes 12-17). These include "G_4T_4 36mer" and "T_4G_4 64mer". Additional elements must be present on dG_4T_4 oligodeoxynucleotides to bring about $(C_4A_4)_n$ RNA synthesis.

Single-stranded $(dG_4T_4)_n$ oligodeoxynucleotides are effective templates if a random sequence is present at the 5' end of the oligodeoxynucleotide, but, in addition, the $(dG_4T_4)_n$ part of the oligodeoxynucleotide must be at least 36 bases long. In the CJD series, all five oligodeoxynucleotides have the same random sequence at their 5' end. CJD-20 and CJD-28, with 20 and 28 bases of dG_4T_4 repeats were poor in supporting $(C_4A_4)_n$ RNA synthesis (lanes 7 and 8). CJD-36 and CJD-44, with 36 and 44 bases of dG_4T_4 repeats strongly supported $(C_4A_4)_n$ RNA synthesis (lanes 9 and 10). In CJD 36U, dU was substituted for T in the 3' terminal 16 bases. This had no discernable effect on $(C_4A_4)_n$ RNA synthesis (lane 11). The random sequence in the five members of the CJD series is in fact an 8-base palindrome. It is conceivable, although unlikely because it is so short, that the random sequences of two molecules might pair, forming a double-helix region, which could improve the efficacy of the template action in $(C_4A_4)_n$ RNA synthesis.

Oligodeoxynucleotides called "short G_4T_4" and "long G_4T_4" have identical 5' random sequences. "Short G_4T_4", with 20 bases of dG_4T_4 attatched to a 5' random sequence does not support $(C_4A_4)_n$ RNA synthesis (lane 5), but "long G_4T_4", with 36 bases of dG_4T_4 attatched to an identical 5' random sequence strongly supports $(C_4A_4)_n$ RNA synthesis (lane 6). There is a six-base palindromic sequence in the 5' random region of "short G_4T_4" and "long G_4T_4", and it is conceivable, although more unlikely than for the

Table I. Oligodeoxynucleotides tested for ability to serve as templates for the synthesis of $(C_4A_4)_n$ RNA by macronuclear extracts.

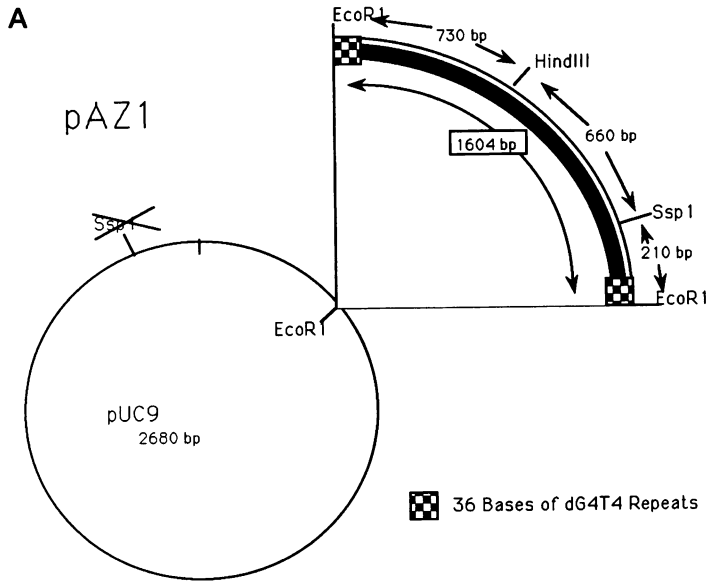
Oligodeoxynucleotide Single-Stranded Templates 5'-3'		Activity	Lane
T_4G_4 16mer	$T_4G_4T_4G_4$	No	12
G_4T_4 16mer	$G_4T_4G_4T_4$	No	13
T_4G_4 32mer	$T_4G_4T_4G_4T_4G_4T_4G_4$	No	14
G_4T_4 32mer	$G_4T_4G_4T_4G_4T_4G_4T_4$	No	15
G_4T_4 36mer	$G_4T_4G_4T_4G_4T_4G_4T_4G_4$	No	16
T_4G_4 64mer	$T_4G_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_4T_4G_4$	No	17
CJD-20	CGGCTTAAGCG$_4$T$_4$G$_4$T$_4$G$_4$	Weak	7
CJD-28	CGGCTTAAGCG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$	Weak	8
CJD-36	CGGCTTAAGCG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$	Yes	9
CJD-44	CGGCTTAAGCG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$	Yes	10
CJD-36U	CGGCTTAAGCG$_4$T$_4$G$_4$T$_4$G$_4$U$_4$G$_4$U$_4$G$_4$	Yes	11
Short G_4T_4	AATTCTGCAG$_4$T$_4$G$_4$T$_4$G$_4$	No	5
Long G_4T_4	AATTCTGCAG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$	Yes	6
RAN-36	AGACACCTTAG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$	Yes	20
RAN-40	AGACACCTTAG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$	Yes	21
Without Oligodeoxynucleotide		No	18,19

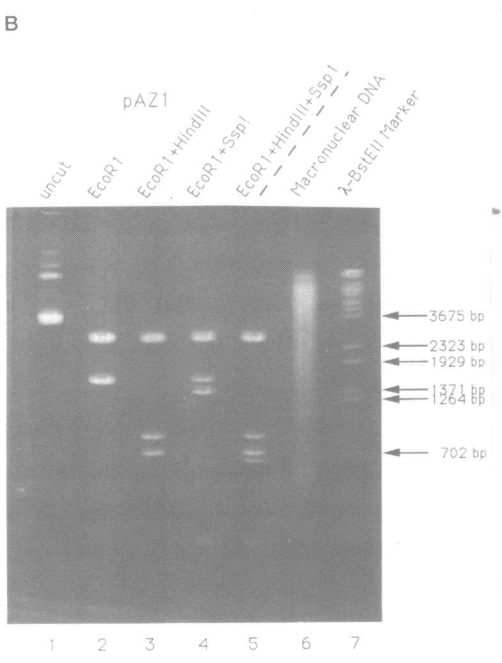
Oligodeoxynucleotide Templates with a Double-Stranded Segment	Activity	Lane
#90 C$_4$A$_4$C$_4$A$_4$C$_4$GGCCG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$3'	Yes	1
3'G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$CCGGC$_4$A$_4$C$_4$A$_4$C$_4$		
#95 C$_4$A$_4$C$_4$A$_4$C$_4$GGCCI$_4$T$_4$I$_4$T$_4$I$_4$T$_4$I$_4$T$_4$I$_3$G3'	Yes	2
3'GI$_3$T$_4$I$_4$T$_4$I$_4$T$_4$I$_4$T$_4$I$_4$CCGGC$_4$A$_4$C$_4$A$_4$C$_4$		
Maxitel C$_4$A$_4$C$_4$A$_4$C$_4$GGGAATTCTGCAGAATTCCCG$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$3'	Yes	3
3'G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$T$_4$G$_4$CCCTTAAGACGTCTTAAGGGC$_4$A$_4$C$_4$A$_4$C$_4$		
Blunt Maxitel C$_4$A$_4$C$_4$A$_4$C$_4$GGGAATTCTGCAGAATTCCCG$_4$T$_4$G$_4$T$_4$G$_4$3'	No	4
3'G$_4$T$_4$G$_4$T$_4$G$_4$CCCTTAAGACGTCTTAAGGGC$_4$A$_4$C$_4$A$_4$C$_4$		

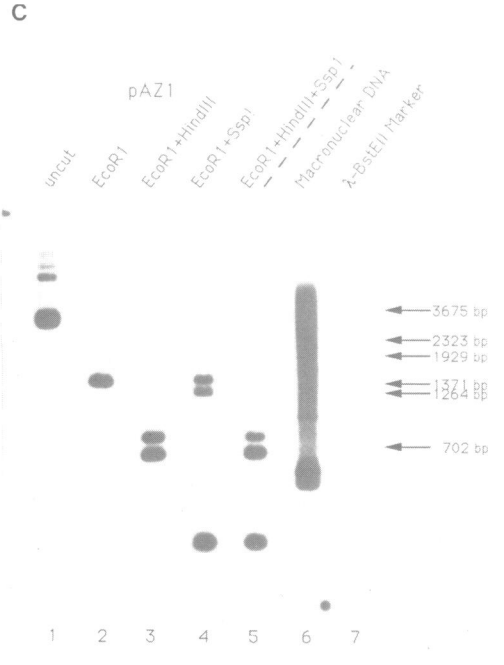
CJD series, that these may also form intermolecular base-pairs that could improve the template ability of these oligodeoxynucleotides.

To determine if secondary structure in the 5' random sequence played any role, we designed RAN-36 and RAN-40, two oligodeoxynucleotides with identical 5' random sequence and 36 and 40 bases of dG_4T_4 repeats respectively. The 5' random sequence of these oligodeoxynucleotides is non-selfcomplementary, and both evoke robust synthesis of $(C_4A_4)_n$ RNA. Thus, single-stranded oligodeoxynucleotides containing dG_4T_4 repeats serve strongly as template for $(C_4A_4)_n$ RNA synthesis if they possess a short 5' random sequence and if the segment of dG_4T_4 repeats is at least 36 bases long.

Oligodeoxynucleotides #90, #95, and "maxitel" all form a dimer structure that possesses the same telomeric arrangement as the natural telomeres of *Oxytricha* macronuclear DNA. These oligodeoxynucleotides are


A
pAZ1
EcoR1
730 bp
HindIII
660 bp
1604 bp
Ssp1
210 bp
EcoR1
Ssp1
EcoR1
pUC9
2680 bp
36 Bases of dG4T4 Repeats


B
pAZ1
uncut
EcoR1
EcoR1+HindIII
EcoR1+Ssp1
EcoR1+HindIII+Ssp1
Macronuclear DNA
λ-BstEII Marker
3675 bp
2323 bp
1929 bp
1371 bp
1264 bp
702 bp
1 2 3 4 5 6 7


C
pAZ1
uncut
EcoR1
EcoR1+HindIII
EcoR1+Ssp1
EcoR1+HindIII+Ssp1
Macronuclear DNA
λ-BstEII Marker
3675 bp
2323 bp
1929 bp
1371 bp
1264 bp
702 bp
1 2 3 4 5 6 7

efficient templates for the reaction (lanes 1-3). Substituting deoxyinosine for deoxyguanosine (in #95, lane 2) has no effect on the reaction. In oligodeoxynucleotides #90 and "maxitel" the length of the dG_4T_4 repeat segment is 36 bases; 20 of these form a duplex with dC_4dA_4 and 16 form a 3' single-stranded extension. "Blunt maxitel" has a 20-base duplex region of dG_4T_4/dC_4dA_4 like #90, #95, and "maxitel", but has no 3' single-stranded $(dG_4T_4)_2$ extension. "Blunt maxitel" has no template ability for $(C_4A_4)_n$ RNA synthesis (lane 4). The experiments cited above with the CJD series, "short G_4T_4", and "long G_4T_4" had suggested that the length of the dG_4T_4 segment was important; it had to be longer than 28 bases to be an effective template. Experiments with oligodeoxynucleotides that have secondary structure like native *O. nova* telomeres suggest that the single-stranded portion of the dG_4T_4 segment need not be longer than 16 bases, and the remainder of the length requirement of dG_4T_4 can be met with dG_4T_4 that is in a duplex with dC_4dA_4.

It is apparent in figure 1 that some of the RNA reaction products in these experiments are longer than the $(dG_4T_4)_n$ segment in the oligodeoxynucleotide templates. For example with "maxitel" (lane 3), the ladder of $(C_4A_4)_n$ RNA is over 50 bases, but there are only 36 bases of dG_4T_4 in the template. This could be the result of slippage or ratcheting consisting of dissociation and reassociation of the RNA with the oligodeoxynucleotide template. Since RNA/DNA hybrids at 14°C are very stable, slippage or ratcheting might be an enzyme mediated process.

The Sequence of the RNA Product

Figure 2b shows a 1% agarose gel of plasmid pAZ1 digested with different restriction endonucleases indicated in the figure above each lane.

Figure 2. Primase reaction product hybridized to a Southern blot.
2a. Gross restriction endonuclease map of plasmid pAZ1. Checkered regions each contain 36 bases of dG_4T_4 repeats. The pUC9 vector has had its Ssp1 site deleted, indicated by the site being crossed out.
2b. Ethidum bromide stained agarose gel of plasmid pAZ1 digested with indicated restriction endonucleases. Lane 6 contains undigested macronuclear DNA from *O. nova*. Lane 7 contains a λ BstEII size marker with sizes indicated in base pairs. Please note that the restriction enzyme Ssp1 (lanes 4 and 5) only cleaved the DNA with about 50% efficiency.
2c. Southern blot of gel in figure 2b probed with the primase reaction product of a reaction with oligodeoxynucleotide #90 in the presence of $\alpha^{32}P$ CTP and ATP. Blot was washed at a final stringency of 2X SSPE at 40°C.

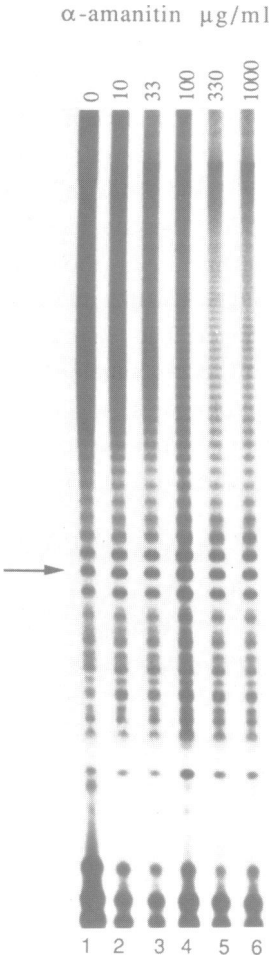


Figure 3. Effect of α-amanitin on the primase reaction. A macronuclear extract was incubated with the oligodeoxynucleotide maxitel (see table 1 for sequence), α^{32}P CTP, 125 µm ATP, and varying concentrations of α-amanitin indicated above each lane. The arrow is a 5' end-labeled 16mer marker consisting of $pdC_4dA_4dC_4dA_4$.

Also included in the gel are a lane of undigested macronuclear DNA from *O. nova* and a λ BstEII marker lane (New England Biolabs). Note that the restriction enzyme Sspl (lanes 4 and 5) only cleaved the DNA with about 50% efficiency. pAZ1 contains the macronuclear actin gene of *O. nova* from plasmid pMAC-4 (described in 16) cloned into the EcoR1 site of a pUC9

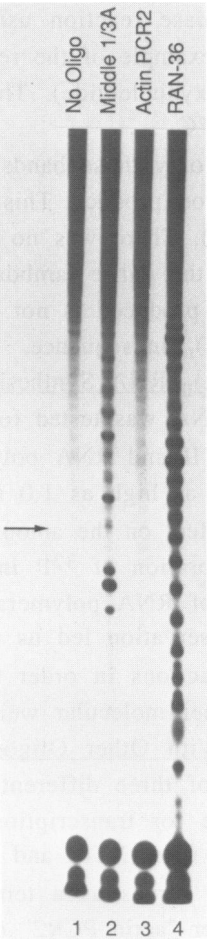


Figure 4. RNA synthesis with random oligodeoxynucleotides and telomeric oligodeoxynucleotides. Macronuclear extracts were incubated with α^{32}P CTP, 125 μm ATP, 125 μm GTP, 125 μm UTP, 50 μg/ml of α-amanitin and the following oligodeoxynucleotides. Lane 1) No oligodeoxynucleotide. Lane 2) Middle 1/3A 5'CCGGATCCACGCGTGATCATGGATATCGTCCTCGGGTTCTCCTTAGGGGGTGTCA3'. Lane 3) Actin PCR2 5'GCCCCATAATAGTATTTGGG3'. Lane 4) RAN-36 5'AGACACCTTAGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG3'. The arrow is a 5' end-labeled 16mer marker consisting of $pdC_4dA_4dC_4dA_4$.

plasmid, the Ssp1 site of which has been removed. A gross restriction endonuclease map of pAZ1 is shown in figure 2a. Figure 2c contains a southern blot of the gel in figure 2b that has been probed with the RNA

reaction product from a primase reaction using oligodeoxynucleotide #90 (see figure 1, lane 1 for an example of the reaction product, and table 1 for the sequence of the oligodeoxynucleotide). The stringency of the final wash of the blot was 2X SSPE at 40°C.

As seen from the blot, only those bands that contain dG_4T_4 sequences hybridized to the RNA reaction product. This includes the entire macronuclear genome (lane 6). There was no hybridization of the RNA reaction product to pUC9 or the entire lambda genome. From this we conclude that the RNA being produced is not a random assembly of CTP and ATP, but is in fact $(C_4A_4)_n$ in sequence.

Effect of α-amanitin on $(C_4A_4)_n$ RNA Synthesis

Synthesis of $(C_4A_4)_n$ RNA was tested for sensitivity to α-amanitin, an inhibitor of RNA polymerase II and RNA polymerase III (figure 3). Concentrations of α-amanitin as high as 1.0 mg/ml in the reaction mixture (lane 6) had no discernable effect on the amount of $(C_4A_4)_n$ RNA product formed, but the heavy incorporation of ^{32}P into higher molecular weight RNA (presumably the result of RNA polymerase II and III activities) was dramatically reduced. This observation led us to use 50 μg/ml of α-amanitin in all subsequent reactions in order to eliminate most of the incorporation of ^{32}P into higher molecular weight RNA.

RNA-Polymerizing Activity With Other Oligodeoxynucleotides as Templates

Figure 4 shows a test of three different oligodeoxynucleotides for the ability to serve as a template for transcription in crude macronuclear extracts in the presence of all four rNTPs and 50 μg/ml of α-amanitin. The random 55mer "middle 1/3A" serves as a template for RNA transcription (lane 2), but the random 20mer "actin PCR2" does not (lane 3). The telomeric dG_4T_4 oligodeoxynucleotide RAN-36 serves as a template as already demonstrated (lane 20 of figure 1 and lane 4 of figure 4). It appears that random single-stranded oligodeoxynucleotides longer than 20 bases can be used as template by an α-amanitin-insensitive activity that transcribes DNA into RNA. However, both oligo $p(dG)_{12-18}$ and poly dG failed to work as templates for polymerization of rCTP over a wide range of oligodeoxynucleotide concentrations in the presence or absence of various other rNTPs (data not shown). The α-amanitin-insensitive activity that synthesizes RNA on random oligodeoxynucleotide templates may be the same activity that synthesizes $(C_4A_4)_n$ RNA on dG_4T_4 templates.

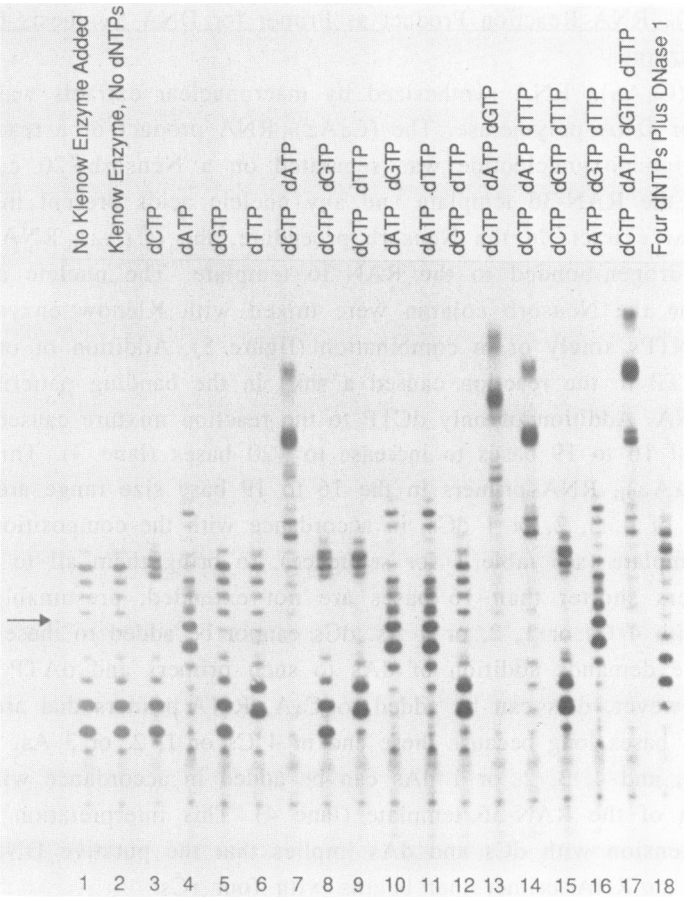


Figure 5. Extension by the Klenow enzyme of RNA made in macronuclear extracts. A macronuclear extract was incubated with α^{32}P CTP, 125 μm ATP, 50 μg/ml α-amanitin, and the oligodeoxynucleotide RAN-36. The reaction product was purified on a Nensorb™20 column. This reaction product was then incubated with the Klenow enzyme and the indicated dNTPS. Lane 1) No Klenow enzyme; control. Lane 2) Klenow enzyme added but no dNTPs. Lanes 3-17) Klenow enzyme and 33μM of the dNTPS indicated above were added to each reaction. Lane 18) Same as lane 17 but RNAse free DNAse I was added to the reaction during the final 10 minutes of incubation. The arrow is a 5' end-labeled 16mer marker consisting of $pdC_4dA_4dC_4dA_4$.

The $(C_4A_4)_n$ RNA Reaction Product as Primer for DNA Synthesis by the Klenow Enzyme

The $(C_4A_4)_n$ RNA synthesized by macronuclear extracts was tested as a primer for DNA polymerase. The $(C_4A_4)_n$ RNA product of a reaction with RAN-36 oligodeoxynucleotide was separated on a Nensorb™20 column along with the RAN-36 template and any nucleic acids present in the macronuclear extract. In the Nensorb procedure, the $(C_4A_4)_n$ RNA product remains hydrogen-bonded to the RAN-36 template. The nucleic acids separated on the Nensorb column were mixed with Klenow enzyme and different dNTPs singly or in combination (figure 5). Addition of only dCTP or only dATP to the reaction caused a shift in the banding pattern of the $(C_4A_4)_n$ RNA. Addition of only dCTP to the reaction mixture caused C_4A_4 molecules of 16 to 19 bases to increase to ≈20 bases (lane 3). This suggests that the $(C_4A_4)_n$ RNA primers in the 16 to 19 base size range are extended by addition of 4, 3, 2, or 1 dCs, in accordance with the composition of the RAN-36 template (see table 1 for sequence), to bring them all to 20 bases. RNA primers shorter than 16 bases are not extended, presumably because they end with 4 Cs or 1, 2, or 3 As. dCs cannot be added to these because the template demands addition of dAs to such primers and dATP is not present. However, dAs can be added to C_4A_4 RNA primers that are 12 to 15 or 20 to 23 bases long because these end in 4 Cs or 1, 2, or 3 As, respectively, and 4, 3, 2, or 1 dAs can be added in accordance with the composition of the RAN-36 template (lane 4). This interpretation of the Klenow extension with dCs and dAs implies that the putative DNA primase synthesizes an RNA primer that begins with four rCs.

When dGTP alone is added to the reaction mixture, no extension of the primer occurs (lane 5), which is consistent with the nature of the RAN-36 template. However, addition of dTTP alone to the reaction causes lengthening by one base to RNAs of 12 and 13 bases. The $(C_4A_4)_n$ RNAs that are lengthened are presumed to end with 4 Cs that are hydrogen bonded to the last 4 dGs that adjoin the random sequence of the RAN-36 template. Because the first base of the random sequence is an A, a dT should be added to the primer. The second base of the random sequence template is a T, so no further bases should be added since dATP is absent. With the addition of dATP alone, the primer bands at 12 and 13 bases also shift to ≈16 bases (lane 4), implying that these smaller RNAs can dissociate and then reassociate in a new position on the template that will allow for addition of dATP to the primer. The fact that bands at both 12 and 13

bases are elongated by one base after addition of dTTP to the reaction mixture implies that smaller RNAs in the reaction mixture may have 5' end heterogeneity.

Addition of dATP and dCTP together to the reaction mixture causes lengthening of the $(C_4A_4)_n$ RNA (lane 7). Addition of all four dNTPs brings about the most lengthening of the input RNA (lane 17). All additions to the RNA are DNAse I sensitive with the exception of the first dNTP added (lane 18).

DISCUSSION

Telomeres in diverse organisms such as *Arabidopsis thaliana*, human, yeast, slime mold, and ciliates have a common motif in their deoxynucleotide sequences (4-6). This may also include a 3' single-stranded extension proven to be present on the gene-sized molecules in hypotrichous ciliates, *Tetrahymena*, and the acellular slime mold *Didymium* (7,18). The presence of the consensus, repeated sequence and a 3' extension presumably contribute to the functional properties of telomeres, in particular prohibition of rejoining of chromosomes at telomeres, attachment of telomeres to the nuclear envelope in some organisms during meiosis, and replication of the ends of the linear DNA molecule in a chromosome (reviewed in 4). Some or all of these telomere properties probably depend on telomere binding proteins (10,11).

In a previous paper we described a model for replication of the ends of the linear, gene-sized DNA molecules in the macronucleus of *O. nova* (13). An updated version of the model is included here in figure 6. We propose that DNA replication begins by synthesis of a $(C_4A_4)_2$ RNA primer, using the 3' 16-base $(dG_4T_4)_2$ single-stranded extension of the telomere as a template. In the model, the primer is subsequently extended by DNA polymerase. This produces a leading DNA strand that ultimately joins to the lagging strand being produced from the opposite end of the same parental template (figure 6c). Telomere terminal transferase (13) then makes the 3' single-stranded extension of $(T_4dG_4)_2$. The length of the terminal extension is proposed to be set by binding of the telomere binding proteins when the proper length telomeric extension has been achieved (10). The model is simple, requiring no scission of strands, ligations, strand switching, strand foldbacks, or other devices. Replication at telomeres requires the telomere terminal transferase described earlier (13) and the DNA primase described in this paper. The body of the DNA

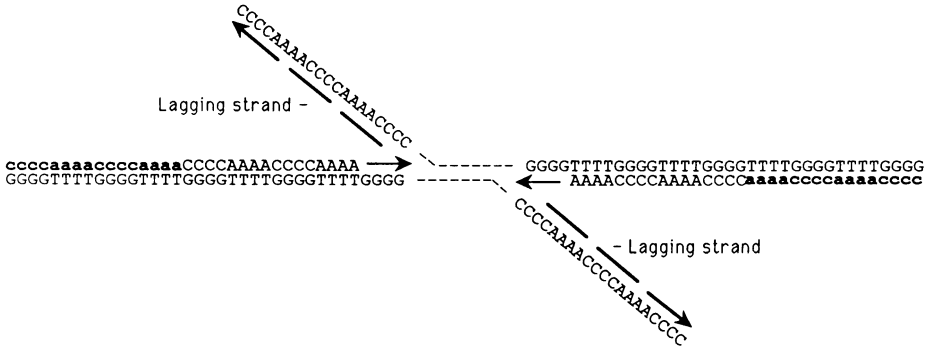
A. Macronuclear DNA molecule with telomere binding proteins removed prior to replication.

```
                  CCCCAAAACCCCAAAACCCC --------- GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG
GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG --------- CCCCAAAACCCCAAAACCCC
```

B. RNA primer synthesized on 3' telomeric extension.

```
ccccaaaaccccaaaaCCCCAAAACCCCAAAACCCC --------- GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG
GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG --------- CCCCAAAACCCCAAAACCCCaaaaccccaaaaccccc
```

C. DNA polymerase begins leading and lagging strand synthesis from each end of the molecule.



D. RNA primer is removed. DNA polymerase completes replication of the molecule. Only one daughter molecule is shown.

```
ccccaaaaccccaaaa
                  CCCCAAAACCCCAAAACCCC --------- GGGGTTTTGGGGTTTTGGGG
GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG --------- CCCCAAAACCCCAAAACCCC
```

E. Telomere terminal transferase makes the 3' telomeric extension. The final length is determined by interaction with telomere binding proteins.

```
                  CCCCAAAACCCCAAAACCCC --------- GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG
GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG --------- CCCCAAAACCCCAAAACCCC
```

Figure 6. Model for replication of telomeres in hypotrichous ciliates.

molecule is replicated by the conventional complex of enzymes identified in other systems.

In this paper we describe the DNA primase that can make $(C_4A_4)_n$ RNA using a dG_4T_4 template. For a single-stranded molecule to function as a template it must have a dG_4T_4 oligodeoxynucleotide sequence at least 36 bases long and must have a short random sequence at its 5' end. Single-stranded oligodeoxynucleotides consisting solely of dG_4T_4 repeats from 16 to 64 bases in length do not function as templates for the $(C_4A_4)_n$ RNA-synthesizing enzyme. Oligodeoxynucleotide structures that mimic a native double-strand telomere (with a 3' 16-base $(dG_4T_4)_2$ single-stranded extension) serve as excellent templates for this reaction.

The sequence of the RNA product of the reaction has been characterized in several ways. The results all indicate that it is $(C_4A_4)_n$. First, the reaction only occurs when a proper dG_4T_4 oligodeoxynucleotide is added in the presence of both CTP and ATP, therefore the RNA is presumed to be made from that dG_4T_4 template. Second, the RNA product of a reaction in the presence of CTP, ATP and an oligodeoxynucleotide possessing 36 bases of dG_4T_4 repeats, with 10 bases 5' of that containing random sequence, can prime DNA synthesis on that same oligodeoxynucleotide by the Klenow enzyme (figure 5). The RNA product must hybridize to the dG_4T_4-containing oligodeoxynucleotide in order to serve as a primer. Third, hybridization of the RNA product to southern blots of gels with DNA bands containing dG_4T_4 repeats shows that the RNA product hybridizes only to DNA bands containing $(dG_4T_4)_n$, and does not hybridize to other DNA bands.

The primase reaction is insensitive to concentrations of α-amanitin that severely inhibit RNA polymerase II and RNA polymerase III in other organisms, implying that the primase activity is not performed by either of these two enzymes (reviewed in 19). Addition of α-amanitin to the primase reaction mixture (figure 3) enormously decreases incorporation of labeled rNTPs into RNA products other than $(C_4A_4)_n$ RNA, apparently reflecting the inhibition of some of the RNA polymerases in the *O. nova* macronuclear extract (presumably RNA polymerases II and III, although nothing is known about RNA polymerase sensitivity to α-amanitin in hypotrichous ciliates). Conceivably, RNA polymerase I could be responsible for the primase activity. Synthesis of RNA on random oligodeoxynucleotide templates occurs in the same α-amanitin-containing, macronuclear extract in which $(C_4A_4)_n$ RNA is synthesized (figure 4). Both syntheses may be

catalyzed by the same enzyme, the synthesis with random templates possibly representing a primase for Okazaki fragments (DNA primases reviewed in 20) and the synthesis with dG_4T_4 templates representing the primase for replication at telomeres.

The two telomeres should provide sufficient origins of replication to replicate an entire macronuclear DNA molecule. These molecules range in size from 400 base pairs to ≈15,000 base pairs with a number average size of 2200 base pairs (12), which is much shorter than the replication units found in eukaryotic chromosomes in general (36,000 base pairs for yeast (21)). At rates of replication typical for eukaryotes (≈3000 bases/minute), the average size DNA molecule in the *O. nova* macronucleus would replicate in 22 seconds and the longest molecules in 2.5 minutes. The model of DNA replication we propose in hypotrichs is supported by the electron microscope observation that replication may begin at each end of the gene-sized molecules in two other hypotrich species (22).

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