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The cohering telomeres of Oxytricha

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ABSTRACT

We have studied the process by which purified Oxytricha macronuclear DNA associates with itself to form large aggregates. The various macronuclear DNA molecules all have the same terminal or telomeric DNA sequences that are shown below.

$$5'\ C_4A_4C_4A_4C_4\text{--mean length----}G_4T_4G_4T_4G_4T_4G_4T_4G_4$$
$$G_4T_4G_4T_4G_4T_4G_4T_4G_4\text{-----2.4 kb------}C_4A_4C_4A_4C_4$$

When incubated at high concentrations, these telomeric sequences cohere with one another to form an unusual structure - one that is quite different from any DNA structure so far described. The evidence for this is the following: 1) These sequences cohere albeit slowly, in the presence of relatively high concentrations of Na^+, and no other cation tested. This contrasts with the rapid coherence of complementary single-chain terminals of normal DNA (sticky ends) which occurs in the presence of any cation tested. 2) If the cohered form is transferred into buffers containing a special cation, K^+, it becomes much more resistant to dissociation by heating. We estimate that K^+ increases the thermal stability by 25^o or more. The only precedent known (to us) for a cation-specific stabilization is that seen in the quadruplex structure formed by poly I. The thermal stability of double helical macronuclear DNA depends on the cation concentration, but not the cation type.

Limited treatment with specific nucleases show that the 3' and 5'-ended strands are essential for the formation of the cohering structure. Once in the cohered form, the telomeric sequences are protected from the action of nucleases. Coherence is inhibited by specific, but not by non-specific, synthetic oligomers, and by short telomeric fragments with or without their terminal single chains. We conclude that the coherence occurs by the formation of a novel condensed structure that involves the terminal nucleotides in three or four chains.

INTRODUCTION

The ciliated protozoa have proven to be a rich source of unexpected findings, perhaps the best example being catalytic RNAs (1). Another example is the unusual organization of their macronuclear DNA. In the case of the hypotrichous ciliate, Oxytricha, the micronucleus, from which the macronucleus is derived, contains 1.1 to 1.2 x 10^9 bp in the two species that have been measured. In contrast, the kinetic complexity of the macro-nuclear DNA (of related species) is only about 2 - 5 x 10^7 bp. Thus, only about 2 to 5% of the sequences found in the micronucleus are represented in the macronucleus. In contrast, the macronucleus contains about 50 times more DNA than the micronucleus. Thus, at a minimum each sequence must be represented an average of (1/.05) x 50 or 1000 times. The macronuclear DNA is a collection of

discrete species ranging in length from 0.5 to 24 kb, with a mean value of about 2.4 kb. Thus, there are on the order of 20,000 different species of DNA molecules, some of which have very high multiplicities and may be seen as sharp bands in the general continuum (2).

The hypotrichous ciliate Oxytricha nova, like other related species, has the peculiarity that all of the terminal, or telomeric, DNA sequences are identical (see Abstract) (2,3). Moreover, these sequences are added during the maturation of the macronucleus (4). It is not known how these telomeres are replicated with such high precision during vegetative growth.

In 1980, Lipps reported that concentrated solutions of purified macronuclear DNA aggregated when incubated at room temperature in the presence of NaCl (5,6). This conclusion rested mainly on the reduced continuum of mobilities of the aggregated form on agarose gels. He proposed that the telomeres fused to form a four-stranded structure.

Many questions remained about these observations and their interpretation. Therefore, we have undertaken a systematic study of this aggregation. The experiments reported below demonstrate that the aggregation occurs by a specific coherence at or near the terminals of the DNA molecules; that coherence is not the result of the participation of proteins or other small molecules; and that nucleotides from three or four chains are involved in the cohering site. Most important, we have found that coherence only occurs in the presence of Na^+, and that the thermal stability of the cohered form is sharply increased by 25^o or more in the presence of K^+. No other cation tested confers this added stabiliity. This observation indicates that the telomeres cohere to form a novel structure unlike any so far reported. This structure may have some general significance.

MATERIALS AND METHODS

Macronuclear DNA

Macronuclear (Mac) DNA from Oxytricha nova was prepared by published procedures (7) and given to us by Prof. D. M. Prescott. This DNA was frequently repurified by phenol extraction and banding in CsCl. Lambda DNA was from Bethesda Research Laboratories.

Standard Coherence Test and Preparation of COH Mac DNA

Mac DNA dissolved in an appropriate buffer was precipitated with 2 volumes of 95% ethanol, rinsed twice with 95% ethanol, then redissolved in 10 mM Tris (pH 7.6), 1 mM EDTA (called Tris-EDTA) to a final DNA concentration of 2 mg/ml. NaCl was added to a final concentration of 1.0 M or 2.0 M NaCl, to form the standard coherence buffer. This DNA solution was heated for 10 minutes at 70^o to melt any cohering ends, the product being called "DIS Mac DNA." The standard coherence test consisted of incubating this solution for 48 to 72 hr at room temperature. The resulting cohered form is called "COH Mac DNA."

Many of these experiments were made possible by the discovery that both COH and DIS Mac DNA can be recovered, without change of state, by the following procedure. The DNA (2 mg/ml) was divided into 5 ul aliquots in individual eppendorf tubes. The samples

were diluted to 50 ul with Tris-EDTA containing 20 mM NaCl and precipitated with 2 volumes of 95% ethanol. The precipitate was cured by standing for 2 hr at room temperature, then centrifuged at 10,000 rpm for 2 minutes. The invisible pellet was redissolved by standing for 2 hours at room temperature in 10 ul of Tris-EDTA containing 200 mM NaCl. The many 10 ul samples were then combined into one tube and centrifuged to remove debris which may have collected during the course of this preparation. The resulting DNA solution containing either DIS or COH Mac DNA was used for the heat stability and other tests. Note: COH Mac DNA must be distributed in about 10 ug samples in order for efficient recovery in the 200 mM NaCl.

Gel Electrophoresis

Agarose gel electrophoresis was performed on horizontal slabs using Boyer's buffer (8). Acrylamide gel electrophoresis was performed using Tris-Borate buffer (89 mM Tris, 89mM boric acid, 2mM EDTA, pH 8.0). The slabs were then stained with ethidium bromide and photographed using conventional procedures (9). Occasionally certain species were recovered from the agarose gel by reversing the polarity and depositing the DNA on DEAE paper (10).

Estimation of Extent of Coherence

In order to estimate the degree of coherence in mixtures of partially cohered Mac DNA, such as that found in experiments involving competitive inhibitors, a series of standard mixtures of COH and DIS Mac DNA were prepared and run. These mixtures ranged from 0:10 to 10:0 (COH:DIS). Comparing the general mobility distribution and the relative density of the high multiplicity species, in both the experimental and standard series, permitted a crude estimate of the "extent of coherence."

Gradient Sedimentation

Sucrose gradients (10-40%, w/v, 10.6 ml) were constructed from the bottom by the method of Noll (11). One solution contained 10%, the other 40% sucrose, while both contained 1 M NaCl in Tris-EDTA. Finally, 0.5 ml of 60% sucrose in the same buffer was introduced to form a bed. A sample of 100 ul containing 10 ug of Mac DNA, or lambda/HindIII standards, in Tris-EDTA, 1 M NaCl was loaded onto the top of the gradient. After centrifugation at 34,000 rpm for 16 hrs in the Beckman SW-41 rotor, the gradient was pressed out with the dense "Fluorinert" (DuPont) through a quartz cuvette placed in a sensitive UV densitometer operated at 254 nm. Equilibrium sedimentation was performed as in CsCl containing Tris-EDTA and centrifuged for 36 hrs. Fractions were collected from the bottom.

Viscosity Measurements

A floating rotor viscometer (12) was used to measure the reduced specific viscosity of COH and DIS Mac DNA. A sample of COH was diluted to about 100 ug.ml (0.01 g/dl) in filtered 1.0 M NaCl in Tris-EDTA. The viscosity of the solution was measured by the time required for 5 rotations of the rotor, which is driven by the inductive effect of a magnet rotating at constant speed. The temperature of the water jacket was increased step-wise and the period of rotation measured for each temperature. The measurements were repeated with pure

solvents. The ratio of these periods is equal to the relative viscosity. After subtracting 1.0 and dividing by the concentration in g/dl, one obtains the reduced specific viscosity, which for DNAs measured at these low gradients (ca 0.001 dynes/cm) and concentration are equal to the intrinsic viscosity.

Synthetic Oligomers

Oligomers of 5'GATCC$_4$A$_4$C$_4$A$_4$ and 5'GATCT$_4$G$_4$T$_4$G$_4$ were prepared by the phosphotriester method (13) and purified on 20% denaturing acrylamide gels followed by DE52 Sephadex column chromatography.

The synthetic telomeres (5'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$_4$) were synthesized with an Applied Biosystems 380 B DNA synthesizer and purified on a 12% denaturing acrylamide gel. This product was dissolved in Tris-EDTA containing 1 M NaCl and heated for 15 min at 90^0 then allowed to cool slowly overnight. Samples were subjected to 5% acrylamide gel electrophoresis. Two bands could be seen that had the expected mobility of telomeric dimers and hairpin monomers. The conversion to telomeric dimers was nearly complete. This was confirmed by treating the dimers with T4 polymerase in the presence of dTTP and dGTP (see below) which removed the 3' tails to produce a duplex molecule 44 bp long. The dimers could be cleaved by HaeIII, as expected, to produce a species of higher mobility equal to that of the putative "hairpin monomers."

Thermal Denaturation

COH Mac DNA in the desired solvent was placed in a cuvette mounted in a water-jacketed holder in a Zeiss PMQII spectrophotometer. The temperature of the DNA solution was measured by a mercury thermometer placed in a reference cuvette filled with buffer.

Enzymes and Reaction Conditions

Proteinase K, restriction enzymes, S1 nuclease, lambda exonuclease and T4 polymerase were purchased from Bethesda Research Laboratories and reactions were carried out according to the instructions supplied, unless otherwise described. T7 gene 3 endonuclease was a gift from F.W. Studier and used under conditions reported (20). T4 polymerase was frequently used to trim the 3'-ended tails from native and synthetic telomeres. The reaction conditions were: 67 mM Tris-HCl,pH 8.0, 6.7 mM $MgCl_2$, 17.7 mM $(NH_4)_2SO_4$, 167 ug/ml bovine serum albumin, 0.5 mM dithiothreitol and 400 uM each of dGTP and dTTP. The triphosphates were added to prevent the net removal of nucleotides from the double-helical portion of the telomere.

RESULTS

Incubation of Mac DNA at concentrations of 1 to 2 mg/ml in Tris-EDTA containing 1 to 2 M NaCl for 48 to 72 hours at room temperature, invariably resulted in the formation of the cohered form called COH Mac DNA. We have observed COH Mac DNA in three different ways: by its increased sedimentation rate through sucrose gradients, by its high specific viscosity, and by its retarded electrophoretic mobility through agarose gels. Agarose gels have

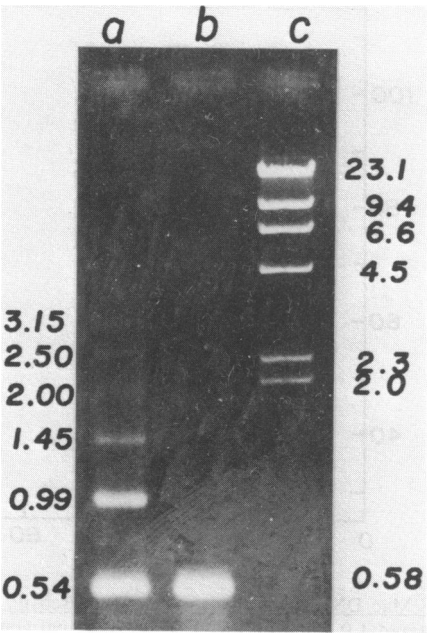

Fig. #1. Agarose gel of cohered 540 bp species. The unusually abundant 540 bp species was purified from gels, then subjected to a standard coherence test and then analyzed by 1% agarose gel electrophoresis in lane a. This lane shows bands at 540, 990 and 1450 bp; the negative shows longer species at 2000, 2500, and 3150 bp. Lane b shows the effect of heating the same sample to 70° for 10 min. Lane c contains lambda DNA cleaved by HindIII as markers.

proven the method of choice because of their convenience. In addition to the generally slower distribution of mobilities, the COH form can be recognized by the absence of the discrete high multiplicity species such as those containing the genes for ribosomal RNAs and the very abundant species, found in some Mac preparations, that had a mobility corresponding to 540 bp. The COH form may dispersed to the DIS form by heating to 60-70° for 10 minutes. The reformation of the COH form from the DIS form may be repeated any number of times.

A DNA-DNA Interaction is Responsible for Coherence

At first, we thought that some protein or other species was involved in this coherence. Therefore, the Mac DNA was extensively treated with Proteinase K and by phenol extraction, yet it displayed unimpaired ability to cohere. If proteins were there, they must be very resistant and tightly bound. Therefore, the DNA was passed through glass filters under conditions known to bind both proteins and DNA molecules bearing terminal proteins (14). The DNA was not retained on the filters and retained full ability to cohere. Finally, DIS Mac DNA was sedimented through sucrose gradients, to separate possible small molecules, and fractions collected: each fraction cohered normally. The same DNA was fractionated on CsCl gradients:

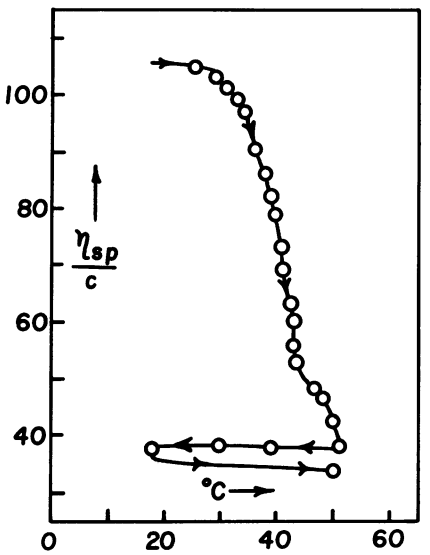

Fig. #2. The COH to DIS Mac DNA conversion as seen by viscosity. A sample of COH Mac DNA was diluted with filtered 1.0 NaCl in Tris-EDTA to a final concentration of 110 ug/ml or 0.011 g/dl. This solution was placed in a floating rotor-type viscometer that was was surrounded by a thermostated water jacket. The viscosity of the solution was measured as described in Methods. The temperature of the water jacket was increased or decreased in a step-wise manner before each measurement; each point required about 5 min. The time course of the experiment is indicated by arrows.

again each fraction cohered normally. These observations make it very unlikely that attached proteins or separate cofactors other than the DNA itself are involved in the cohering structures. This conclusion is further supported by sensitivity of terminal nucleotides to limited nuclease treatment as shown later.

Coherence Occurs by the Joining of Molecular Ends

In order to test whether the cohering structure actually involves the terminals (in contrast to merely being required for its formation), we turned to the unusually abundant 540 bp species. This species was isolated from gels using the DEAE paper method (10). As with all other high-multiplicity species, this band cannot be seen in COH Mac DNA, presumably because it is randomly incorporated into concatemers. This strongly implies that it is typical of the Mac DNA molecules in this respect. When the purified 540 bp species is allowed to cohere with itself, a characteristic oligomeric ladder is seen (Fig. 1). Multimeric ladders of this type are typical of linear DNAs that differ in multiples of a common length. In this case the modular length is about 500 bp. Thus, one can be reasonably sure that coherence occurs by the fusion of the telomeric regions of the Mac DNA - probably the telomeric sequences themselves.

Another line of evidence can be drawn from the measurement of the intrinsic

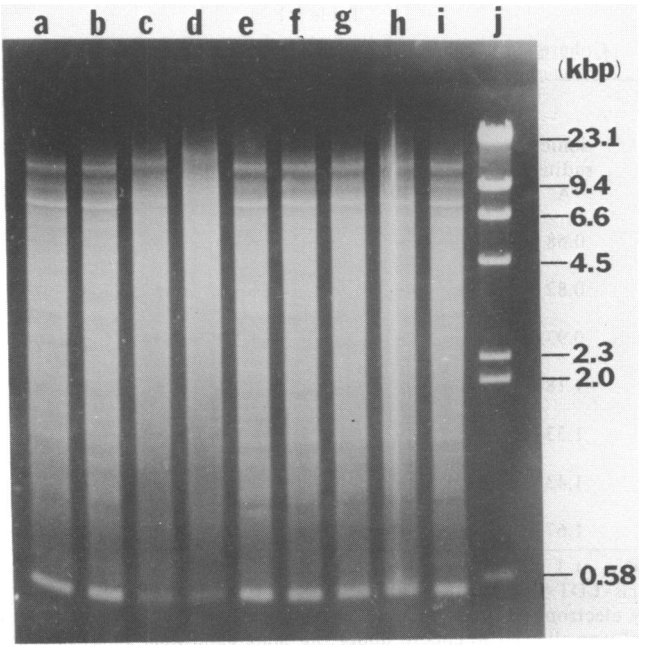

Fig. #3. NaCl promotes coherence but KCl does not. Mac DNA was allowed to cohere at room temperature for 2 days in Tris-EDTA containing 20, 50, 250 and 1000 mM NaCl (lanes a-d) or the same concentrations of KCl (lanes e-h). Lanes (i) and (j) show DIS Mac DNA and lambda/Hind III respectively. Note that 250 and 1000 mM NaCl allow the formation of COH, but equivalent concentrations of KCl will not. Results with other cations are shown in Table #1.

viscosity of solutions containing COH. As seen in Fig. 2, the reduced specific viscosity measured at very low shear rates is about 100 - 110 dl/g. This is the value expected for linear DNA molecules that are 28 kb (Fig. 11) in length (15). This suggests that the cohered form is essentially a linear, not a highly branched, structure. A single network point would be expected to significantly reduce the intrinsic viscosity (16). Fig. 2 shows that as the temperature increases, the viscosity falls to 35 dl/g, a value consistent with an average molecular length of 4.2 kb which is estimated by sedimentation rate (Fig.11). After dispersal by heat, the viscosity does not change significantly upon recooling or subsequently reheating, as shown at the bottom of Fig. 2. No special significance should be attributed to the apparent transition temperature. These experiments require several hours (about 4-5 min per point) and differ sharply from the much briefer heat treatments at higher temperatures.

Coherence is Promoted by Na^+ but Not by Other Cations

Mac DNA was subjected to a standard coherence test in the presence of various concentrations of Na^+ or K^+ and then examined by agarose gels. The results shown in Fig. 3, demonstrate that Na^+ promotes coherence but that K^+ does not. Seven different cations were

Table #1

Coherence is promoted by Na^+ but not by other cations.

		Macronuclear DNA				Lambda DNA			
	ionic radius	mM				mM			
Cation	A	20	50	250	1000	20	50	250	1000
Li^+	0.68	-	-	-	-	+	+	+	+
Mg^{++}	0.82	-	-	-	-	+	+	+	+
Na^+	0.97	-	-	+	+	+	+	+	+
Ca^{++}	1.18	-	-	-	-	+	+	+	+
K^+	1.33	-	-	-	-	+	+	+	+
NH_4^+	1.43	-	-	-	-	+	+	+	+
Cs^+	1.67	-	-	-	-	+	+	+	+

DIS Mac DNA at 1 mg/ml was adjusted to contain the concentrations of cations shown above in Tris-EDTA buffers. After 48 hours at room temperature, the samples were analysed by electrophoresis through 1% agarose gels. A Hind III digest of lambda DNA at 0.3 ug/ml was allowed to cohere under the same conditions in parallel tubes, and the joining of the terminal fragments observed. The formation of COH is marked by a (+); the joining of lambda ends is marked by a (+). For reference, the second column includes the crystal ionic radius of the cation (Handbook of Chemistry and Physics, 57th ed. R.C.Weast, ed. 1978).

tested for their ability to promote coherence, but only Na^+ would do so. In contrast, any cation will permit the coherence of the sticky ends of lambda DNA (see Table #1). Mac DNA will not cohere in a solution containing both 1 M Na^+ and 1 M K^+, a fact that suggests that K^+ can inhibit COH formation.

The Coherence is Very Slow

Table #1 reveals another feature of coherence: it is very slow. The concentration of lambda DNA is only 0.3 ug/ml, while that of the Mac DNA is 1 mg/ml (3000-times higher). The average length of the Mac DNA is only 2.4 kb, while that of the lambda DNA is 48 kb (20-times longer). Thus, the concentration of Mac telomeres is 60,000-times higher that that of the lambda sticky ends. Other experiments indicate that the lambda sticky ends are nearly all joined after 5 to 6 hrs incubation, whereas 48 to 72 hrs is required to cohere the telomeres. Thus, we can estimate that telomeric coherence is at least 100,000-times slower than the association of sticky ends. Apparently, only very rare collisions result in cohered structures.

K^+ Increases the Thermal Stability of the Cohered Form

COH Mac DNA was prepared as usual in NaCl and then mixed with the cohered right and left BamH1 arms of lambda. This mixture was diluted into 100 mM KCl or 100 mM NaCl

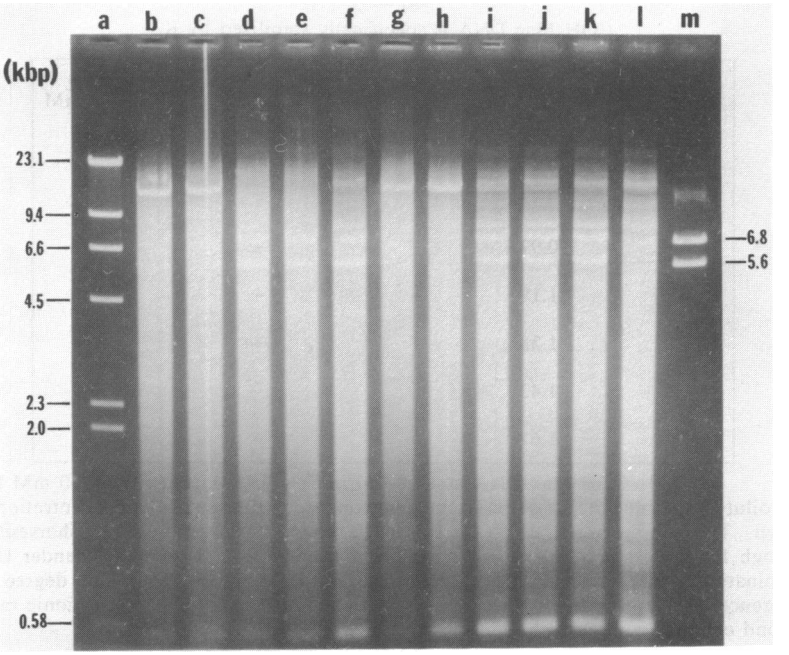

Fig. #4. Thermal stabilty of COH Mac DNA and lambda cohesive ends. Cohered lambda Bam H1 fragments were separated and mixed with COH Mac DNA dissolved at 0.5 mg/ml in Tris-EDTA containing 200 mM NaCl. The mixture was divided into 2 ul aliquots and diluted to 20 ul with Tris-EDTA containing 100 mM KCl (lanes b-f) and 100 mM NaCl (lanes g-k). The samples in lanes b and g were not heated. The samples in c and h were heated to 50^{o} for 10 min; those in d and i at 60^{o}; those in e and j to 70^{o}; those in f and k at 80^{o}. The DIS Mac DNA was run in lane l and the unjoined lambda ends were run in lane m.

and aliquots heated to increasing temperatures for 10 min. The melting of the cohered BamH1 segments served as an internal control. As shown in Fig. 4, the lambda segments separate by exposure to 60^{o} in either K^{+} or Na^{+}. The COH Mac DNA in Na^{+} is partially dispersed by exposure to 50^{o}. In contrast, COH in K^{+} is not completely dispersed by exposure to 80^{o}. Thus the thermal stability of the cohering site in 90 mM KCl, 20 mM NaCl has been increased by nearly 30^{o} above that in 110 mM NaCl.

We desired to know if any other cation would stabilize the cohered form. Table #2 shows the results of seven different cations: only K^{+} increased the thermal stability.

The Stability of COH in Solutions Containing K^{+} and Na^{+}

We were interested to know if the K^{+}-stabilized cohered structure involved a covalent linkage. This seems unlikely to us because heating to 80^{o} is seen to partially dissociate the cohered form (Fig.4). This conclusion is reenforced by the observations that the addition of Na^{+} to K^{+}-stabilized COH Mac DNA results in its destabilization, and that the addition of K^{+}

Table #2
COH Mac DNA is specifically stabilized by K^+

Cation	Radius A	20 mM	100Mm	1000 mM
Li^+	0.68	-	-	+
Mg^{++}	0.82	-	-	-
Na^+	0.97	-	-	-
Ca^{++}	1.18	-	-	+
K^+	1.33	+	+++	+++
NH_4^+	1.43	-	-	-
Cs^+	1.67	-	-	-

COH Mac DNA at 1 mg/ml in Tris-EDTA containing 200 mM NaCl was diluted 10-fold into Tris-EDTA buffers containing cations at the concentrations shown. The samples were heated to 70^0 for 10 min and assayed by electrophorsesis through 1% agarose gels, stained with ethidium bromide and photographed under UV illumination. The extent of coherence was estimated: (+++) indicates a high degree of coherence; (++) substantial; (+) weak; (-) completely dispersed. The crystal ionic radius (second column) is described beneath Table #1.

to COH Mac DNA in Na^+ results in its stabilization (Table #3). These results are more consistent with the idea that K^+ is chelated by the cohering structure, and that this site may be occupied by Na^+, to produce a less stable structure. Roughly speaking, Table #3 shows that a transition from the more stable to the less stable form occurs when the K^+ and Na^+ concentrations are about equal.

The Thermal Stability of COH Mac DNA in NaCl and KCl

At this point, it was possible that the cohering structures involved a significant portion of the Mac DNA. If this were the case, it would be likely that the dispersal of the COH structure would result in a change, likely an increase, in the UV absorption. Therefore, we performed conventional thermal denaturation experiments on COH Mac DNA in K^+ or Na^+, following the transition by the absorbance at 260 nm. As seen in Fig. 5, the transitions were exactly the same, even though the Na^+-stabilized COH is known to be dispersed by a 10-minute exposure to 60^0. This means that the cohering structure involves only a small portion of the DNA - probably just the telomeres themselves.

Nuclease Experiments

Lambda exonuclease is a processive nuclease that releases 5'-phosphorylated nucleotides from indented 5'terminals (17). While not absolute, this nuclease has a strong preference for terminals bearing a 5' phosphate (18). As Fig. 6A shows, DIS is very sensitive to the nuclease, suggesting that the 5'terminal is available to the nuclease, and that it probably

Table #3
The stability of COH Mac DNA in solutions containing both Na^+ and K^+

#1	NaCl	10 mM	NaCl	13.3 mM	NaCl	47mM
KCl	66	+++	133	++++	33	+
conc	132	+++	200	++++	46	++
(mM)	198	+++	256	++++	60	+++
	264	+++	331	++++	73	+++
	330	+++	397	++++	80	+++
#2	KCl	33 mM	KCl	66 mM	KCl	133 mM
NaCl	47	+	10	+++	13	++++
conc.	60	-	76	++	80	++++
(mM)	75	-	142	+	145	+++
	87	-	208	-	211	+++
	100	-	274	-	277	+++

COH Mac DNA at 1 mg/ml was diluted 10-fold with Tris-EDTA, then 1 M NaCl or 1 M KCl was added to produce the composition shown. The three experiments fall into two series: series #1 begins with the NaCl concentrations shown, and contains samples of increasing KCl, while series #2 begins with the KCl concentrations shown, and subsequent samples contain increasing concentrations of NaCl. A sample of 15 ul was heated to 70^0 for 10 min and the extent of coherence was estimated as described.

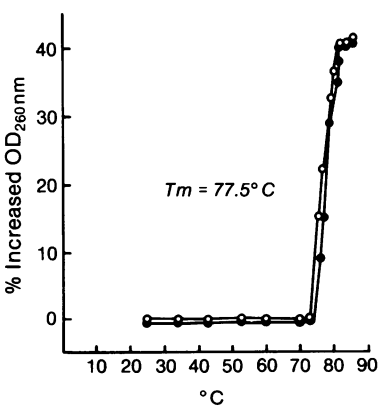

Fig. #5. Thermal stability of COH Mac DNA in NaCl and KCl. COH Mac DNA was transfered to Tris-EDTA buffer containing either 66 mM KCl and 10 mM NaCl, or 76 mM NaCl. Each sample containing about 50 ug/ml of DNA was placed in a cuvette and the absorbance at 260 nm measured as the temperature was increased from 20 to 85 deg. Note that the only visible transition for both samples occurs at 77.5^0 with the same hyperchromicity.

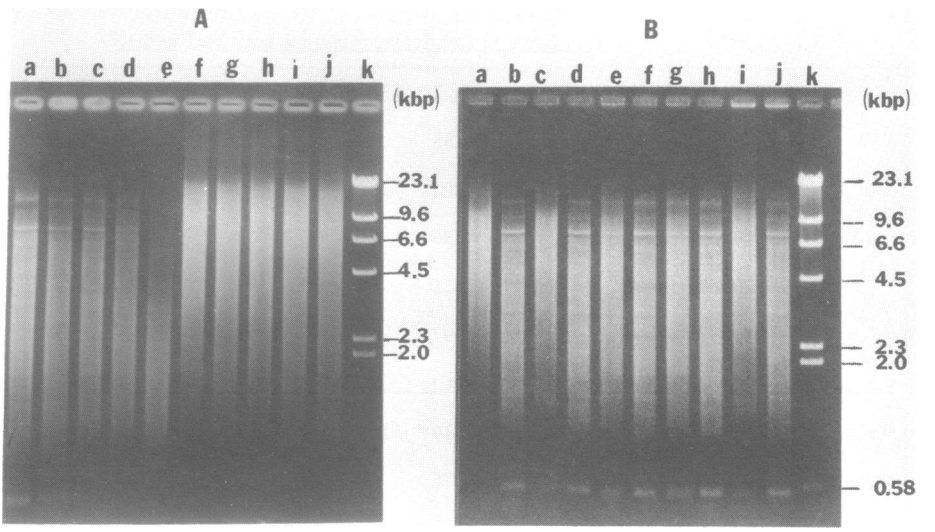

Fig. #6. Lambda exonuclease is effective in abolishing the ability of Mac DNA to recohere, only if treatment occurs when the Mac DNA is dispersed. Panel A, lanes (a-e) shows the results of treating DIS Mac DNA with 0, 0.25, 0.50, 1.0 and 2.0 U/ug DNA of lambda exonuclease at 30° for 30 min. Lanes (f-j) shows the results the same treatment of the cohered form. As can be seen, the DIS form is sensitive to the nuclease, while the cohered form is not. Panel B shows the ability or failure to recohere after treatment with 0.25 U/ug of lambda exonuclease (same as that used in lane b, panel A). Lanes c and d were produced as follows: COH DNA was treated with nuclease at 30° for 30 min. After deproteinization and heating to disperse the DNA, the sample was subjected to a standard coherence test. This sample was run in lane c. Lane d contains the same sample as lane c after a dispersive heat treatment. The sample in lanes a and b received no enzyme. Lanes e-j are identical experiments performed on DIS DNA. The samples in e and f were exposed for 6 min and those in g and h for 11 min. The sample in lanes i and j received no enzyme. The coherence displayed in lane c, and the sharp reduction in coherence seen in lanes e or g means that the DIS form is very sensitive to lambda exonuclease.

carries a 5' phosphate. In contrast, COH appears quite resistant.

Fig. 6B shows the results of treating COH and DIS with minimal amounts of lambda exonuclease and then testing the sample for its ability to cohere. Before running, each sample was divided in two; one of which received a dispersing heat treatment. This experiment shows that a low level of nuclease will not damage the ability to recohere, if the COH form is treated, but will do so if the DIS form is treated. This means that the $5'C_4A_4$ sequences are essential for the formation of COH. The observation that the COH form is not sensitive was expected, because this nuclease requires a molecular end in order to initiate hydrolysis (19).

T7 gene 3 endonuclease cuts single-stranded DNA or X or Y-shaped double-helical DNA at the branch point in the transition region between paired and unpaired nucleotides (20). COH and DIS was exposed to increasing levels of this enzyme, then heated to form DIS, then

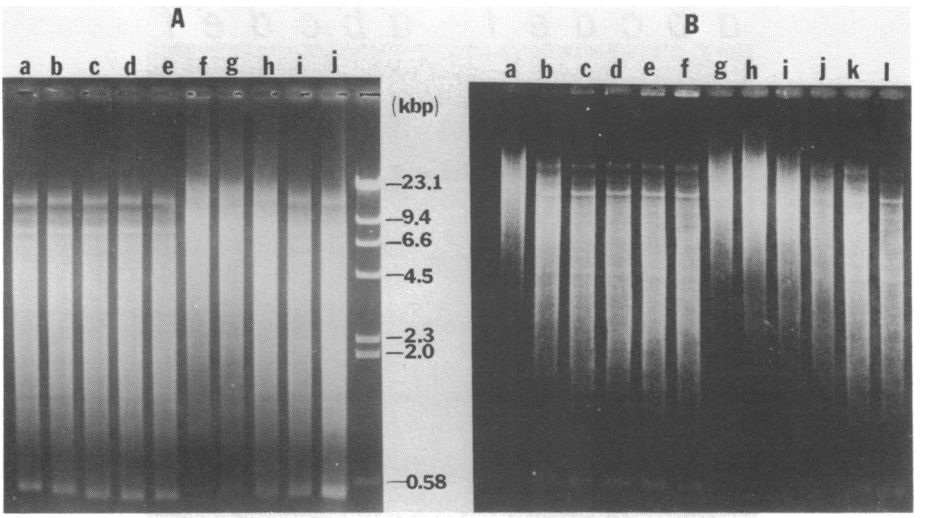

Fig. #7. The treatment of DIS and COH Mac DNA with S1 nuclease abolished its ability to subsequently cohere. Panel A lanes (a-e) shows DIS Mac DNA treated with 0, 0.10, 1.0, 3.0 and 5.0 U/ug DNA of S1 nuclease at 30^0 for 30 min. Lanes (f-j) shows COH Mac DNA treated with the same amounts of S1 under identical conditions. The DNA concentration in all cases was about 10 ug/ml. The normal S1 buffer contains 50 mM NaCl along with the 30 mM NaAcetate, pH 4.6 and 1 mM $ZnSO_4$. However, in these experiments, the NaCl was increased to 270 mM NaCl to prevent the dispersion of COH Mac DNA. The S1 is active, because the 540 bp species can be seen to increase in mobility. An aliquot of each of these reactions was then deproteinized, heated to disperse any COH forms and allowed to cohere under standard conditions then analysed by electrophoresis. The results are shown in Panel B. Lanes (a-e) correspond to the lanes with the same letter in Panel A (lane f is an additional sample that was treated with 10 U/ug). Lanes (g-k) correspond with lanes (f-j) in Panel A (lane k is an additional sample that was treated with 10 U/ug) The last lane l is a DIS control.

tested for its ability to recohere. Both COH and DIS lost the ability to recohere at about the same levels of nuclease. This is consistent with the idea that an unpaired region is present in or near the COH site.

S1 nuclease in known to cleave single chained regions and mismatched regions of sufficient length in the DNA duplex (21). The left hand side of Fig. 7A shows the effect of S1 nuclease on DIS Mac DNA. We can be sure that the enzyme is active because the 540 bp species is seen to increase in mobility as the concentration of enzyme increases. The right hand side of Fig. 7A shows the effect of the same levels of nuclease on the COH form. COH is very resistant to S1, requiring more than 30-times the concentration of nuclease to break it. When S1 breaks COH, it is dispersed into the same characteristic multiplicity of species as seen in untreated DIS, including the 540 bp species at the bottom of the gel. This suggests that the S1 is breaking at or near the cohering terminals.

Fig. 7B shows the results of a recoherence test performed on samples of the reactions

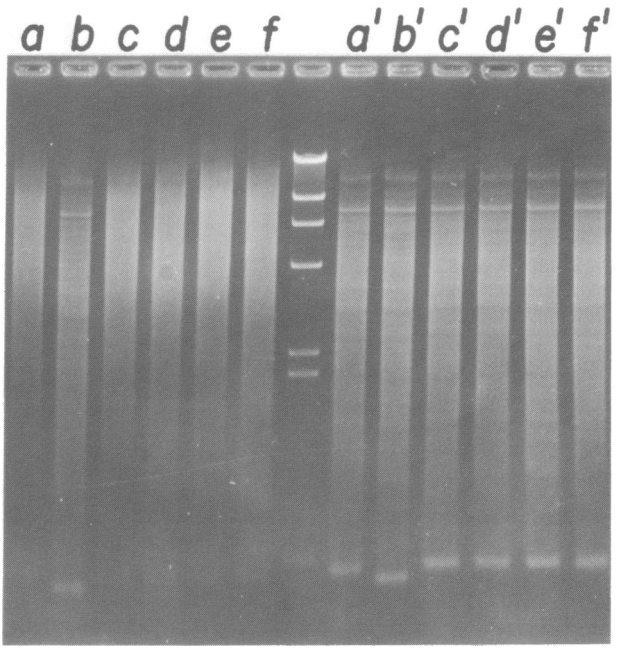

Fig. #8. The action of T4 polymerase on DIS and COH Mac DNA.
A total of six samples were constructed containing COH Mac DNA. The first two (a and b) were heated to produce DIS; the others were not. Samples b, d and f received T4 polymerase, the others received buffer. All tubes received dTTP and dGTP, and tubes e and f received dCTP and dATP as well. After incubation at 37^{o} for 60 min (tubes e and f for 15 min), the DNA was precipitated, deproteinized, dried, resuspended in 2x coherence buffer and subjected to a standard coherence test. The results are shown in lanes a-f. All samples cohered except that in lane b. The six samples shown to the right of the lambda/HindIII markers (a'-f') are the same as those shown on the left, except that each has been heated to 70^{o} for 10 min to disperse any cohered forms that may be present. Here again only lane b' shows any evidence of exonuclease action: the 540 bp species is migrating faster than usual, indicating that the 3'-ended chains have been removed.

shown in Fig. 7A. That is, each sample was deproteinized, concentrated by ethanol precipitation, redissolved, heated and subjected to a standard coherence test. The results show that as little as 0.1 U/ug of S1 will damage the DIS DNA so that it cannot recohere. In the COH form, as much as 3 to 5 U/ug DNA is required to destroy the ability to cohere. This offers another line of support to the conclusion that more than 30-times the concentration of S1 is required to break the cohered form.

T4 Polymerase A highly specific way to remove the 3'ended G_4T_4 tails is to treat with T4 polymerase in the presence of dTTP and dGTP (see ref. 22). This effectively stops hydrolysis at the C_4A_4:G_4T_4 duplex blunt end. Fig. 8 shows the results of a recoherence test after treatment of DIS and COH in the presence of various combinations of nucleotide triphosphates. As can be seen, the COH form is very resistant to the exonucleolytic action of

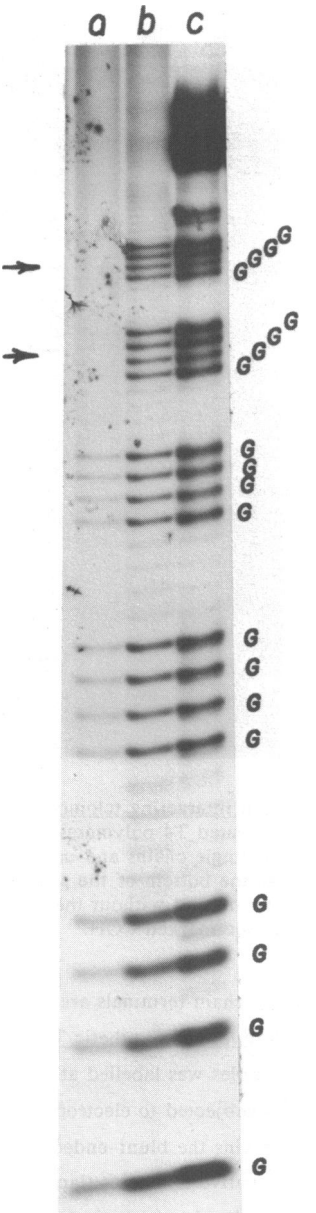

Fig. #9. Comparative Sequence Gels: G-reaction. Three samples of DNA were prepared: a) Mac DNA that had been treated with T4 polymerase in the presence of G and T triphosphates; b) DIS mac DNA; c) synthetic telomeric dimers. The 3' ends of these DNAs were labelled with ^{32}P cordycepin, then treated to cleave at G (using the Maxam-Gilbert reagents), and electrophoresed in adjacent lanes on a standard sequencing gel. The arrows point to the missing G_4 clusters.

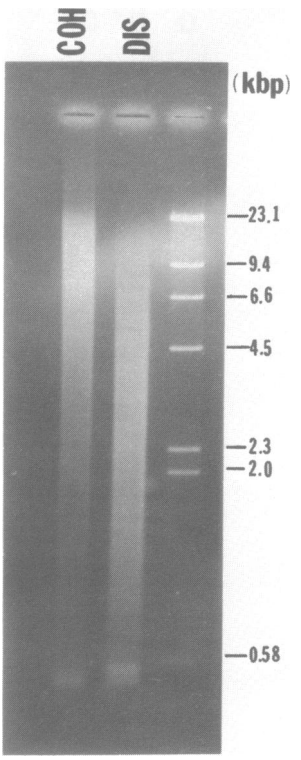

Fig. #10. For coherence to occur, both interacting telomeres must have a 3'ended terminal single chain. Purified 540 bp species was treated T4 polymerase in the presence of G and T triphosphates to remove the terminal single chains and mixed with DIS Mac DNA. A sample was run in the lane marked DIS. At the bottom of the gel, one may see two bands corresponding to the 540 bp species with and without their terminal single chains. After coherence, a sample was run in the lane marked COH.

T4 polymerase. However, the single-chain terminals are very sensitive in the DIS form.

Sequencing Gels of Blunt-ended, Native, and Synthetic Telomeric Dimers

Each of three different samples was labelled at the 3' end by ^{32}P-labelled cordycepin, treated to partially cleave at G, and subjected to electrophoresis on standard sequencing gels. As seen in Fig. 9, lane a, which contains the blunt-ended telomere sample, is missing two G_4 clusters as would be expected. The native telomeres (lane b) give a series of oligomeres that migrate together with those produced by the synthetic telomeres (lane c) except the synthetic telomeres have a final GG corresponding to the HaeIII site in the middle of the telomeric dimer. From these gels we conclude that: 1) the T4 polymerase stops rather precisely after removing the 3'$G_4T_4G_4T_4$ tails; 2) the synthetic sequence is the one expected; 3) it is very unlikely that either native G's or T's bear modification groups of significant size, otherwise the

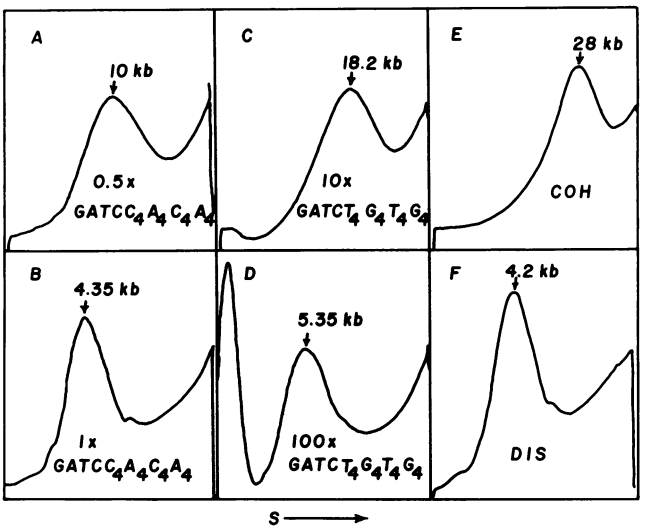

Fig. #11. Synthetic oligomers containing C_4A_4 or G_4T_4 can inhibit the formation of COH Mac DNA. Sucrose gradients were prepared and run and calibrated with respect to fragment length as described in Materials and Methods. The completely dispersed DNA is shown in panel (F) showing a peak at 4.2 kb. If allowed to cohere under standard conditions the COH form sediments more rapidly as shown in panel (E), corresponding to a length of 28 kb. If the DIS DNA is allowed to cohere in the presence of one molecule of $GATCC_4A_4C_4A_4$ for every two telomeres (0.5x), the cohered form has a reduced sedimentation rate corresponding to 10 kb, panel (A). At 1x, this value is reduced to 4.35 kb, panel (B). Coherence can be prevented by the addition of $GATCT_4G_4T_4G_4$, but the stoichiometric ratio must be increased to 10x, panel (C), for a visible effect, and 100x for a near-complete competition, panel (D).

mobility of these oligomeres would not correspond to those derived from the synthetic telomeres.

Are Both Tails Necessary?

Are the single-chain tails required on both participating telomeres in order for coherence to occur? This was tested by preparing some "blunt-ended" 540 bp species by treating with T4 polymerase in the presence of dGTP and dTTP. The resulting product migrated somewhat faster than the normal 540 bp species. When added to DIS Mac DNA, it can be seen as an extra band at the bottom of the gel (Fig. 10). When this mixture is subjected to a standard coherence test, the normal 540 species is incorporated into the COH form, while the 540 species lacking the single chains is left behind. Thus, both telomeres must have their single chain tails for coherence to take place.

Inhibition of Coherence

The standard coherence test was performed in the presence of a variety of competing DNAs. These were: 1) synthetic oligomers that were complementary to or identical with the $3'G_4T_4G_4T_4$ single chain tails; 2) "short telomeres" and their "blunt-ended" variants described

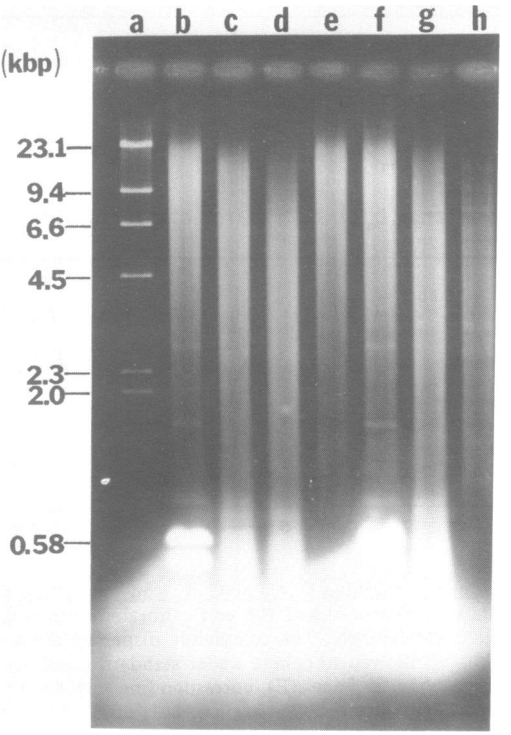

Fig. #12. The sequence-specific inhibition of coherence by native telomeres and blunt-ended native telomeres. Competing short telomeres were prepared as described in the text and added to DIS Mac DNA. After a standard coherence test, samples were run on 1% agarose gels. Lane a: lambda/Hind III markers. Lane b: 10x (wgt/wgt) pUC9/Hae III; COH forms normally. Lane c: 10x blunt-ended telomers; some (<50%) inhibition of coherence is evident. Lane d: 5x native telomeres; near total inhibition of coherence is observed. Lane e: COH Mac prepared with no added inhibitors. Lane f: 20x pUC9 DNA/Hae III; showing normal coherence. Lane g: 20x blunt-ended telomeres; again inhibition of coherence is observed, perhaps more pronounced (60-70% inhibition) than seen in lane c, but not as complete as seen in Lane h: which shows DIS Mac DNA containing no inhibitors.

below; 3) synthetic telomeres; and their "blunted-ended" variants.

Synthetic Oligomeres We were curious to know if the coherence could be blocked by synthetic oligomeres that were complementary to the 3'$G_4T_4G_4T_4$ tails. Accordingly, the synthetic oligomer 5'GATC$C_4A_4C_4A_4$ was mixed with DIS Mac DNA in varying proportions and subjected to a standard coherence test. Although gels were run, we show instead in Fig. 11 the UV absorbance profiles of sucrose gradients that were pressed upward through the photometer. Here it can be seen that as little as 0.5x (0.5 moles of oligomer for each mole of telomere) of the C_4A_4-containing oligomer resulted in a substantial reduction of the sedimentation rate of the cohered material. At 1.0x the inhibition of coherence was almost

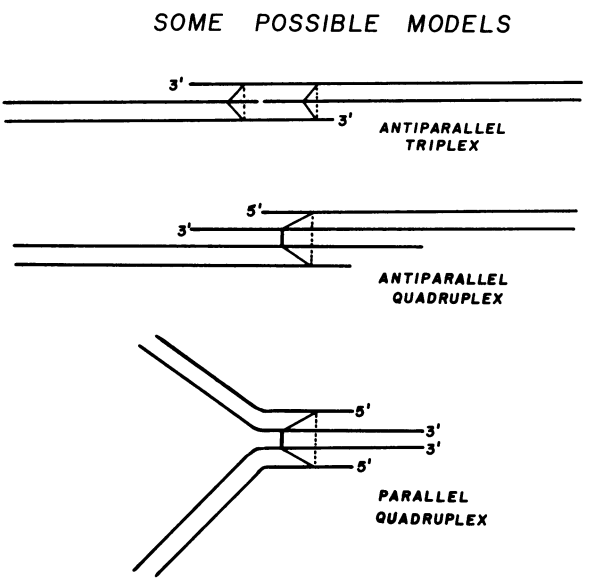

Fig. #13 Some Possible Models Consistent with Observations.
The first two structures show an antiparallel association of duplex telomeres to form a triplex and a quadruplex structure. The third shows a parallel association of identical telomeric sequences to form a quadruplex structure. These drawings are highly schematic and nothing about the detailed structure is implied here.

complete. Thus, this single-chain tail must be free, and not united with a complementary sequence in order for coherence. Other experiments show that once formed, the addition of excess C_4A_4-containing oligomer will not disperse the cohered form.

The synthetic oligomer having a sequence identical to that in the single chain tail is very ineffective in preventing coherence. As shown in Fig. 11, it is necessary to include 10x of 5'GATCT$_4$G$_4$T$_4$G$_4$ in order to cause a visible reduction of sedimentation rate, and a 100x excess to obtain substantial inhibition. Even though a 100x excess is needed to effectively block coherence, we are confident that this is a sequence-specific effect because a 150x excess of an oligomer of unrelated sequence had no effect on coherence; neither did comparable levels of poly U or poly UG.

Competing Telomeres In order to observe coherence in the presence of competing telomeres, it is necessary to remove them from the high molecular weight region of the gel. This is accomplished by treating Mac DNA with the multicutting nucleases HaeIII, AluI, and RsaI so that the average fragment size is 150 to 600 bp and moves to the bottom of the gel. Blunt-ended competing telomeres were produced in the same way, but starting with Mac DNA that had been treated with T4 polymerase plus G and T triphosphates under conditions that favor a complete reaction - ie. complete elimination of the ability to cohere, missing G clusters

(Fig. 9) and the faster form of the 540 bp species (Fig.7,8,10). Under these circumstances, one would predict that none of the tails survive. However, none of these experiments could rule out 5% of the telomeres still bearing tails.

The unseparated mixture of restriction segments was added at various levels to DIS Mac DNA which was then subjected to a standard coherence test. It was found that a 5x molar ratio of "competing native telomeres" was necessary in order to achieve nearly complete inhibition of coherence. If blunt-ended telomeres were used, then a 20x excess was required to achieve a 60-70% inhibition. In contrast, unrelated blunt-ended restriction segments, from plasmid DNA for example, had no effect at any level in blocking coherence. Some of these results are shown in Fig. 12. This competition by blunt-ended telomeres is considered to be a real effect. If this competition were to be ascribed to a fraction of telomeres that retain tails, then this fraction would have to be 25%.

Similar experiments were performed with synthetic telomeric dimers and their blunt-ended variants that had been cleaved with HaeIII. The synthetic telomeres were effective as competitors but a two-fold higher concentration excess was required to achieve the same level of inhibition. Thus, a 10x and 40x excess were required for the tailed and blunt-ended variants. This discrepancy may be related to the fact that the synthetic telomeres are completely without "interior" sequences that may be needed for stabilization.

DISCUSSION AND MODELS

We have reexamined the aggregation of concentrated solutions of Oxytricha macronuclear DNA first reported by Lipps (5,6). Our results indicate that aggregation is not the result of proteins or other separable cofactors. Nor is it an artifact of gel electrophoresis, since viscosity and sedimentation experiments confirm this DNA:DNA association. Gel and hydrodynamic measurements show that aggregation is by a coherence that occurs at or near the terminals of the DNA molecules. Studies with lambda exonuclease, S1 nuclease, and T4 polymerase (acting as an exonuclease) show that both the 3' and 5'-ended strands are necessary for the formation of cohered terminals, and once cohered, these terminals are strongly protected from the action of these nucleases. Thus, it is very likely that these nucleotides actually form part of the cohered structure.

The cationic requirements for the formation of the cohered form are very strict: of seven cations tested, only Na^+ would allow coherence. Once formed, it was discovered that the stability of the cohered form was increased by 25^o or more in the presence of K^+. None of six other cations would do so. The only other known example of cation specificity of this type is seen with the quadruplex poly I (23). In this case K^+, Rb^+, Cs^+ and NH_4^+ (24) all confer 25^o or more increase in melting temperature. In our case, the specificity for increased stability is more stringent since NH_4^+ and Cs^+ showed no effect (Rb^+ was not tested). We propose that K^+ is coordinated by the bases thereby confering additional thermal stability to the COH structure.

The Na^+-specificity for formation and the K^+-specificity for stability allows one to conclude directly that the cohering structure is not mainly based upon the Watson-Crick double helix. The familiar double helix reforms in the presence of any cation and no special cation confers added stability. Miles has interpreted the stabilization of poly I by specific cations as being the result of a specific chelation of the cation between the planes of the bases, in such a way as to have favorable coordination distances with oxygen atoms that lie within the plane of the bases (23). Cation coordination structures of this type have been reviewed by Truter (25).

In the present case, there are only two ways the terminals can unite (Fig. 13): 1) in an antiparallel condition, or 2) in a parallel condition (favored by Lipps, see ref. 5). In either case, three or four chains could actually be involved in the cohering structure. A quadruplex model would provide more coordination ligands for K^+, but none of these possibilities can be ruled out at present. The antiparallel models are appealing because the G_4T_4 single chains might find a home by associating with the double helix to form a triplex, but detailed models are difficult to construct. The parallel model leaves the G_4T_4 chains to unite with themselves in a parallel condition, while the quadruplex can assume the structure first proposed by McGavin (26) and studied by others subsequently (27,28).

The telomeric sequences contain clusters of A_4, which are known to bend the double helix (29). Because they are spaced at intervals of something less than one turn of the double helix, the two bends must produce a helical twist of the double helix. We speculate that this helical twist permits the fusion of two double helices to form the 4-stranded structure.

We expect that the contrasting parallel or antiparallel models can be distinguished by protein film electron microscopy. Restriction segments bearing cohered telomeres should display a sharp kink at their centers corresponding to the parallel association of two double helices. Lipps sought to visualize such kinks in electron micrographs of cohered Mac DNA (5). Unfortunately, such kinks, if present at all, could not be distinguished from randomly located artifacts, so no firm conclusion can be drawn from this work.

If either of these structures is the case, it should be possible to produce synthetically long telomeres of the required sequence, and to form substantial quantities of COH that will be required for structural and stability studies. This material could also serve as an immunogen for the preparation of antibodies that might permit a search COH structures in a variety of cells.

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