Volume 15 Number 15 1987 Nucleic Acids Research

S1 nuclease sensitivity of a double-stranded telomeric DNA sequence

Marcia Budarf* and Elizabeth Blackburn

Department of Molecular Biology, University of California, Berkeley, CA 94720, USA

Received March 24, 1987; Revised and Accepted July 10, 1987

ABSTRACT We examined structural properties of poly $d(C_4A_2)\cdot d(T_2G_4)$, the telomeric DNA sequence of the ciliated protozoan Tetrahymena. Under conditions of high negative supercoiling, poly $d(C_4A_2)\cdot d(T_2G_4)$ inserted in a circular plasmid vector was preferentially sensitive to digestion with S1 nuclease. Only the C_4A_2 strand was sensitive to first-strand S1 cutting, with a markedly skewed pattern of hypersensitive sites in tracts of either 46 or 7 tandem repeats. Linear poly $d(C_4A_2)\cdot(T_2G_4)$ showed no preferential S1 sensitivity, no circular dichroism spectra indicative of a Z-DNA conformation, no unusual Tm, and no unusual migration in polyacrylamide gel electrophoresis. The S1 nuclease sensitivity properties are consistent with a model proposed previously for supercoiled poly d(CT)·d(AG) (Pulleyblank et al., Cell 42:271-280, 1985), consisting of a double-stranded, protonated, right-handed underwound helix. We propose that this structure is shared by related telomeric sequences and may play a role in their biological recognition.

INTRODUCTION

The specialized biological properties of telomeres, the ends of eukaryotic chromosomes, imply that telomeric DNA sequences share recognizable structural features (reviewed in 1, 2). First, all eukaryotes examined to date have a similar type of telomeric sequence: tandemly repeated short G+C-rich sequence units, with an absolute or very strong strand bias of G versus C residues (3; reviewed in 1, 2). For example, the telomeres of the macronuclear DNA molecules of the ciliated protozoan Tetrahymena each consist of at least 50 tandem repeats of the sequence CCCCAA·TTGGGG, abbreviated here as poly $d(C_4A_2)\cdot d(T_2G_4)$, with the G_4T_2 strand oriented 5' to 3' toward the end of the DNA molecule (4, 5). Second, like another ciliate, Oxytricha, whose telomeres consist of short poly $d(C_4A_4)\cdot d(T_4G_4)$ stretches (6, 7), the macronuclear telomeric DNA of Tetrahymena is packaged into non-nucleosomal complexes (8, 9, 10). Third, a telomere-specific terminal transferase-like activity has been identified and characterized in Tetrahymena cell free extracts (11). This novel enzymic activity adds telomeric GGGGTT repeats, by de novo synthesis, to extend a DNA primer consisting of the G-rich

strand of a telomeric sequence, and it is thought to be important for the complete replication of telomeres (11). Furthermore, telomeric sequences not only of Tetrahymena but also of other eukaryotes such as yeast are specifically recognized and act as efficient primers for this activity (11, 12). Finally, telomeric DNA sequences of ciliated protozoa are able to specify telomere function when they are introduced on purified linear molecules into yeast cells (13, 14), although the telomeric sequence of yeast, poly $d(C_{1-3}A)\cdot d(TG_{1-3})$ (15), is different from the telomeric sequences of ciliates.

The ability of nonhomologous telomeric sequences to be recognized in heterologous systems strongly suggests that these simple DNA sequences have characteristic structural properties. Conformations of double-stranded DNA which differ from the normal B-helix have been reported for several homocopolymeric sequences which are similar though not identical to those of telomeres -i.e. G+C-rich sequences with a strong strand bias of G and C residues (16-25). However, the biological roles of many of these sequences (16-22) are not well understood. We therefore wished to determine whether telomeric DNA, which has a known biological function, has any distinguishing structural properties.

As a first step toward understanding the basis for biological recognition of telomeric DNA, we examined properties of the cloned telomeric DNA sequence of the ciliate Tetrahymena. Several different criteria were tested to determine whether poly $d(C_4A_2)\cdot d(T_2G_4)$ had any structural or conformational properties different from those of normal B-helix DNA.

In this paper we report that poly $d(C_4A_2)\cdot d(T_2G_4)$ inserted into a supercoiled plasmid is hypersensitive to cleavage by S1 nuclease. A strand-specific, markedly skewed pattern of S1 nuclease-sensitive sites was observed. Our findings suggest that this telomeric DNA sequence can assume a distinguishable, probably double-stranded structure, unlikely to result from strand slippage, in torsionally stressed DNA. In contrast, poly $d(C_4A_2)\cdot d(T_2G_4)$ duplex DNA in linear form showed no unusual properties, by the criteria of S1 nuclease sensitivity, melting temperature, circular dichroism and electrophoretic mobility in polyacrylamide gels.

MATERIALS AND METHODS

Construction of plasmids containing inserts of poly $d(C_4A_2)\cdot d(T_2G_4)$

Plasmid pCA2 was constructed from a parent plasmid, pTrel, which contains ~4 kb of the telomeric region of the palindromic rDNA of Tetrahymena

thermophila, including 56 terminally located tandem C_4A_2 repeats, inserted between the BamHI and PvuII sites of pBR322 (26). Details of the cloning and sequence analysis of pTrel have been described elsewhere (26). To subclone the poly $d(C_4A_2)\cdot d(T_2G_4)$ region, pTrel was digested with DdeI, which cuts the rDNA telomere-associated region at a position 150 bp away from the C_4A_2 repeats, and pBR322 at position 2286, 217 bp away from the end of the C_4A_2 repeats in the rDNA insert. The C_4A_2-containing DdeI fragment was digested with Bal31 nuclease (New England Biolabs), removing ~150 bp from each end, then with HhaI to remove all but 10 bp of the pBR322 sequence adjoining the C_4A_2 repeats. After S1 nuclease treatment to produce blunt ends, BamHI linkers were ligated to the fragment and it was inserted into the BamHI site of pBR322 to produce the recombinant plasmid pCA2 (Fig. 1a).

Plasmid pCA3 was constructed by subcloning C_4A_2 repeats and the neighboring rDNA region from another parent plasmid, p11b1 (27), which contains a telomeric region of an 11 kb linear rDNA species found transiently in newly formed macronuclei of Tetrahymena (28). We used a spontaneous deletion of plasmid p11b1 containing 7 instead of the original 40 terminally-located C_4A_2 repeats, joined to ~4 kb of unrearranged T.thermophila rDNA (12, 27). This rDNA insert of p11b1 is between the BamHI and PvuII sites of pBR322, with the poly $d(C_4A_2)\cdot d(T_2G_4)$ in the same orientation relative to the vector as in pTrel. Digestion of p11b1 with ClaI removed ~2 kb of rDNA sequence and 350 bp of the flanking pBR322 sequence. The fragment containing the remaining pBR322 vector and 7 C_4A_2 repeats joined to ~2 kb of telomere-associated rDNA was recircularized to form pCA3 (Fig. 1a).

DNA Samples

Plasmids were prepared by the modified alkaline method (29) or by the cleared lysate-Triton X100 procedure (30), as noted in the Results. The plasmids were further purified by cesium chloride-ethidium bromide equilibrium centrifugation.

DNA fragments from restriction digests were separated by agarose gel electrophoresis and purified by the glass bead method of Vogelstein and Gillespie (31).

Enzyme Digestions

S1 nuclease digestions were performed in 30 mM sodium acetate, 50 mM sodium chloride, 1 mM $ZnCl_2$, pH 4.6 with 50 µg DNA/ml and 50 units/ml of S1 nuclease (purchased from Bethesda Research Laboratories) at 30°C for 20 hrs, unless otherwise indicated. Samples were then either run immediately on agarose gels or phenol extracted, ethanol precipitated and resuspended in 10

mM Tris·HCl, 1 mM EDTA, pH 7.5. Restriction digests were performed according to the directions of the manufacturer (New England Biolabs).

Agarose Gel Electrophoresis, Southern Blotting and Hybridization

S1 treated and control samples were analyzed by agarose gel electrophoresis, using Tris-acetate buffer, followed by bidirectional transfer of DNA to nitrocellulose filters (32). Hybridization of filters was as described by Botchan et al (33). ^{32}P-labeling of hybridization probes was according to the procedure of Maniatis et al (34).

End Labeling of DNA

Supercoiled and relaxed circles from S1 treated and control samples were separated by agarose gel electrophoresis. Supercoiled and relaxed circles were isolated (31), digested with the appropriate restriction enzyme and treated with bacterial alkaline phosphatase (Worthington). Restriction fragments were separated by agarose gel electrophoresis, and the appropriate fragments were isolated (31) and 5' end labeled using polynucleotide kinase (P.-L. Biochemicals) and carrier free [γ^{32}P]-ATP. [γ^{32}P]-ATP was prepared according to Schendel and Wells (35). ^{32}P-labelled restriction fragments were secondarily cut with the desired restriction enzyme and fragments were again separated by agarose gel electrophoresis. These isolated fragments were denatured by boiling in 80% formamide and analyzed on high resolution denaturing polyacrylamide gels (7 M urea-6% polyacrylamide) (36). Markers were prepared by performing Maxam and Gilbert sequencing reactions (36) on the equivalently [^{32}P] end-labeled fragments.

Spectroscopic Analysis of Poly d(C_4A_2)·d(T_2G_4)

Melting point determinations were carried out on the pCA2 BamHI fragment containing poly d(C_4A_2)·d(T_2G_4) (see Figure 1a and b). The DNA was in 0.2 M NaCl, 0.1 mM EDTA, 10 mM sodium phosphate pH 7.0, and its melting profile was determined using a Gilford 250 spectrophotomer with temperature programmer. Circular dichroism spectra were determined on the same BamHI fragment in the same buffer, or in 10 mM Tris HCl, pH 7.0, 1 mM EDTA buffer, containing NaCl concentrations of 0.2 M, 1.0 M, 2.0 M, 3.0 M or 4.0 M, using a Carey Model 60 spectropolarimeter equipped with a Model 6001 CD attachment.

RESULTS

Sequence Analysis of pCA2 and pCA3

The inserts containing poly d(C_4A_2)·d(T_2G_4) in plasmids pCA2 and pCA3 were sequenced. Figure 1b shows the sequence of the BamHI fragment insert in pCA2. Figure 1c shows the sequence of the 7 C_4A_2 repeats and their immediately flanking rDNA and pBR322 vector sequences in pCA3.

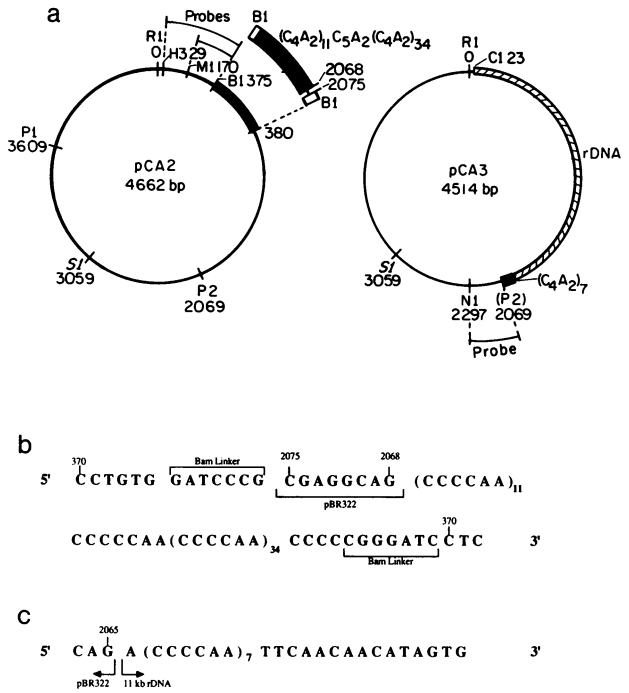


Figure 1. Maps and Sequences of pCA2 and pCA3.

a. Plasmids pCA2 and pCA3 were constructed as described in Materials and Methods. Thin line = pBR322 vector DNA; filled bar = C_4A_2 repeats; open rectangles = BamHl linkers; hatched bar = T.thermophila 11 kb rDNA. Restriction enzyme sites used in this work: Rl = EcoRl; H3 = HindIII; Bl = BamHI; P2 = PvuII (site destroyed in plasmid construction in pCA3); Pl = Pstl; Nl = NdeI; Ml = MspI; Sl = major Sl nuclease sensitive site of pBR322 (37). Numbers next to marked restriction sites refer to positions in the pBR322 vector. Fragments used as hybridization probes in Figs. 2, 3 and 4 are shown on the maps of pCA2 and pCA3 respectively.

b. DNA sequence of the insert (between Bam linkers) containing poly $d(C_4A_2)\cdot d(T_2G_4)$ in pCA2. The numbers refer to positions in the pBR322 vector.

c. DNA sequence of the end of the insert containing $(C_4A_2)_7$ and neighboring 11 kb rDNA sequence in pCA3.

In addition to 45 C_4A_2 repeats, pCA2 contained a single C_5A_2 sequence, within the otherwise homogeneous tandem C_4A_2 repeats (Figure 1b). The same C_5A_2 sequence was also present in the pTrel parent plasmid. It is not known whether this variant repeat unit was present in the rDNA or was a cloning artifact. We note that of the 11 different plasmids containing poly $d(C_4A_2)\cdot d(T_2G_4)$ from Tetrahymena or Glaucoma (26, 27) telomeres which have been cloned and sequenced in this laboratory, this single C_5A_2 is the only

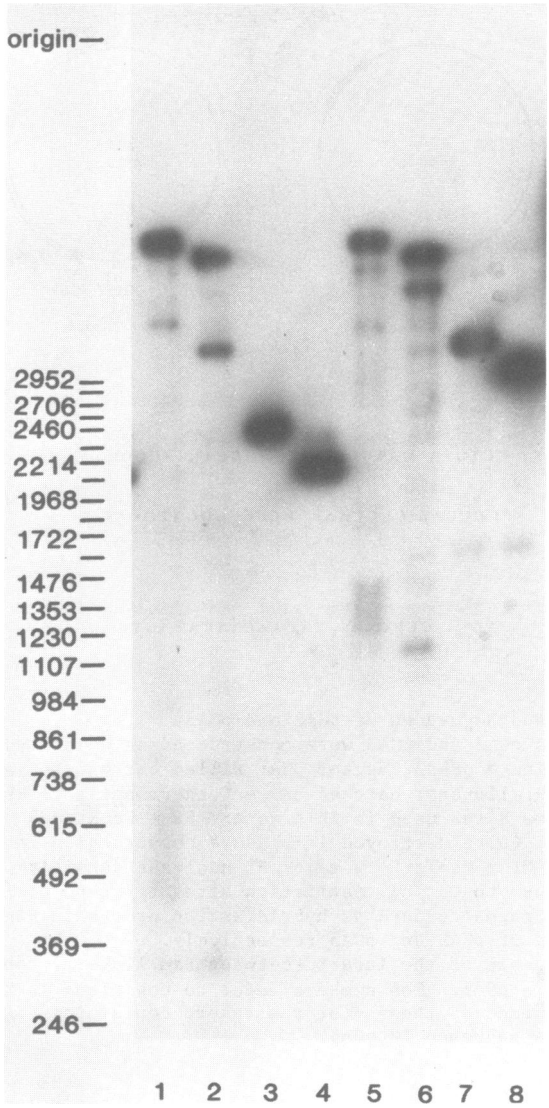


Figure 2. Double-stranded S1 Nuclease Digestion of pCA2.
Autoradiogram of products of S1 nuclease digestion of pCA2 (lanes 1, 3, 5 and 7) and the parent vector pBR322 (lanes 2, 4, 6 and 8). Supercoiled plasmids (lanes 1, 2, 5, and 6), or plasmids first linearized with PvuII (lanes 3, 4, 7 and 8) were digested with S1 nuclease as described in Materials and Methods, following the conditions of Lilley (37), and subsequently with EcoRI (lanes 1-4) or PstI (lanes 5-8). The hybridization probe of the Southern blotted fragments was the nick-translated HindIII-BamHI fragment of pBR322 described in Fig. 1a and in the Materials and Methods. Markers (shown in bp on left) were a 123-bp multiple ladder.

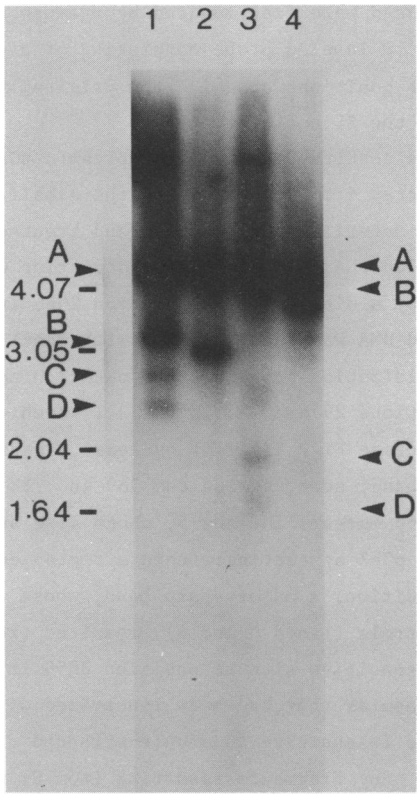


Figure 3. Autoradiogram of Products of Double-Stranded S1 Nuclease Digestion of pCA3

Supercoiled plasmids pCA3 (lanes 1 and 3) and pBR322 (lanes 2 and 4) were digested with S1 nuclease as described in Materials and Methods and subsequently with ClaI (lanes 1 and 2) or PstI (lanes 3 and 4). The hybridization probe was the nick-translated NdeI-PvuI fragment of pBR322 described in Fig. 1a and in the Materials and Methods. A-D (arrowheads) indicate bands in lanes 1 and 3. Band A is the linearized pCA3; bands B-D are described in the text.

variant out of a total of ~300 repeat units analyzed in these plasmids (15, 26, 27).

Sensitivity of Double Stranded Poly $d(C_4A_2)\cdot d(T_2G_4)$ to S1 Nuclease

We first determined the approximate locations of S1 cleavage sites in plasmids pCA2 and pCA3, and in the vector plasmid pBR322 as a control. Each plasmid was treated with S1 nuclease as described in Materials and Methods, using digestion conditions selected to linearize the plasmid. The S1 nuclease treated DNA was then digested with a suitable restriction enzyme, and the

plasmid fragments produced were fractionated by agarose gel electrophoresis, and hybridized with a ^{32}P labeled probe consisting of a pBR322 sequence. This allowed localization of positions at which the originally supercoiled plasmid had been linearized by the S1 nuclease.

Figure 2 shows the results obtained with plasmid pCA2. Purified pCA2 and pBR322 were each extracted from host cells by the alkaline plasmid preparation method as described in Materials and Methods, and treated with S1 nuclease either as the supercoiled DNA, or after prior digestion with PvuII to linearize the plasmid by a single cut at position 2066 in the pBR322 vector. The S1 nuclease treated DNA was then digested with EcoR1 (lanes 1-4) or PstI (lanes 5-8). The hybridization probe was the pBR322 HindIII-BamHI fragment extending between positions 29 and 375 in pBR322, which flanks the poly $d(C_4A_2)\cdot d(T_2G_4)$ insert (see Fig. 1a). S1 nuclease digestion of supercoiled pCA2 produced a broad band, seen between the 369 and 738 bp markers in lane 1, and the 1230 and 1476 bp markers in lane 5, whose size and width showed that S1 nuclease linearized pCA2 by cutting within a region encompassing the C_4A_2 repeat stretch. In addition, a major sharp band, whose counterpart is also seen in the pBR322 controls (lanes 2 and 6), resulted from linearization at the major S1 nuclease sensitive site at position 3050 in pBR322 (Fig. 1a) (37). In contrast, plasmids that had been linearized with PvuII prior to S1 nuclease digestion were insensitive to double-stranded cuts by this nuclease (lanes 3, 4, 7 and 8). Any fragments resulting from S1 nuclease sensitivity of the C_4A_2 repeats in the previously linearized pCA2 samples (lanes 3 and 7) would have had the same sizes as those in lanes 1 and 5.

A similar analysis was carried out on pCA3, which has only 7 tandem C_4A_2 repeats. Figure 3 shows the results of an experiment in which supercoiled pCA3 was treated with S1 nuclease under conditions which produced linearized pCA3 as a prominent product. The hybridization probe was the NdeI-PvuII fragment of pBR322 which extends between positions 2297 and 2069 in the pBR322 vector adjacent to the C_4A_2 repeats in the insert (Fig. 1a). The band marked D in lanes 1 and 3 of Fig. 3 corresponds to cutting of pCA3 in its C_4A_2 repeats. As was found for pCA2, the major S1 nuclease sensitive site in the pBR322 vector was also cut by S1 nuclease digestion of pCA3 producing band B. Band C results from cutting pCA3 within an A + T-rich sequence (26, 27) in the rDNA insert.

These results showed that as few as 7 tandem C_4A_2 repeats in supercoiled plasmids were sensitive to S1 nuclease. Under the conditions of these experiments, the previously characterized major and minor S1 sensitive sites

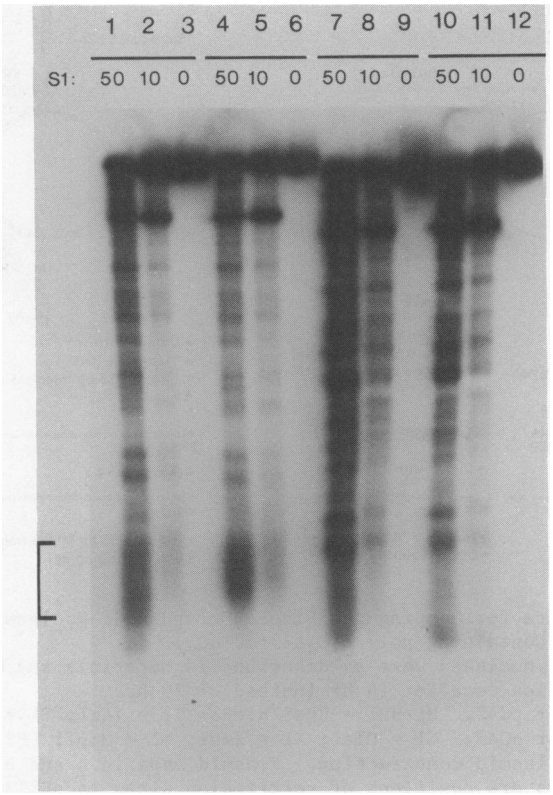


Figure 4. Double-Stranded S1 Nuclease Digestion of Plasmids Prepared by Alkaline or Lysis-Triton X-100 Procedures.

Autoradiogram of S1 nuclease products. Supercoiled plasmids pCA2 (lanes 1-6) and pBR322 (lanes 7-12) were purified from host E.coli cells by either the alkaline method of Birnboim and Doly (29) (lanes 4-6, 10-12) or a lysis-Triton X-100 method (30) (lanes 1-3, 7-9) , and digested with S1 nuclease at 50 or 10 µg/ml or no S1 nuclease, as indicated. The S1 treated DNA was subsequently digested with HindIII. The hybridization probe was the nick-translated MspI-BamHI fragment of pBR322 shown in Fig. 1a. Bands corresponding to the minor S1 nuclease sensitive sites of pBR322 (37) are visible. Bracket indicates broad band resulting from S1 cutting sites in poly $d(C_4A_2) \cdot d(T_2C_{14})$ in pCA2.

of supercoiled pBR322 (37) were also cut, and the major sensitive site remained the preferred site in plasmids containing C_4A_2 repeats.

To determine whether the sensitivity of C_4A_2 repeats to S1 nuclease was dependent on the method of preparation of the plasmid DNA, pCA2 was prepared by either an alkaline method (29) or a cleared lysate-Triton X100 method which does not involve exposure of the DNA to high pH (30). Plasmids prepared by

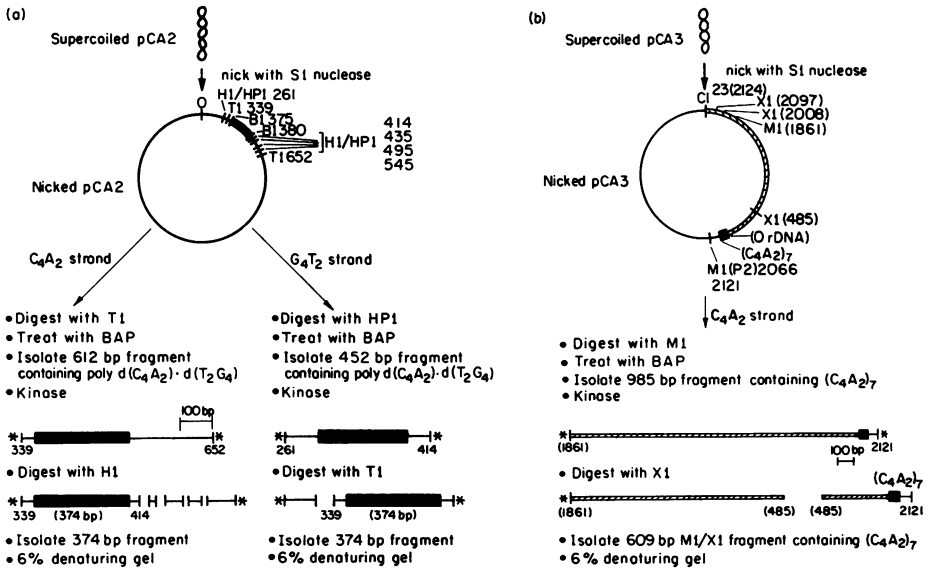


Figure 5. Procedure for Mapping the Single-Stranded S1 Nuclease Cleavage Sites in Plasmids Containing poly $d(C_4A_2)\cdot d(T_2G_4)$.
Digestions with S1 nuclease were as described in Materials and Methods, except that digestion were for 16 hr instead of 20 hr.

a. Scheme for pCA2. H1/HP1 = HhaI/HinP1; T1 = TaqI; B1 = BamHI.

b. Scheme for pCA3. C1 = ClaI; X1 = XmnI; M1 = MspI; (P2) = PvuII site destroyed during plasmid construction. Plasmid maps in a and b are drawn as in Fig 1a. Numbers are positions of restriction sites in pBR322 vector sequence. Numbers in parentheses are positions of restriction sites in the T.thermophila rDNA insert, numbered as in (26).

both methods were digested in parallel with S1 nuclease at two different concentrations, and analyzed as described above. As shown in Fig 4 for pCA2 and pBR322, the same patterns of S1 nuclease sensitive sites were found for each plasmid prepared by both methods. The autoradiogram shown in Fig. 4 was scanned densitometrically, to confirm that there were no significant differences between the two types of DNA preparations (data not shown). Thus, the sensitivity of C_4A_2 repeats in supercoiled plasmids is not detectably influenced by the conditions of plasmid isolation.

Fine Structure Mapping of the S1 Nuclease Sensitive Sites in Poly $d(C_4A_2)\cdot d(T_2G_4)$ in Supercoiled Plasmids

The gel-blotting-hybridization experiments shown in Figs 2-4 did not permit exact mapping of the S1 nuclease cleavage sites in the poly $d(C_4A_2)\cdot d(T_2G_4)$ sequence, nor any determination of the relative sensitivities of the two DNA strands in this region. For precise mapping of first-strand S1

sensitive sites, we chose S1 digestion conditions known to generate predominantly nicked circular plasmids from supercoiled substrates. The preferred site(s) of S1 attack of supercoiled pCA2 and pCA3 were assayed by the procedures outlined in Fig 5. The end-labelling procedure on restriction fragments generated after S1 nuclease digestion labelled only the restriction enzyme-cut ends, but did not label the S1 cleavage sites themselves. This was shown in control experiments, in which relaxed circles from S1 treated samples were first treated with phosphatase, end labeled using polynucleotide kinase, then digested with restriction enzymes. Samples treated in this manner had only very low levels of ^{32}P incorporation, and on sequencing gels showed no specific bands (see Figs. 6, 7 and 8). Thus, under the conditions used for end labeling there was no detectable incorporation of ^{32}P at the cuts made by S1 nuclease. Analysis of the end-labelled fragments on a sequencing gel with appropriate markers of the same sequence allowed us to determine precisely the phosphodiester bonds on each strand susceptible to S1 nuclease.

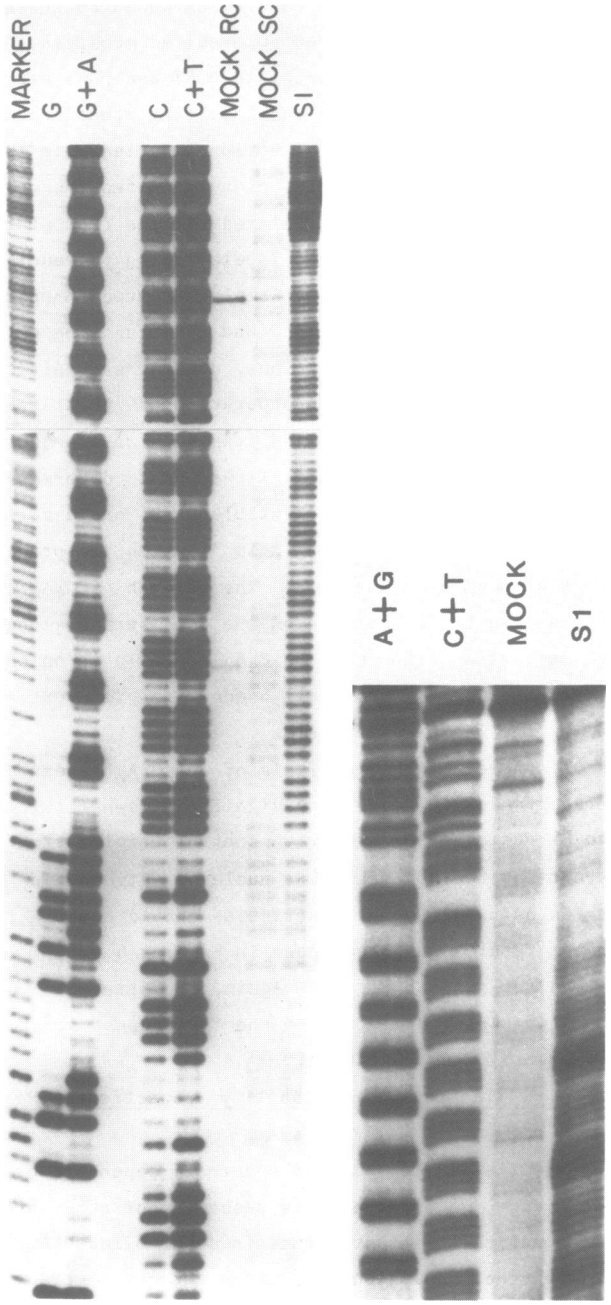
Figures 6 and 7 show DNA sequencing gels in which the products of S1 nuclease digestion of pCA2 were analyzed. The stretch of C_4A_2 repeats was preferentially sensitive to S1 nuclease on the C_4A_2 strand along its entire length, with the exception of the most 3' C_4A_2 repeat unit on this strand (Figure 6). Within the C_4A_2 repeats, all bonds were preferentially cleaved, the A-A bonds being the least susceptible.

In contrast to these results, analysis of the G_4A_2 repeat strand of pCA2 by a similar strategy did not reveal any significant sensitivity of the G_4T_2 repeat region to S1 nuclease compared with control samples treated identically, except for the absence of S1 nuclease (Figure 7). Two different labeling strategies using different restriction enzyme combinations (data not shown) gave similar results to those shown in Figures 6 and 7. A similar experiment is shown for pCA3 in Fig. 8. Again, the stretch of C_4A_2 repeats was sensitive to S1 nuclease digestion of the C_4A_2 repeat strand, with the exception of the most 3' C_4A_2 repeat unit.

Spectroscopic and Gel Electrophoretic Mobility Properties of Poly $d(C_4A_2)\cdot d(T_2G_4)$

Because poly $d(C_4A_2)\cdot d(T_2G_4)$ showed supercoil-dependent hypersensitivity to S1 nuclease cleavage, we examined this sequence for other properties that might indicate the nature of its structure in supercoiled DNA.

To determine whether a torsionally driven structural transition was detectable in pCA2, we analyzed the topoisomer profile of this plasmid, along with the parent vector pBR322 as a control, using the two-dimensional gel


MARKER
G
G+A
C
C+T
MOCK RC
MOCK SC
S1
A+G
C+T
MOCK
S1

electrophoresis system of Peck and Wang to separate topoisomers (38). Under the gel electrophoresis conditions used, i.e. pH 8.0 in the absence of Mg^{++} at 25° (38), no mobility shift was observed as a function of superhelical density in two dimensional gel electrophoresis of topoisomer series of pCA2 or pBR322 (data not shown).

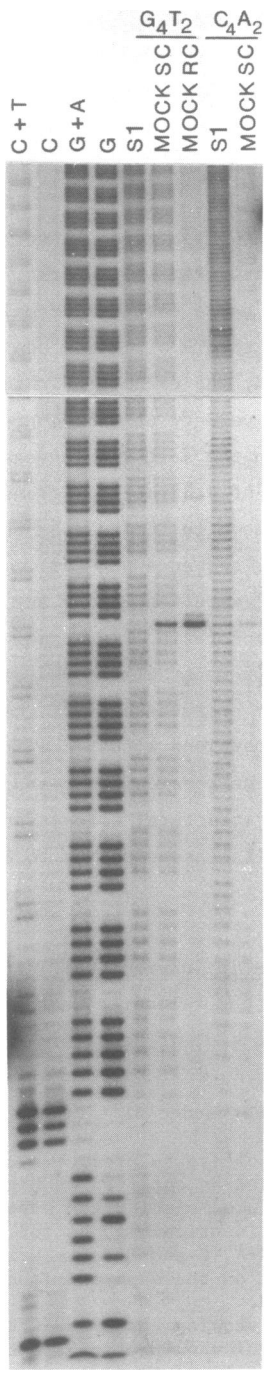
We also analyzed physical properties of the linear BamHI fragment of pCA2 containing the poly $d(C_4A_2)\cdot d(G_4T_2)$ region. Over 90% of this fragment consists of tandem C_4A_2 repeats (Fig 1b). First, the melting profile of this fragment was determined. It had a sharp major melting point transition, at the temperature expected for the Tm of linear DNA of the base composition of poly $d(C_4A_2)\cdot d(T_2G_4)$. Second, the circular dichroism spectra of the fragment were determined under a wide variety of NaCl concentrations (see Materials and Methods). However, even when the salt was increased to 4 M NaCl, no circular dichroism changes indicative of a structural transition were observed. The conditions used were those shown to induce a B- to Z-DNA transition in linear alternating purine-pyrimidine sequence DNA (39). Finally, various linear restriction fragments of pCA2 were tested for any aberrant mobility properties in polyacrylamide gel electrophoresis. The conditions of electrophoresis were those used by others to detect bent helical DNA structures (40). At 25° we observed no aberrant mobility properties of fragments containing poly $d(C_4A_2)\cdot d(T_2G_4)$ in various positions within the fragment. These findings suggest that, in the absence of torsional stress, linear poly $d(C_4A_2)\cdot d(T_2G_4)$ does not readily undergo any structural transition from a B DNA-like structure, as expected from its insensitivity to S1 nuclease.

DISCUSSION

Conformation of Telomeric DNA in Supercoiled Plasmids

We have shown here that the telomeric sequence of Tetrahymena, when inserted into a supercoiled plasmid, is sensitive to cleavage by S1 nuclease.

Figure 6. Mapping of Single-Stranded S1 Cleavage Sites on the C_4A_2 Strand of poly $d(C_4A_2)\cdot d(T_2G_4)$ in pCA2.
S1 = pCA2 digested with S1 nuclease to produce nicked circles, and the isolated nicked circles treated as outlined in Fig. 5a. Mock RC and SC are controls consisting of the nicked circles and supercoiled circles respectively, isolated after identical incubation but with no S1 nuclease and treated as for lane S1. G, G+A, C and C+T are Maxam-Gilbert sequencing reaction products carried out on the same end-labeled fragment used for the analysis of S1 cleavage sites.
Left panel: Autoradiogram showing the 5' end region of the C_4A_4 repeats.
Right panel: A different gel showing the 3' end region of the C_4A_2 repeats. MOCK = nicked circle control.


C + T
C
G + A
G
S1
MOCK SC
MOCK RC
S1
MOCK SC
G4T2
C4A2

Fine structure mapping of the first-strand S1 cleavage sites showed that only the C_4A_2 strand of poly $d(C_4A_2)\cdot d(T_2G_4)$ was cleaved above background levels. The cleavage sites showed a skewed distribution along the stretch of telomeric DNA sequence, the most 3' C_4A_2 repeat unit not being susceptible. The distribution of S1 nuclease sensitive bonds is summarized in Fig 9.

A number of homopolymeric or simple sequence DNAs can exist in S1 nuclease sensitive structures which are distinguishable from normal B-DNA, under both torsionally stressed or unstressed conditions. A structure consistent with our observations is a right-handed helical form less tightly wound than B-DNA (i.e., one with a higher number of base pairs per helical turn than B-DNA). Such a helical form has been proposed to account for the supercoil- and low pH-dependent sensitivity to S1 nuclease of poly d(CT)·d(GA) (20). This structure relies on protonated C residues forming Hoogsteen base-pairs with G residues, in syn conformation, on the complementary strand, and results in an S1 nuclease-sensitive double-stranded DNA form with an estimated 16 to 20 basepairs per helical turn (20). A similar right-handed helical structure was determined by X-ray crystallography for a short double-stranded DNA complexed with a peptide antibiotic (41).

The pattern of S1 nuclease sensitive sites in poly d(CT)·d(GA) in a supercoiled plasmid (20) was strikingly similar, in both its strand bias and 5' skewed distribution, to that we observe with poly $d(C_4A_2)\cdot d(T_2G_4)$. The low pH and high supercoil density conditions we used for S1 digestion of the Tetrahymena telomeric sequence are those shown to favor the protonated double-stranded form proposed for poly d(CT)·d(GA) (20). A superhelix density-dependent structural transition from B DNA to the protonated double-stranded form resulted in reduction of torsional strain, and S1 sensitivity of the sequence which had undergone the transition (20). High pH and absence of Mg^{++}, conditions used in our analysis of gel electrophoretic mobility shifts of supercoiled pCA2, were shown not to favor transition to this form (20), consistent with our failure to observe such a transition.

The pattern of S1 nuclease sensitivity we observe for the telomeric repeated sequence also has some features in common with those observed for

Figure 7. Mapping of S1 Nuclease Cleavage Sites on the T_2G_4 Strand of poly $d(C_4A_2)\cdot d(T_2G_4)$ in pCA2.
The plasmid pCA_2 was digested with S1 nuclease to produce nicked circles, and the isolated nicked circles treated as outlined in Fig. 5a. Lanes are marked as in Fig. 6.

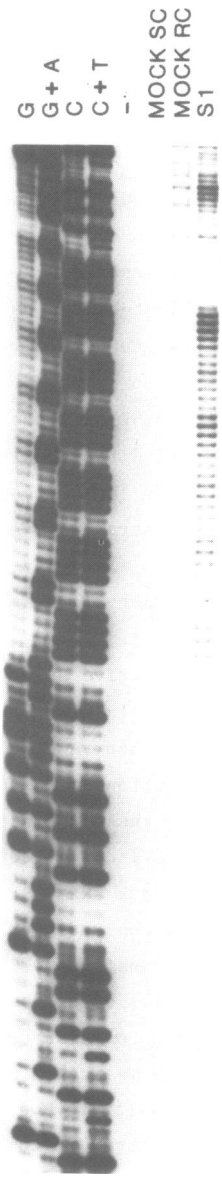


Figure 8. Mapping of Single-Stranded S1 Nuclease Cleavage Sites on the C_4A_2 Strand in the $(C_4A_2)_7 \cdot (T_2G_4)_7$ region of pCA3.
Plasmid pCA3 was incubated with or without S1 nuclease and subsequently treated as outlined in Fig. 5b. Lanes are marked as in Fig. 6. It is not known whether the cluster of S1 nuclease sensitive sites in the rDNA sequence on the 3' side of the $(C_4A_2)_7$ sequence (see Figure 1c) results from its proximity to the C_4A_2 repeats.

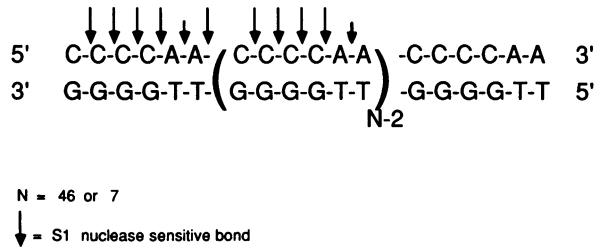


Figure 9. Summary of S1 Nuclease Nicking Patterns in the poly $d(C_4A_2)\cdot d(T_2G_4)$ of pCA2 and pCA3.
Length of arrows approximately indicates relative nicking frequency of the bonds indicated on the C_4A_2 repeat strand.

poly dC·dG or polypyrimidine.polypurine tracts in supercoiled plasmids. The S1 nuclease sensitivity of these sequences, as judged by linearization at the poly dC or poly pyrimidine tract, was dependent on the DNA being in a supercoiled state, with no sensitivity being seen in the relaxed circular or linear forms (21-24). In some reports (22, 24), only one strand, the poly-C or pyrimidine-rich strand, was found to be sensitive. A skewed distribution of S1 sensitive sites, strongest toward the 5' end of the poly-C sequence on that strand, was reported for a poly dG·poly dC tract (23). This strand bias and skewed distribution resemble those we observe for the telomeric sequence.

The pattern of S1 sensitive sites we find for the telomeric DNA cannot readily be explained by local slippage, which has been invoked to explain the S1 sensitivity of both strands of polypurine.polypyrimidine tracts in supercoiled DNA (19, 24, 25). The transitory local strand melting that would be required for strand slippage might be expected to produce sensitive sites on both strands, and such a slippage model does not easily account for the skewed arrangement of hypersensitive sites on the C_4A_2 strand. From calculations of the energy requirements for poly dG·dC and related sequences to melt in a supercoiled molecule, it was concluded that such denaturation would be energetically very unfavorable (42). Together, our observations and these calculations argue that the form of telomeric DNA in supercoiled molecules accounting for its S1 sensitivity does not involve extensive unpairing of bases.

Relevance of the S1 Nuclease-Sensitive DNA Conformation to Telomeric DNA Sequences In Vivo

Does the S1 nuclease sensitivity of telomeric DNA in supercoiled molecules result from a DNA conformation involved in telomere recognition in

vivo? The similarity of all known telomeric sequences to various G+C-rich polypurine.polypyrimidine sequences, which can assume a supercoil-dependent, S1 nuclease sensitive conformation, suggests that the family of related telomeric sequences could also assume a common non-B-DNA conformation. While the conformational state of the telomeric regions of chromosomes in vivo is not known, several lines of evidence suggest that internal regions of chromosomes are torsionally constrained (reviewed in 43). The presence of evolutionarily conserved sets of topoisomerase I binding site sequences in Tetrahymena rDNA suggests that these short linear DNA molecules also are under torsional constraint in vivo (44). However, no direct information is available about the in vivo conformational state of these and other short, naturally occurring linear molecules in ciliates, or of linear yeast plasmids, all of which are known to carry functional and recognizable telomeric sequences. Relevant to the question of conformation of nuclear DNA in vivo, we note that in two reports of the S1 sensitivity of poly d(CT)•d(AG), the sensitivity, and hence the structural state giving rise to the sensitivity, was not dependent on supercoiling (19, 45).

Could a non B-DNA conformation be stabilized or induced by telomere specific binding proteins? Such proteins could stabilize the non B-DNA conformation, perhaps by intercalation of amino acid side-chains, in the same way that intercalation of side chains of a peptide antibiotic stabilizes an underwound right handed DNA structure involving Hoogsteen pairing determined by X-ray crystallography (41). The existence of sequence-specific proteins bound to telomeric DNA is supported by two lines of evidence. In chromatin of the ciliates Tetrahymena and Oxytricha (8-10), telomeric DNA sequences are packaged as non-nucleosomal complexes. Recently, two different types of telomere sequence - specific biding proteins, from yeast and Oxytricha, have been found by in vitro binding assays (46, 47). Thus, maintenance of linear DNAs in a torsionally constrained state could involve interactions between proteins bound to telomeres.

*Present address: Department of Cytogenetics, Children's Hospital of Philadelphia, 34th and Civic Center Blvd, Philadelphia, PA 19104, USA

REFERENCES

1. Blackburn, E.H. and Szostak, J.W. (1984) Ann. Rev. Biochem. 53, 163-194.
2. Blackburn, E.H. (1984) Cell 37, 7-8.
3. Ponzi, M., Pace, T., Dove, E. and Frontali, C. (1985) EMBO J. 4, 2991-2996.

4. Blackburn, E.H. and Gall, J.G. (1978) J. Mol. Biol. 120, 33-53.
5. Blackburn, E.H., Budarf, M.L., Challoner, P.B., Cherry, J.M., Howard, E.A., Katzen, A.L., Pan, W.-C. and Ryan, T. (1983) Cold Spring Harbor Symp. Quant. Biol. 47, 1195-1207.
6. Oka, Y., Shiota, S., Nakai, S., Nishida, Y., Okuba, S. (1980) Gene 10, 301-306.
7. Klobutcher, L.A., Swanton, M.T., Donini, P., Prescott, D.M. (1981) Proc. Natl. Acad. Sci. USA 78, 3015-3019.
8. Blackburn, E.H. and Chiou, S.-S. (1981) Proc. Natl. Acad. Sci. USA 78, 2663-2557.
9. Budarf, M.L. and Blackburn, E.H. (1986) J. Biol. Chem 261, 363-369.
10. Gottschling, D.E. and Cech, T.R. (1984) Cell 38, 501-510.
11. Greider, C.W. and Blackburn, E.H. (1985) Cell 43, 405-413.
12. Blackburn, E.H. Larson, D., Greider, C., Challoner, P. and Spangler, E. (1987) UCLA Symp. Mol. Cell. Biol., New Series Vol. 51, 293-302 .
13. Szostak, J.W. and Blackburn, E.H. (1982) Cell 29, 245-255.
14. Pluta, A.F., Dani, G.M., Spear, B.B. and Zakian, V.A. (1984) Proc. Natl. Acad. Sci. USA 81, 1475-1479.
15. Shampay, J., Szostak, J.W. and Blackburn, E.H. (1984). Nature 310, 154-157.
16. Arnott, S., Chandrasekaran, R., Birdsall, D.L., Leslie, A.G.W. and Ratcliff, R.L. (1980) Nature 283, 743-745.
17. Singleton, C.K., Klysik, J., Stirdivant, S.M. and Wells, R.D. (1982) Nature 299, 312-316.
18. Cross, D.S., Huang, S.-Y. and Garrard, W.T. (1985) J. Mol. Biol. 183, 251-265.
19. Hentschel, C.C. (1982) Nature 295, 609-622.
20. Pulleyblank, D.E., Haniford, D.B. and Morgan, A.R. (1985) Cell 42, 271-280.
21. Cybvig, K., Clark, C.D., Aliperti, G. and Schlesinger, M. (1983) Nucl. Acids Res. 11, 8495-8508.
22. Shen, C.-K.J. (1983) Nucl. Acids Res. 11, 7899-7910.
23. Schon, E., Evans, T., Welsh, J. and Efstratiadis, A. (1983) Cell 35, 837-848.
24. Nichol, J.M. and Felsenfeld, G. (1983) Cell 35, 467-477.
25. Yu, Y.-T., Manley, J.L. (1986) Cell 45, 743-751.
26. Challoner, P.B., Amin, A.A., Pearlman, R.E. and Blackburn, E.H. (1985) Nucl. Acids Res. 13, 2661-2680.
27. Challoner, P.B. and Blackburn, E.H. (1986) Nucl. Acids Res., 14, 6299-6311
28. Pan, W.-C. and Blackburn, E.H. (1981) Cell 23, 459-466 (1981).
29. Birnboim, H.C. (1983) Methods in Enzymol. 100, 243-255.
30. Courey A.J. and Wang, J.C. (1983) Cell 33, 817-829.
31. Vogelstein, B. and Gillespie, D. (1979) Proc. Natl. Acad. Sci. USA 76, 615-619.
32. Smith, G.E. and Summers, M.D. (1980) Analyt. Biochem. 109, 123-129.
33. Botchan, M., Topp, W. and Sambrook, J. (1976) Cell 9, 269-287.
34. Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975) Proc. Natl. Acad. Sci. USA 72, 1184-1188.
35. Schendel, P.F. and Wells, R.D. (1973) J. Biol. Chem. 248, 8319-8320.
36. Maxam, A.M. and Gilbert, W. (1980) Methods in Enzymol. 65, 499-560.
37. Lilley, D.M. (1980) Proc. Natl. Acad. Sci. USA 77, 6468-6472.
38. Peck, L.J. and Wang, J.C. (1983) Proc. Natl. Acad. Sci. USA 80, 6206-6210.
39. Pohl, F.M. and Jovin, T.M. (1972) J. Mol. Biol. 67, 375-396.
40. Wu, H.-M. and Crothers, D.M. (1984) Nature 308, 509-515.
41. Quigley, G.J. (1986) Science 232, 1255-1258.

42. Cantor, C.R. and Efstratiadis, A. (1984) Nucl. Acids Res. 12, 8059-8072.
43. Lilley, D.M.J. (1983) Nature 305, 276-277.
44. Bonven, B.J., Gocke, E. and Westergaard, O. (1985) Cell 41, 541-551.
45. Htun, H., Lund, E. and Dahlberg, J. (1984) Proc. Natl. Acad. Sci. USA 81, 7288-7292.
46. Berman, J. and Tye, B.-T. (1986). Proc. Natl. Acad. Sci. USA 83, 3713-3717.
47. Gottschling, D.E. and Zakian, V.A. (1986). Cell 47, 195-205.