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# A Tandemly Repeated Sequence at the Termini of the Extrachromosomal Ribosomal RNA Genes in *Tetrahymena*

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The extrachromosomal genes coding for ribosomal RNA (rDNA) in the ciliated protozoan *Tetrahymena thermophila* were studied with respect to sequences occurring at their termini. The linear rDNA molecules had previously been shown to be palindromic (Karrer & Gall, 1976; Engberg *et al.*, 1976). Within the terminal rDNA fragment produced by restriction endonuclease digestion, a tandemly repeated hexanucleotide sequence 5′ (C-C-C-C-A-A)$_n$ 3′ was found, where $n$ is between 20 and 70. This fragment was heterogeneous in length as judged by gel electrophoresis. The repeating sequence was preferentially synthesized when rDNA was used as the template by *Escherichia coli* DNA polymerase I. Initiation occurred at specific single-strand discontinuities, probably one-nucleotide gaps, found every few repeats on the C-C-C-C-A-A strand. At least one discontinuity is present on the G-G-G-G-T-T strand. Experiments with T4 DNA polymerase suggested that there are no free cohesive ends on the rDNA of the kind found in bacteriophage λ DNA. The orientation of the strands carrying the repeating hexanucleotide sequence was determined, and a model for the termini of the rDNA based on these findings is presented.

## 1. Introduction

The genes coding for ribosomal RNA (rDNA) are amplified to form extrachromosomal DNA in the developing oocytes of a number of animals (Brown & Dawid, 1968; Gall, 1968; for review see Tobler, 1975). Extrachromosomal rDNA is also found in the slime mold *Physarum* (Vogt & Braun, 1976) and in the ciliated protozoan *Tetrahymena*. rDNA from *Tetrahymena* can be extracted as extrachromosomal linear molecules of uniform length (Gall, 1974). Each molecule consists of two identical halves arranged in the form of a palindrome, or inverted repeat, with one copy of the ribosomal RNA genes on each half of the molecule (Karrer & Gall, 1976; Engberg *et al.*, 1976).

*Tetrahymena* has two different nuclei: a polyploid, metabolically active macronucleus which divides amitotically during vegetative cell division, and a diploid micronucleus, which divides mitotically and functions as a germinal nucleus. At conjugation the macronucleus is destroyed and replaced by a division product of the micronucleus (for review see Elliott, 1973). The macronucleus contains extrachromosomal rDNA molecules, approximately 200 copies per haploid genome (Engberg & Pearlman, 1972). By contrast the micronuclear genome contains only a single chromosomally integrated rDNA copy (Yao & Gall, 1977). Amplification of the rDNA presumably occurs after conjugation when the macronucleus is formed.



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The sequence and molecular structures at the termini of the extrachromosomal, linear, palindromic rDNA are therefore of considerable interest. This sequence in the integrated copy in the micronucleus is covalently bonded to the neighboring chromosomal sequence, and is therefore involved in amplification and processing. In addition, subsequent replication of the amplified rDNA molecules must involve the terminal sequences in a special way. Replication of extrachromosomal rDNA proceeds bidirectionally from the center of the linear molecules towards the ends (Truett & Gall, 1977). Therefore, determination of the structure of the termini of the linear rDNA molecules should be useful for defining the processes involved in the completion of their replication in vegetatively growing cells, and in understanding the mechanism of amplification.

The results described in this paper show that at each end of the palindromic, extrachromosomal rDNA molecules there is a tandemly repeating hexanucleotide sequence, which has specific discontinuities, probably one-nucleotide gaps, on one strand at intervals. A possible model for the structure of the terminal regions of the rDNA molecules based on these findings is presented.

## 2. Materials and Methods

### (a) *Cell culture and purification of rDNA*

*Tetrahymena thermophila*, previously classified as syngen 1 of *Tetrahymena pyriformis* (Nanney and McCoy, 1976), strains BIV, BVI or BVII, was grown in proteose peptone medium and rDNA was extracted by a modification of the procedure of Kavenoff & Zimm (1973) as described by Karrer & Gall (1976), except that treatment of cell extracts with α-amylase was omitted. No differences were detected between the rDNAs of strains BIV, BVI or BVII.

### (b) *Labeling of cells with* $^{32}P$

*T. thermophila*, strain BIV or BVII, was grown at room temperature in 100 ml of 2% (w/v) proteose peptone (Difco), 0·2% (w/v) glucose and 0·1% (w/v) yeast extract (Difco) to a cell density of $1 \times 10^5$ to $2 \times 10^5$ cells/ml (early log phase of growth). The cells were gently pelleted by centrifugation at 600 *g* for 4 min, washed once with sterile 10 mM-Tris·HCl (pH 7·1), and resuspended in 200 ml of defined medium (Rasmussen & Modeweg-Hansen, 1973), from which inorganic phosphate was omitted and 10 mM-Tris·HCl (pH 7·1) was added. Carrier-free $H_3{}^{32}PO_4$ (HCl-free; New England Nuclear) was added to a final concn of 10 to 25 μCi/ml. The cells were grown for 17 h in this medium at room temperature, and harvested by centrifugation. rDNA was extracted from the labeled cells. The spec. act. of the rDNA was greater than $3 \times 10^5$ cts/min per μg. In some experiments, the cells were labeled with $^{32}P$ in defined medium for 3 h, then a 5% (w/v) stock solution of the proteose peptone medium was added to a final concn of 1%, and growth continued for 17 h. The spec. act. of the rDNA purified from cells labeled in this way was $1 \times 10^5$ cts/min per μg.

### (c) In vitro *labeling of rDNA by* Escherichia coli *DNA polymerase I*

DNA was incubated with DNA polymerase I from *E. coli* under conditions suitable for *in vitro* repair synthesis from single-stranded nicks or breaks (Kelly *et al.*, 1970). Incubations were performed as described by Maniatis *et al.* (1975), and unless otherwise specified contained, in 100 μl, 1 to 2 μg rDNA, 2 units of DNA polymerase I from *E. coli* (grade I, Boehringer Mannheim, GmbH) and 2 μM-deoxynucleoside triphosphates (dNTPs), 3 unlabeled (PL Biochemicals, Inc. Milwaukee, Wisc.), and one [α-$^{32}P$]dNTP (spec. act. 100 to 150 Ci/mmol, New England Nuclear, Mass.). Incubations were for 1 h at 15°C unless otherwise specified. Reactions were stopped by addition of EDTA and extraction with phenol, and excess triphosphate was removed by 2 or 3 precipitations with ethanol in the presence of carrier RNA.

(d) *Digestion with restriction endonucleases*

The restriction endonucleases *Eco*RI and *Hin*dII + III were generously provided by T. Barnett; *Bam*HI, *Alu*I and *Hin*dIII were purchased from New England Biolabs (Beverly, Mass.). DNA was digested with *Eco*RI and *Bam*HI as described by Karrer & Gall (1976). For the other restriction enzymes, digestions were for 1 to 4 h at 37°C and incubations (15 to 50 $\mu$l) contained from 50 to 100 $\mu$g DNA/ml, 6·6 mM-Tris·HCl (pH 7·4), 6·6 mM-$MgCl_2$, 6·6 mM-2-mercaptoethanol and 60 mM-NaCl. In many experiments carrier RNA was present in concentrations up to 100 $\mu$g/ml in the restriction digestion mixture, with no detectable effect on the DNA digestion.

(e) *Gel electrophoresis*

DNA restriction fragments were separated by electrophoresis in 1% or 1·4% agarose gels, with 80 mM-Tris-acetate (pH 8·0), 4 mM-EDTA and 10 mM-sodium acetate as the running buffer, in an EC470 vertical slab gel apparatus (E-C Apparatus Corp., St. Petersburg, Flor). For autoradiography, the wet gel was wrapped in Saranwrap and placed in contact with a Kodak No Screen (NS 54T) X-ray film. DNA was eluted from agarose gels for further analysis as described by Yao & Gall (1977). Endonuclease IV digestion products were fractionated by electrophoresis on a 10% polyacrylamide gel containing 8·3 M-urea, and DNA was eluted from the gel, as described by Galibert *et al.* (1974).

(f) *Synthesis reaction with T4 DNA polymerase*

$\lambda$cI857Sam7 DNA (kindly provided by T. Barnett) and rDNA were incubated together or separately with T4 DNA polymerase (kindly supplied by J. W. Sedat). Incubations, as described by Goulian *et al.* (1968) for the synthesis reaction, were in 30 $\mu$l of 67 mM-Tris·HCl (pH 8·0), 6·7 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, 16 mM-ammonium sulfate, 3 unlabeled dNTPs at 66 $\mu$M and one [$\alpha$-$^{32}$P]dNTP at 10 $\mu$M; T4 DNA polymerase and $\lambda$ DNA at 70 $\mu$g/ml and/or rDNA at 18 $\mu$g/ml were present. Reactions were from 5 to 60 min at 14°C. To test for incorporation of label at nicks, freshly diluted DNAase I (Worthington) was added to $10^{-9}$ g/ml to the synthesis reaction.

(g) *Depurination analysis of* [$^{32}$*P*]*DNA*

DNA was depurinated as described by Burton (1967). Pyrimidine tracts were fractionated either in two dimensions as described by Ling (1972) or in one dimension by electrophoresis at pH 3·5 on DE81 paper (Whatman) (Galibert *et al.*, 1974). When several [$^{32}$P]DNA restriction fragments separated by agarose gel electrophoresis were to be analyzed, they were located by autoradiography and depurinated without prior elution from the gel. The gel band was excised, its volume estimated from its dimensions (typically 0·8 cm × 0·3 cm × 0·3 cm), and 2·8 vol. 89% formic acid containing 2·7% diphenylamine were added. After incubation for 17 h at 37°C (the gel dissolved within an hour), $\frac{1}{2}$ vol. distilled water was added and the aqueous phase (often cloudy after addition of the water) was extracted 5 times with 6 vol. ether, then lyophilized. The residue dissolved readily in 10 to 20 $\mu$l of water to give a viscous solution (owing to the presence of residual gel material) which was applied to the origin of DE81 paper. The origin was washed for several minutes with 70% ethanol, dried, and the pyrimidine tracts fractionated by electrophoresis at pH 3·5 (Galibert *et al.*, 1974). Separation was reproducible and many DNA fragments could be conveniently analyzed at once by this procedure.

(h) *Nuclease digestions and fingerprinting of* [$^{32}$*P*]*DNA*

[$^{32}$P]DNA was denatured for nuclease digestions by heating the DNA in the absence of $Mg^{2+}$ to 100°C for 1 min, then chilling to 0°C. Digestions with T4 endonuclease IV (Sadowski & Bakyta, 1972), kindly supplied by T. Maniatis, were as described by Galibert *et al.* (1974) using conditions of low ionic strength. Oligonucleotide digestion products were fingerprinted in 2 dimensions as described by Brownlee & Sanger (1969), except that homochromatography was carried out on 20 cm × 20 cm PEI-cellulose thin-layer plates (Schleicher and Schuell, Keene N. H.). Partial digestions of denatured [$^{32}$P]DNA by micrococcal nuclease were carried out at 25°C in 30 $\mu$l containing 0·5 $\mu$g DNA, 20 $\mu$g

carrier RNA, 25 units of micrococcal nuclease (Worthington), 20 μg bovine serum albumin/ml, 20 mM-Tris·HCl (pH 8·9), 2 mM-$CaCl_2$, and 0·2 mM-EDTA. Portions were withdrawn at different times during the digestion and applied to the origin of a 20 cm × 40 cm DEAE-cellulose thin-layer plate (MN plate, Brinkman, Inc.) for homochromatography, or to DE81 paper for electrophoresis at pH 3·5. Oligonucleotides were eluted for depurination and nearest-neighbor analysis as described by Galibert *et al.* (1974).

(i) *Labeling with polynucleotide kinase*

5 to 10 μg rDNA was denatured and treated with 0·5 unit of bacterial alkaline phosphatase (Worthington BAPC, heated at 95°C for 10 min to inactivate nucleases) in 20 μl containing 5 mM-dithiothreitol, 5 mM-$MgCl_2$ and 10 mM-Tris·HCl (pH 8·0) for 30 min at 37°C. After removal of phosphatase by 3 extractions with phenol, and extracting the aqueous phase 4 times with ether, the rDNA was labeled with 2 units of polynucleotide kinase (New England Biolabs) for 1 h at 37°C in 30 μl of 10 mM-Tris·HCl (pH 8·0), 5 mM-dithiothreitol, 5 mM-$MgCl_2$ and 10 μM-[$\gamma$-$^{32}$P]ATP (spec. act. > 1000 Ci/mmol; prepared as described by Maxam & Gilbert (1977) and generously supplied by O. Hagenbüchle). After 3 precipitations with ethanol in the presence of $Mg^{2+}$, excess EDTA was added and the labeled rDNA was denatured and fingerprinted, or depurinated.

(j) *Ligation of rDNA*

rDNA was treated with T4 ligase (purchased from New England Biolabs) under conditions described by Meyer *et al.* (1975). 0·3 μg rDNA was incubated in 50 μl for 3 h at 24°C in the presence of 0·3 unit of T4 ligase, or under the same conditions but with the enzyme omitted as a control, before stopping the reaction by addition of EDTA, extraction with phenol, removal of phenol by extraction with ether, and precipitation with ethanol. The ligated and control rDNAs were then incubated with DNA polymerase I as described above.

## 3. Results

(a) *Template properties of rDNA for* Escherichia coli *DNA polymerase I*

(i) *Location of preferential synthesis*

The rDNA in the macronucleus of *T. thermophila* consists of linear palindromic molecules of molecular weight $13{\cdot}6 \times 10^6$ (Karrer & Gall, 1976). Each half of the molecule contains one cleavage site, symmetrically located about the center of the molecule, for each of the restriction endonucleases *Eco*RI and *Bam*HI. Digestion of the rDNA by either of these endonucleases produces a large central fragment and two identical terminal fragments of molecular weights $1{\cdot}6 \times 10^6$ and $2{\cdot}4 \times 10^6$ for *Eco*RI and *Bam*HI, respectively (Karrer & Gall, 1976). Untreated rDNA was investigated for any structural features that would allow DNA polymerase I from *E. coli* to use it as a template for synthesis. The purified rDNA was incubated with DNA polymerase I and all four deoxynucleoside triphosphates, one labeled in the $\alpha$ position with $^{32}$P, under conditions to favor "nick-translation" (Kelly *et al.*, 1970). To find the distribution of incorporated radioactivity along the molecule, the rDNA was digested with the restriction endonucleases *Eco*RI or *Bam*HI. The DNA fragments produced were separated by agarose gel electrophoresis (Fig. 1). With each restriction enzyme, the fragment known to map at the ends of the palindromic rDNA molecule was specifically and preferentially labeled. This was observed regardless of which dNTP of the four was the labeled one. The dimeric form of the end restriction fragment, which is derived from digestion of circular and concatameric rDNA molecules (Karrer & Gall, 1976), was often observed in different preparations. Like the end fragment from the monomeric linear form of the rDNA, it too was preferentially labeled.

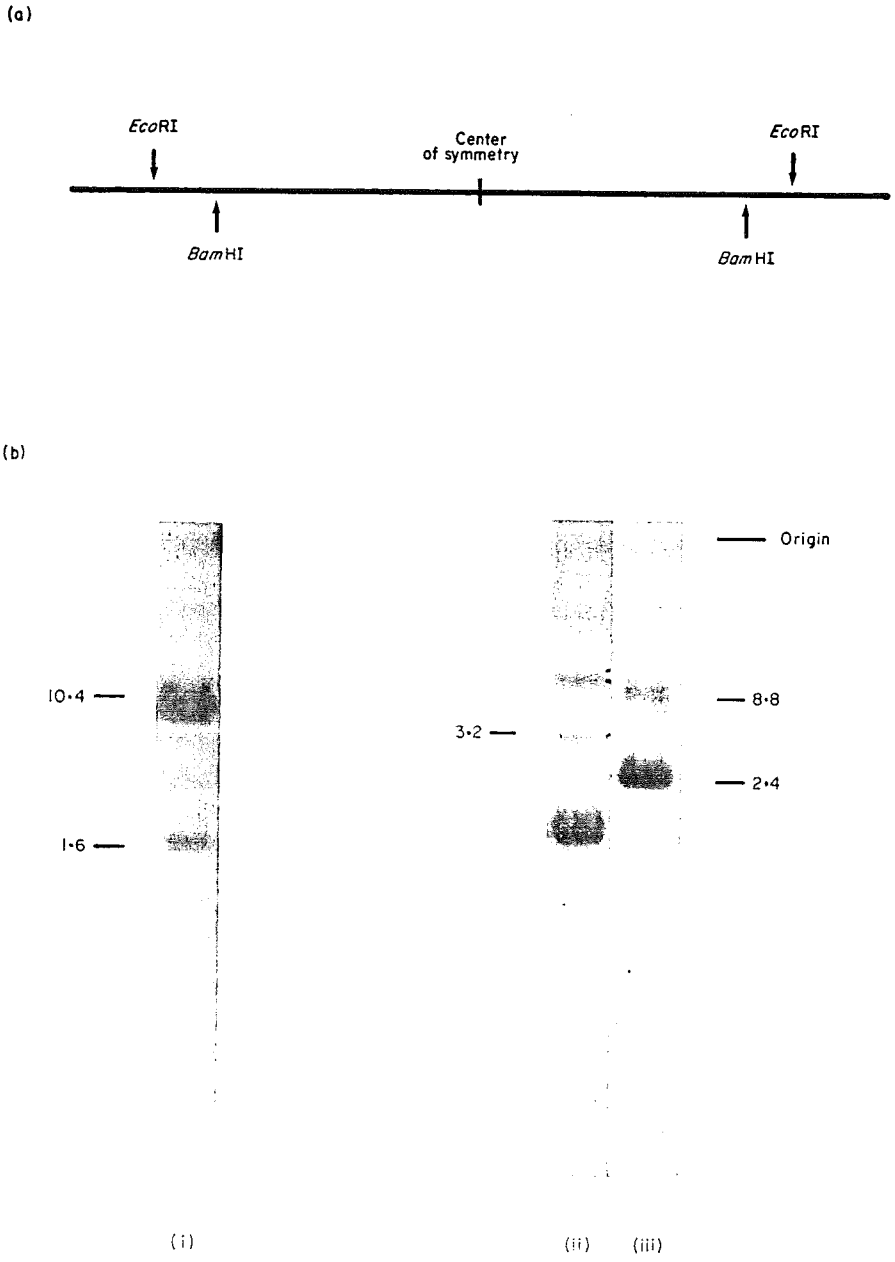


FIG. 1. (a) Schematic representation of a linear extrachromosomal rDNA molecule showing cutting sites for *Eco*RI and *Bam*HI (Karrer & Gall, 1976). (b) Autoradiograms of labeled rDNA restriction fragments. rDNA was labeled either *in vivo* with $^{32}P$, and digested with *Eco*RI (i) or *in vitro* with [$\alpha$-$^{32}P$]dATP in the presence of the other 3 dNTPs by DNA polymerase I, and cut with either *Eco*RI (ii) or *Bam*HI (iii). DNA fragments were separated by electrophoresis in a 1·4% agarose slab gel as described in Materials and Methods. Molecular weights ($\times 10^{-6}$) are indicated.

These findings suggested that the rDNA contains 3′ hydroxyl groups on one or both strands, from which strand elongation by polymerase I was initiated. By measuring the amount of incorporated label, we determined that much more extensive synthesis was occurring at or near the ends of the rDNA molecules than would be expected if the linear rDNA had cohesive ends of the type found, for example, in bacteriophage λ DNA (Strack & Kaiser, 1965; Wu & Taylor, 1971). To locate the site of synthesis more accurately in the labeled rDNA molecule, the end *Eco*RI fragment, and its dimeric form, were eluted separately from the agarose gel and redigested with the restriction endonuclease *Alu*I. The resulting new fragments were separated by agarose gel electrophoresis and located by autoradiography (Fig. 2). The site of preferential incorporation lay in a heterogeneous fragment 360 to 520 base-pairs in length resulting from digestion of the monomeric end *Eco*RI restriction fragment with *Alu*I. That this fragment was at the ends of the rDNA was deduced from the following observations. Digestion of the dimeric end *Eco*RI fragment with *Alu*I resulted in the appearance of a preferentially labeled heterogeneous fragment of twice the molecular weight of the *Alu*I fragment coming from digestion of the monomeric end *Eco*RI fragment. A portion of the dimeric end *Eco*RI fragment was digested after heating it to 79°C for one minute and chilling to 0°C. About half of this dimeric *Eco*RI fragment broke down to monomeric form (Fig. 2). This preparation digested with *Alu*I showed approximately equal amounts of the 360 to 520 base-pair band and its dimeric form (Fig. 2).

These results show that preferential synthesis by polymerase I on rDNA takes place at the terminal regions of the palindromic molecules. The smallest restriction fragment identified, in which all the preferential synthesis occurs, comes from digestion with *Alu*I, thus locating the region of initiation of synthesis within the terminal 360 to 520 base-pairs of the rDNA molecule. The heterogeneity observed for the terminal restriction fragment of the rDNA was not a result of incubation with DNA polymerase I and dNTPs. The terminal restriction fragment of rDNA detected by staining with ethidium bromide after agarose gel electrophoresis was broader than marker DNA fragments of the same size or smaller. The same heterogeneity was observed for the terminal restriction fragment when rDNA was labeled uniformly *in vivo* with $^{32}P$. Upon gel electrophoresis, the width of the band consisting of the terminal restriction fragment corresponded to a difference of approximately 160 base-pairs between the fastest and slowest migrating components of the band. This was most clearly seen with *Hin*dIII, *Hin*dII + III (data not shown) and *Alu*I digests, in which the terminal restriction fragment is smaller than the terminal *Eco*RI fragment.

(ii) *Specificity of initiation of synthesis*

rDNA was incubated with DNA polymerase I and one [α-$^{32}P$]dNTP at a time, to determine whether the first residue incorporated on the rDNA template was nucleotide specific. The labeled DNA was digested with *Eco*RI and the fragments separated by agarose gel electrophoresis (Fig. 3(a)). The only significant incorporation of label occurred with [α-$^{32}P$]dCTP, and this was in the end fragment and its dimeric form. These fragments were eluted from the gel and analyzed for nearest-neighbor transfer of the $^{32}P$ label. Label transferred from the incorporated [$^{32}P$]dCMP to 5′ neighboring residues was found in 3′ dAMP and 3′ dCMP. These findings showed that initiation of DNA synthesis occurred at a specific sequence or sequences.

Combinations of more than one dNTP were then tested for specific incorporation

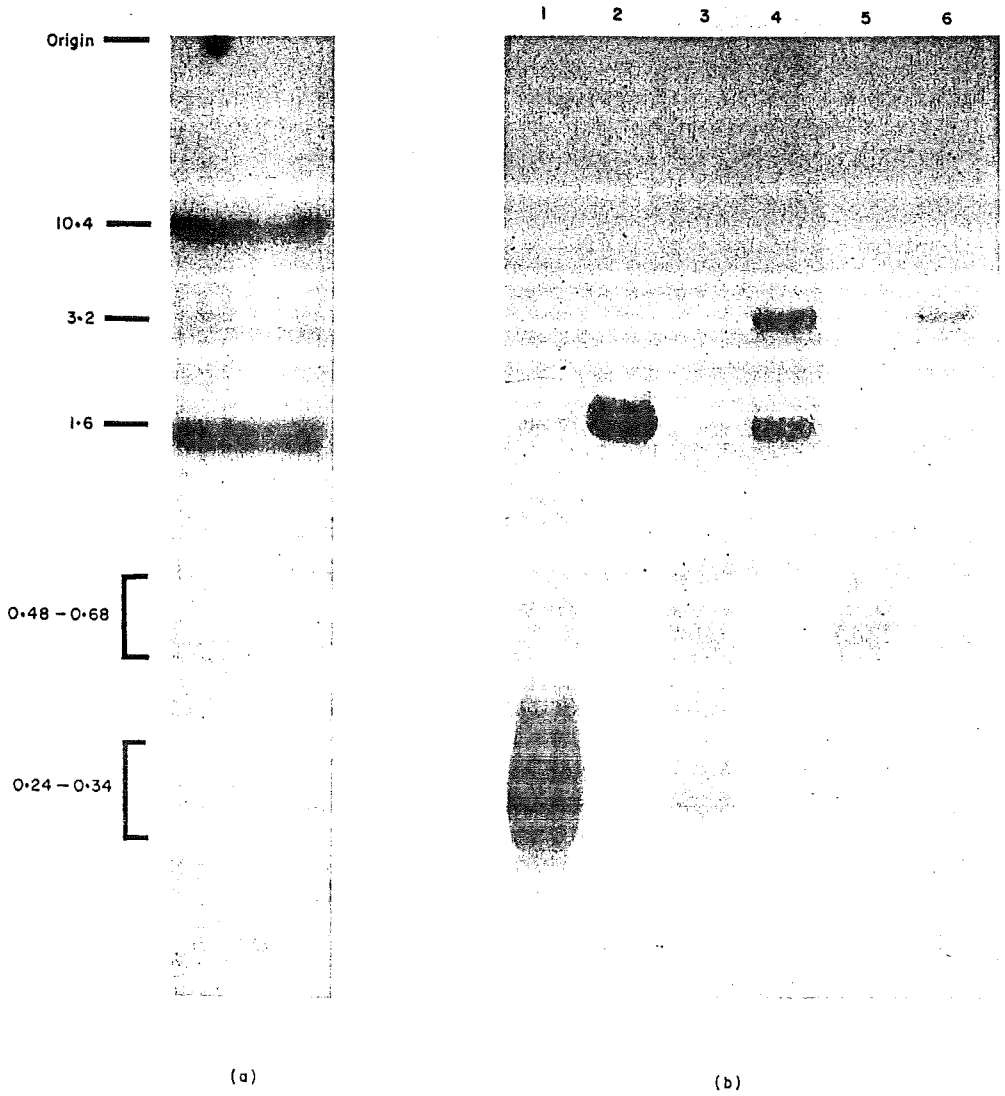


FIG. 2. *Alu*I digestion of labeled rDNA end *Eco*RI fragment and its dimeric form.

(a) rDNA was labeled with [α-$^{32}$P]dGTP in the presence of the other 3 dNTPs by DNA polymerase I for 20 min at 15 C, then digested with *Eco*RI. The fragments were fractionated by gel electrophoresis on a 1·4% agarose gel and autoradiographed. (b) The end fragment and its dimeric form ($M_r$ $1{\cdot}6 \times 10^6$ and $3{\cdot}2 \times 10^6$, respectively) were eluted separately, digested with *Alu*I, rerun on a 1·4% agarose gel and autoradiographed. The monomeric end *Eco*RI fragment was digested with *Alu*I (lane 1) or rerun as a control (lane 2). The dimeric form of the end *Eco*RI fragment was rerun without further treatment (lane 6), digested with *Alu*I (lane 5), or, after heating to 79°C for 1 min and chilling to 0 C, rerun (lane 4) or digested with *Alu*I (lane 3). The molecular weights of heterogeneous *Alu*I fragments were measured by calibration against the *Hae*III restriction fragments of simian virus 40 DNA (not shown).

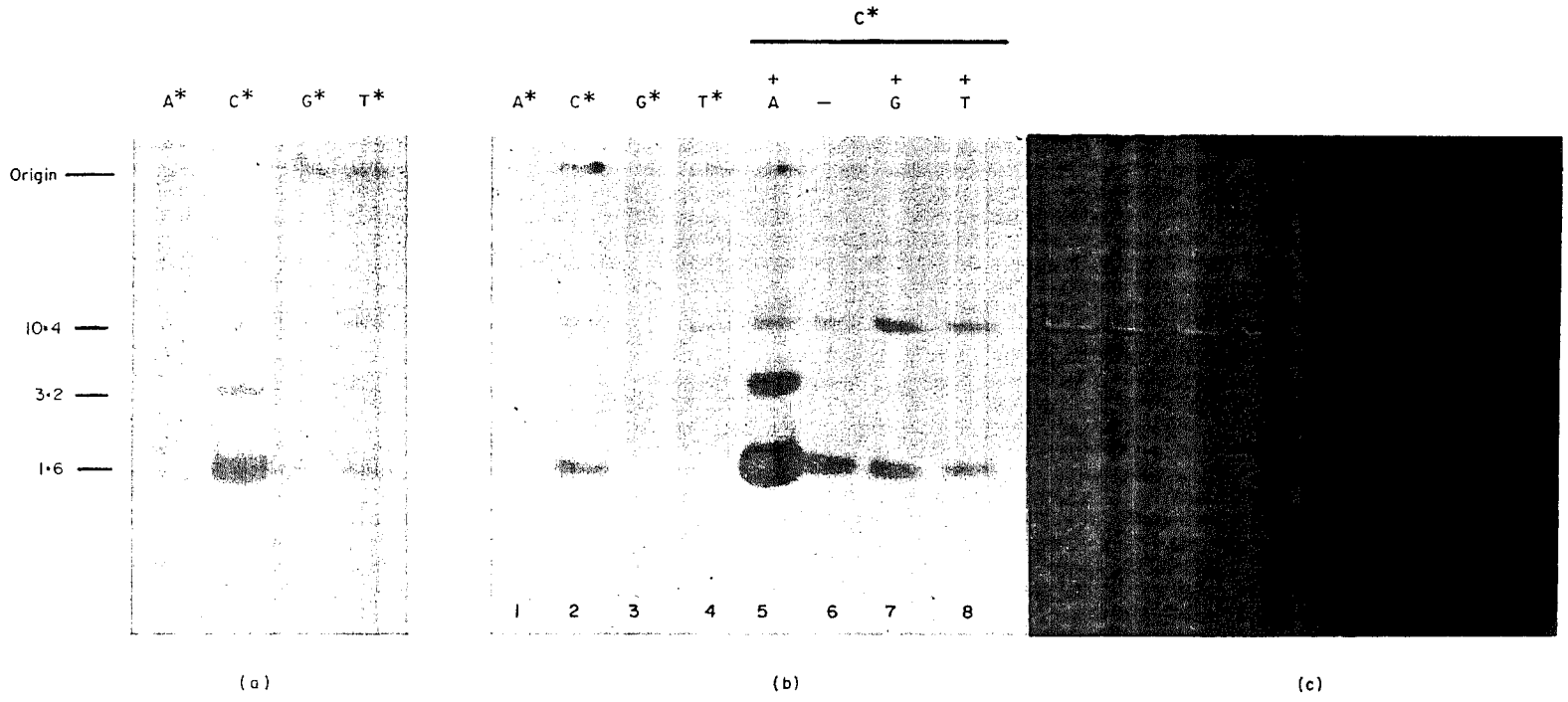


FIG. 3. Specific labeling of rDNA by DNA polymerase I and 1 or 2 dNTPs at a time.

(a) rDNA was incubated with DNA polymerase I and only one [$\alpha$-$^{32}$P]dNTP (10 $\mu$M), with no unlabeled dNTPs, for 10 min at 15°C. The labeled rDNA was digested with *Eco*RI, and the fragments separated by electrophoresis on a 1·4% agarose gel and autoradiographed. A*, C*, G* or T* indicate which [$\alpha$-$^{32}$P]dNTP was used to label the rDNA. Molecular weights ($\times 10^{-6}$) of the fragments are indicated.

(b) Autoradiogram of *Eco*RI fragments of rDNA separated by 1·4% agarose gel electrophoresis. From left to right, lanes 1 to 4: *Eco*RI restriction fragments of rDNA after labeling as in (a). Lanes 5 to 8: rDNA was labeled under the same conditions, except that one other unlabeled dNTP, at a concn of 2 $\mu$M, was present as indicated in addition to 2 $\mu$M [$\alpha$-$^{32}$P]dCTP.

(c) The same gel as (b), stained with ethidium bromide to visualize the amounts of DNA in the restriction fragments.

into rDNA by polymerase I. It was found that the specific incorporation of [$\alpha$-$^{32}$P]-dCTP into the end restriction fragment increased several-fold when dATP was added (Fig. 3(b)). Addition of either dGTP or TTP had no effect on incorporation of [$\alpha$-$^{32}$P]dCTP into the end restriction fragment (Fig. 3(b)). However, when dGTP was present in combination with TTP, the amount of incorporation of label into the end restriction fragment was about half that occurring with dCTP plus dATP under the same conditions (data not shown).

(iii) *Determination of the sequences preferentially synthesized*

rDNA was incubated with DNA polymerase I and all four dNTP precursors for one hour at 15°C. Under these conditions, most of the incorporated label from either [$\alpha$-$^{32}$P]dATP or [$\alpha$-$^{32}$P]dCTP was found in the repeating hexanucleotide sequence 5′ C-C-C-C-A-A 3′. This sequence was determined by depurination and nearest-neighbor analysis of the *in vitro* labeled rDNA, and by analysis of the products of digestion with T4 endonuclease IV (Sadowski & Bakyta, 1972; Galibert *et al.*, 1974).

Depurination fingerprints of label incorporated by polymerase I in the presence or absence of low amounts of DNAase I are shown in Figure 4, where they are compared to a depurination fingerprint of rDNA labeled uniformly *in vivo* with $^{32}$P. In the absence of DNAase I, $C_4$ was virtually the only depurination product of the DNA labeled with [$\alpha$-$^{32}$P]dCTP (Fig. 4(a)). Addition of DNAase I to introduce nicks into the rDNA allowed synthesis of many other sequences by polymerase I, as evidenced by the increase in complexity of the depurination fingerprint of this labeled DNA, and the decrease in the amount of $C_4$ made relative to other sequences (Fig. 4(b)). Although the depurination product $C_4$ represents only a small percentage of the total radioactivity in the uniformly labeled rDNA (Fig. 4(c)) it is clear that multiple molar amounts of this pyrimidine tract are present, and in excess of the number expected on a random basis. When the [$^{32}$P]rDNA labeled *in vivo* was digested with *Eco*RI or *Hin*dII + III, $C_4$ was detected only in the end restriction fragment.

Figure 5(a) shows the products of partial digestion of the labeled rDNA with endonuclease IV, separated by polyacrylamide gel electrophoresis under denaturing conditions. The oligonucleotide digestion products consisted largely of a series of bands which on calibration with marker oligonucleotides formed a set of multiples of six nucleotides, the smallest band being 18 nucleotides and the largest 60 nucleotides in length. Figure 5(b) shows a two-dimensional fingerprint of the products of partial digestion with endonuclease IV. Again, the products fell into a series of related spots, along with minor amounts of other products of closely related composition and size. Table 1 gives the analysis of the total labeled sequence and of the major products of digestion. The only pyrimidine tract labeled in the undigested preferentially synthesized sequence was $C_4$, which was labeled only with [$\alpha$-$^{32}$P]dCTP and [$\alpha$-$^{32}$P]dATP, giving the sequence

$$5' \begin{matrix} A \\ G \end{matrix} \text{C-C-C-C-A 3'}.$$

In endonuclease IV digestion products present in minor amounts, the pyrimidine tracts $C_3$ and $C_2$ were identified, but these depurination products were not found in the undigested material. Nearest-neighbor analysis showed that only the dinucleotide sequences C-A, A-A, C-C and A-C were present in the labeled material. From these data, and from the ratios of nearest-neighbors and the absence of any other dinucleotide

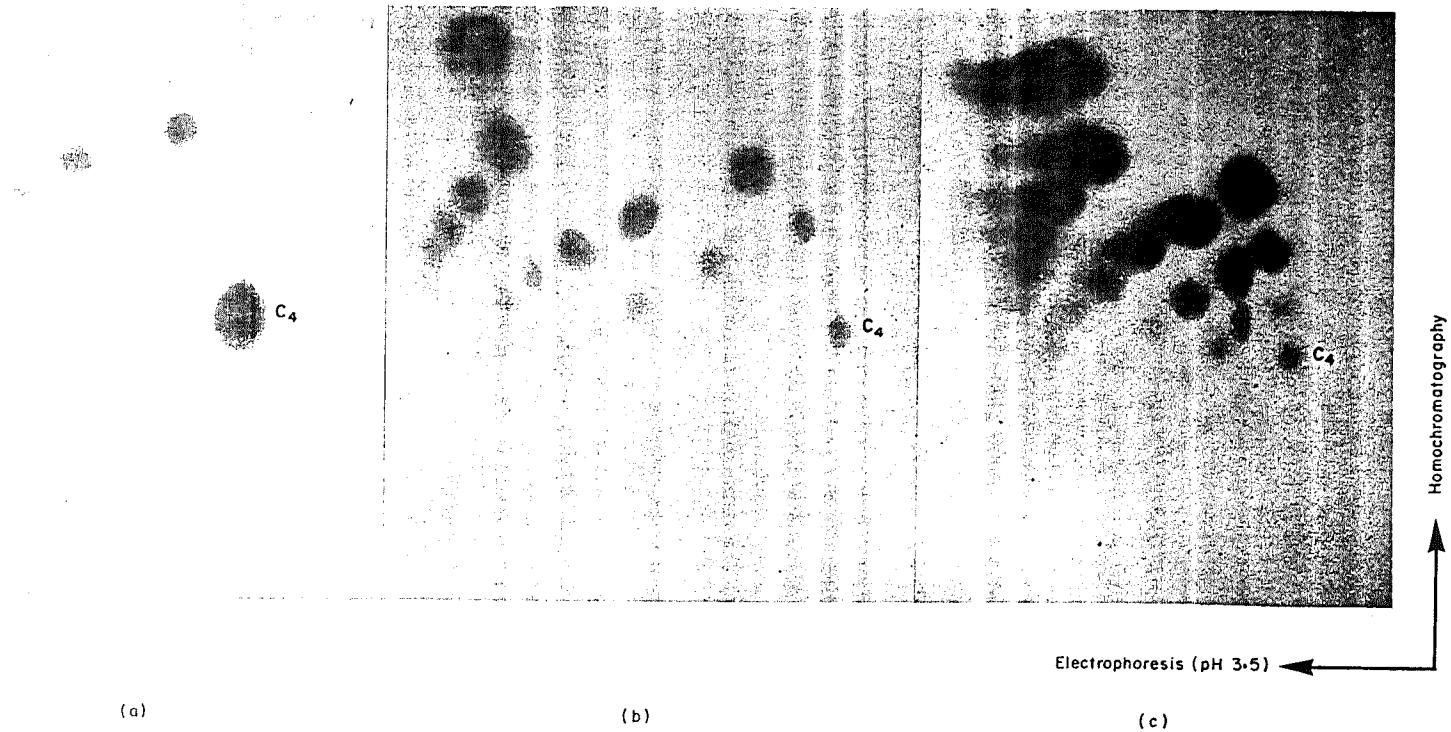


FIG. 4. Autoradiograms of pyrimidine tracts of rDNA labeled *in vitro* by DNA polymerase I or *in vivo* with $^{32}P$.

(a) rDNA was incubated with DNA polymerase I and all 4 dNTPs, including [$\alpha$-$^{32}P$]dCTP, for 1 h at 15°C, then depurinated and fingerprinted as described in Materials and Methods. The composition of the pyrimidine tract pCpCpCpCp was independently verified by digestion with micrococcal nuclease and spleen phosphodiesterase (Galibert *et al.*, 1974).

(b) Depurination fingerprint of rDNA labeled as for Fig. 4(a), except that [$\alpha$-$^{32}P$]dATP was the labeled dNTP, and $10^{-9}$ g of DNAase I/ml was included in the polymerase I incubation. This substantially stimulated incorporation of label into rDNA.

(c) Depurination fingerprint of rDNA purified from *T. thermophila* strain BVII cells grown in $^{32}P$ as described in Materials and Methods. For assignment of pyrimidine tract composition to spots in the 2-dimensional fingerprints, see Ling (1972). $C_4$ is present in multiple molar amounts per molecule.

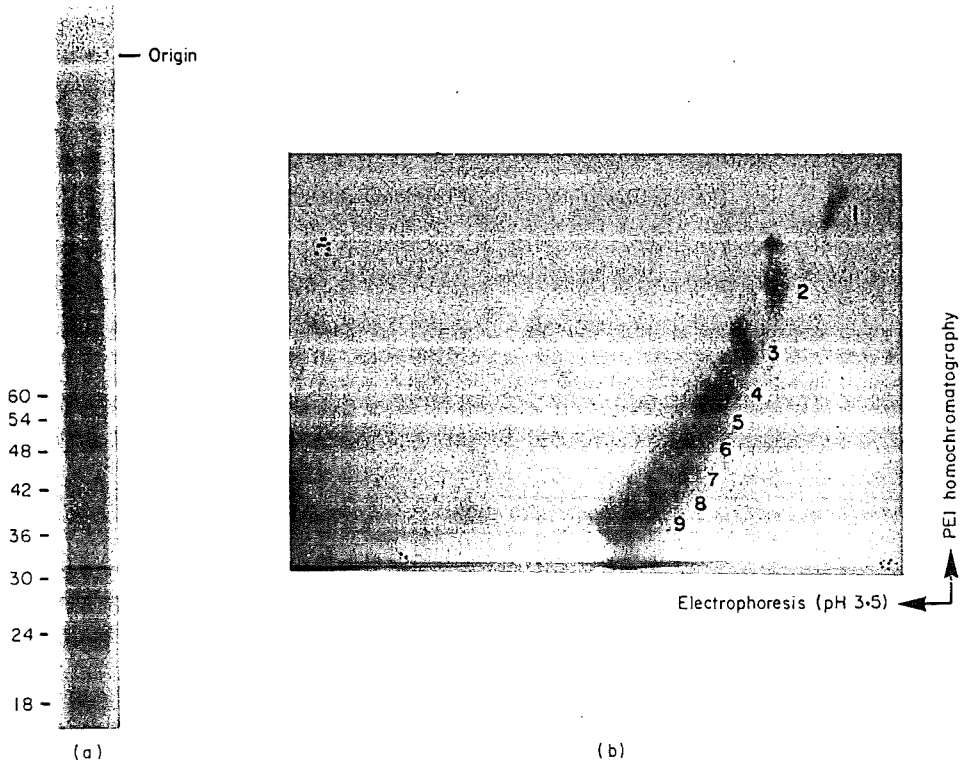


FIG. 5. Endonuclease IV digestion products of the sequence preferentially labeled on rDNA template. rDNA was labeled by DNA polymerase I as described, and restricted with *Eco*RI. After agarose gel electrophoresis the end restriction fragment was eluted and precipitated with ethanol and 20 $\mu$g of RNA as carrier. The labeled DNA was denatured and digested with endonuclease IV in 50 $\mu$l containing 10 mM-$MgCl_2$, 5 mM-2-mercaptoethanol, 20 mM-Tris·HCl (pH 8·5), and endonuclease IV, at 37°C for 17 h. Digestion was stopped by addition of EDTA, the reaction phenol-extracted, and precipitated with ethanol.

(a) Autoradiogram of C-labeled endonuclease IV digestion products separated on a 10% polyacrylamide gel containing 8·3 M-urea. Estimated sizes of the fragments that fell into a series of multiples of 6 nucleotides are indicated by the numbers. Fragments whose sizes did not fall into the series were variably observed, and probably represent digestion products of other sequences that were labeled in the end *Eco*RI fragment.

(b) Autoradiogram of a 2-dimensional fractionation of A-labeled endonuclease IV digestion products. The spots numbered 1 to 9 indicate prominent oligonucleotides whose analyses are given in Table 1.

tide sequences, the sequence of the major endonuclease IV digestion products was deduced to be:

$$5'\ pCpCpCpCpApAp(CpCpCpCpApAp)_nCpCpCpCpApA_{OH}\ 3',$$

where $n$ is from 1 to 8. It was concluded that the most frequent site of cutting of the repeated sequence by endonuclease IV was at the A–C bonds, giving rise predominantly to a series of products that were multiples of the sequence 5′ C-C-C-C-A-A 3′. This is in agreement with published reports on the specificity of this enzyme (Sadowski & Bakyta, 1972; Sedat *et al.*, 1976). Less frequent cutting between C-C residues produced the minor digestion products resolved in the two-dimensional fractionation shown in Figure 5(b).

TABLE 1

*Analysis of sequence preferentially synthesized on rDNA template by DNA polymerase I*

| Label | T4 endonuclease IV digestion product | Analysis | | | Deduced sequence |
|---|---|---|---|---|---|
| | | Depurination | Nearest-neighbor | | |
| A | 1 to 9 (Fig. 5(b)) | pCpCpCpCp(A), $P_i$ | Ap(A) 1 | Cp(A) 1† | |
| C | (not shown) | pCpCpCpCp | Ap(C) + | Cp(C) +++ | $(pCpCpCpCpApA_{OH})_n$ |
| A | Undigested | pCpCpCpCp(A), $P_i$ | Ap(A) | Cp(A) | |
| C | Undigested | pCpCpCpCp | Ap(C) | Cp(C) | Repeated 5′(C-C-C-C-A-A)3′ |

rDNA was labeled with DNA polymerase I and all 4 dNTPs, including either [α-$^{32}$P]dCTP or [α-$^{32}$P]dATP, for 1 h at 15°C, as described in Materials and Methods.

† Measurements of ratios were made in separate determinations of spots 1 to 9 (Fig. 5(b)).

+,+++, Indicate ratios from relative intensities of X-ray film autoradiogram spots, when insufficient radioactivity present for quantitation.

*n*, 3 to 10 for the major T4 endonuclease IV digestion products identified.

$P_i$, inorganic phosphate.

The endonuclease IV digestion products show that at least ten hexanucleotide repeats are present in tandem in the rDNA. In rDNA labeled with G or T, no radioactivity was detectable in these endonuclease IV digestion products. This suggests that interspersion of this sequence with G or T residues does not occur and is consistent with the finding that extensive synthesis of repeating C-C-C-C-A-A occurs in the absence of dGTP or TTP. With only dCTP plus dATP present in the incubation, over 95% of the incorporated label was found in this sequence. Approximately 20 hexanucleotide repeats were synthesized per half rDNA molecule. This estimate of the number of repeats made came from measuring the specific activity of the terminal restriction fragment after labeling rDNA with polymerase I and [α-$^{32}$P]dCTP and dATP only. It was consistent with another estimate made by measuring radioactivity in the depurination product $C_4$ (pCpCpCpCp) after incorporation of [α-$^{32}$P]dATP into rDNA in the presence of the other three dNTPs. For each of the different restriction enzymes tested, all the repeating hexanucleotide sequence was located in the end restriction fragment.

The sequence of the repeating hexanucleotide was also confirmed by partial digestion by micrococcal nuclease of rDNA labeled with either C or A, and analysis of the products by depurination and nearest-neighbor analysis (data not shown).

Under conditions in which most of the A or C label, in the presence of all four dNTP precursors, was incorporated into a simple repeating hexanucleotide sequence (1 h of synthesis at 15°C), G or T label was incorporated into complex sequences, as judged by depurination analysis and endonuclease IV digestion. However, a time-course of incorporation of G or T label into rDNA by polymerase I at 15°C showed that for times up to ten minutes, virtually all of the label was incorporated into the repeating hexanucleotide sequence 5′G-G-G-G-T-T 3′. With longer times of synthesis,

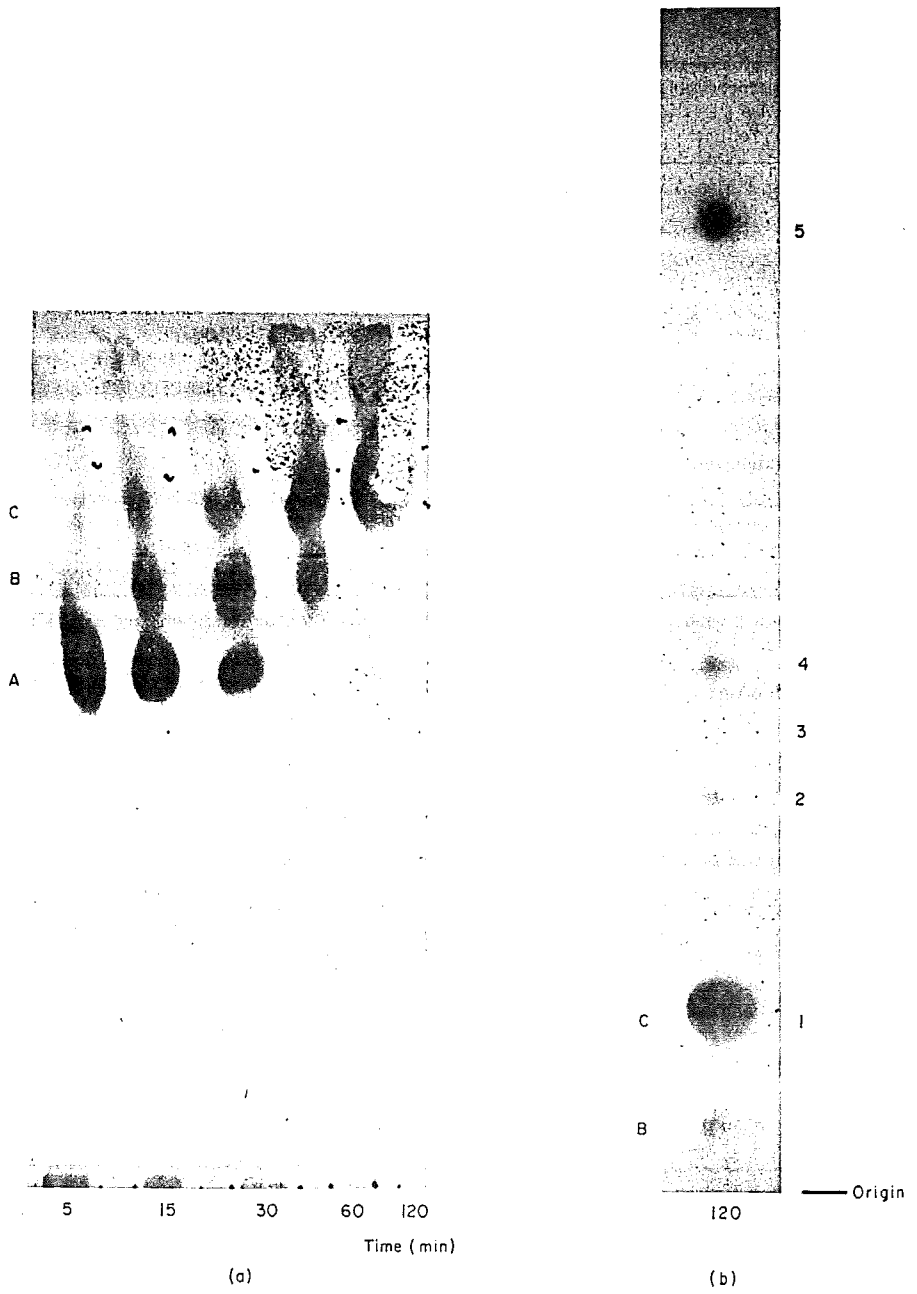


FIG. 6. Partial micrococcal nuclease digestion of G-labeled sequence synthesized early on rDNA template by DNA polymerase I. rDNA was labeled by DNA polymerase I and all 4 dNTPs including [$\alpha^{32}$P]dGTP for 10 min at 15°C. The labeled DNA was digested with micrococcal nuclease under partial digestion conditions and fractionated as described in Materials and Methods. (a) Autoradiogram of oligonucleotides produced after 5, 15, 30, 60 and 120 min digestion with micrococcal nuclease and fractionated by homochromatography on a DEAE-cellulose thin-layer plate. (b) Autoradiogram of a portion of the 120-min digestion sample shown in (a), separated by electrophoresis at pH 3·5 on DE81 paper. Spots A, B and C (a) and 1 to 5 (b) were eluted and analyzed by depurination and nearest-neighbor analysis (see Table 2).

TABLE 2

*Analysis of sequence synthesized early on rDNA template by DNA polymerase I*

| Label | Micrococcal nuclease digestion product | | Analysis: Depurination | | Analysis: Nearest-neighbor | | Deduced sequence |
|---|---|---|---|---|---|---|---|
| G | Fig. 6(a) | A | Tp(G) | $P_i$ | Tp(G) | Gp(G) | TpGpGpGpGpTp |
| | | | 1·0 | 2·7 | + | ++(+) | |
| | | B | Tp(G) | $P_i$ | Tp(G) | Gp(G) | TpGpGpGp(G) |
| | | C | Tp(G) | $P_i$ | Tp(G) | Gp(G) | TpGpGp |
| | Fig. 6(b) | 1 | Tp(G) | $P_i$ | Tp(G) | Gp(G) | TpGpGp(G) |
| | (= C in Fig. 6a) | | 1·0 | 2·1 | 1·0 | 1·9 | |
| | | 2 | | $P_i$ | | Gp(G) | GpGp |
| | | 3 | — | | | — | not identified |
| | | 4 | Tp(G) | $P_i$ | Tp(G) | Gp(G) | TpGp(G) |
| | | | | | + | + | |
| | | 5 | | $P_i$ | | Gp(G) | Gp |
| G | Undigested | | pTpTp(G) | $P_i$ | Tp(G) | Gp(G) | |
| | | | | | + | ++ | |
| T | Undigested | | pTpTp | | Tp(T) | Gp(T) | |
| | | | | | 1·2 : | 1·0 | Repeated 5′(G-G-G-G-T-T)3′ |

rDNA was labeled with DNA polymerase I and all 4 dNTPs, including either [$\alpha$-$^{32}$P]dGTP or [$\alpha$-$^{32}$P]TTP, for 10 min at 15°C as described in Materials and Methods.

+,++, Indicate relative yields as judged by intensities of X-ray film autoradiogram spots. $P_i$, inorganic phosphate.

increasing amounts of the repeating sequence 5′ C-C-C-C-A-A 3′ were made, and a higher proportion of the G or T label was found in complex sequences.

The sequence labeled by G or T in the first ten minutes of synthesis was determined as follows. The labeled DNA was analyzed by depurination and nearest-neighbor analysis. In addition, the labeled DNA was partially digested with micrococcal nuclease, followed by analysis of the digestion products. Figure 6 shows autoradiograms of the oligonucleotides produced during the course of digestion with micrococcal nuclease. Analyses of these products and of undigested labeled material are given in Table 2. The only pyrimidine tract labeled with [$\alpha$-$^{32}$P]dGTP or [$\alpha$-$^{32}$P]TTP was pTpTp, giving the sequence 5′ $\begin{matrix}\mathrm{A}\\\mathrm{G}\end{matrix}$ T-T-G 3′. From the nearest-neighbor analyses the repeating sequence 5′ G(G)$_{1-2}$T-T-G 3′ was deduced. Quantitation of the dinucleotide sequence G-G relative to T-G did not allow unambiguous distinction between the repeating sequences

(i) 5′ T-G-G-G-G-T 3′ and (ii) 5′ T-G-G-G-T 3′.

Sequence (i) was shown to be correct by partial digestion with micrococcal nuclease. Since micrococcal nuclease is very inactive toward dinucleotides (Sulkowski & Laskowski, 1962), the major G-labeled digestion products TpGpGp(G) and Gp(G) found after 120 minutes of digestion can only have arisen from cleavage of the oligonucleotide TpGpGpGpGpTp (spot A, Fig. 6(a)) into TpGpGp(G) (spot 1, Fig. 6(b)), Gp(G) (spot 5, Fig. 6(b)) and GpTp (unlabeled). No Gp(G) should have been detected if sequence (ii) were correct. Under the conditions used for partial digestion with micrococcal nuclease, the T–T bond was the most readily cleaved, in

agreement with the results of Sulkowski and Laskowski (1962) on the sequence specificity of this enzyme. Confirmatory evidence for the repeating hexanucleotide sequence (i) was obtained by subjecting the rDNA labeled with [$\alpha$-$^{32}$P]dGTP and TTP only to depyrimidation analysis. The labeled rDNA was treated with osmium tetroxide followed by piperidine hydrolysis (J. W. Sedat, personal communication). The major labeled purine tract identified was pGpGpGpGp (data not shown).

The incorporation of labeled nucleotides by DNA polymerase I into the termini of rDNA molecules therefore consists of two phases. With all four dNTPs present, synthesis of the repeated sequence 5′ G-G-G-G-T-T 3′ is detected first, followed and overtaken by synthesis of the complementary repeated hexanucleotide sequence. In the presence of only labeled dGTP and TTP, polymerase I synthesized approximately ten hexanucleotide repeats per rDNA end, as measured by the specific activity of the labeled rDNA. These findings are consistent with the presence of one or more discontinuities, containing 3′ hydroxyl groups, on both strands of the DNA, within the repeating hexanucleotide sequence. The results from labeling rDNA by polymerase I with one dNTP at a time indicate that there are more sites for initiation of synthesis on the 5′ C-C-C-C-A-A 3′ strand than on its complement. The spacing of these discontinuities is discussed in a later section.

### (b) *Template properties of rDNA for T4 DNA polymerase; absence of free cohesive ends*

Under suitable conditions, T4 polymerase will carry out strand elongation, from a 3′ hydroxyl terminus, only on gapped or partially single-stranded DNA templates (Goulian *et al.*, 1968). Purified rDNA, alone or mixed in a known ratio with bacteriophage $\lambda$ DNA, was incubated with T4 polymerase and all four dNTP precursors, one labeled with $^{32}$P. The DNA was then digested with *Eco*RI, the fragments identified by staining with ethidium bromide, and the distribution of incorporated label determined by autoradiography and counting. Conditions were found under which T4 polymerase initiated synthesis on linear $\lambda$ DNA only at the single-stranded cohesive ends. Under these conditions, no synthesis was observed at single-stranded nicks introduced by DNAase I into the $\lambda$ DNA. rDNA was a very poor template for T4 polymerase under these conditions. In the same reaction mixture in which the single-stranded ends of $\lambda$ DNA were quantitatively converted to duplex DNA, less than 2 mol of any of the four labeled nucleotides were incorporated per rDNA molecule. However, when *E. coli* polymerase I was added and the incubations were allowed to continue, extensive incorporation of label took place. The label incorporated by T4 polymerase on rDNA template was analyzed by depurination and nearest-neighbor analysis. The only sequences detected were consistent with the synthesis of very low amounts of the repeating hexanucleotide sequences described in the previous section. Thus it appeared that no different specific sequences were synthesized on the rDNA as a result of cohesive single-stranded ends such as those found on $\lambda$ DNA. These results strongly suggest that there are no extensive single-stranded regions on the rDNA that can be converted to duplex DNA by T4 polymerase, although the presence of small gaps is not precluded by the data.

### (c) *Effect of ligation on template properties of rDNA*

rDNA was treated with T4 ligase to test the effect on subsequent incorporation of labeled nucleotides by polymerase I. The control and ligated rDNA were incubated

with DNA polymerase I and all four dNTPs, then digested with *Eco*RI and the fragments separated by agarose gel electrophoresis. Radioactivity in the fragments was determined by cutting out the bands from the gel and counting. Whereas ligation reduced the incorporation into the middle restriction fragment of the rDNA by a factor of ten, synthesis in the end restriction fragment was reduced by only half. Analysis by depurination of the sequences labeled in the end restriction fragment suggested that the discontinuities at which strand elongation is preferentially initiated are resistant to ligation, as would be expected either for nicks bounded by 5′ hydroxyl groups, or for gaps, although the experiments with T4 polymerase show that any such gaps must be infrequent or short.

### (d) *Spacing of the single-stranded discontinuities*

Since the results described above showed that there are one or more specific discontinuities within the repeating hexanucleotide sequence at the termini of the rDNA molecules, it was predicted that complete denaturation of the rDNA might lead to the release of small single-stranded fragments derived from this region. Because the repeating sequence comprises no more than 2% of the molecule, in order to visualize the fragments, the rDNA was labeled by two procedures.

(1) 3′ termini were labeled by *E. coli* polymerase I with [$\alpha$-$^{32}$P]dCTP alone. As described above, this nucleotide is specifically incorporated into the terminal regions of rDNA. rDNA was then denatured and fractionated in two dimensions by electrophoresis at pH 3·5 in 7 M-urea on cellulose acetate, followed by homochromatography. Figure 7 shows an example of a fingerprint obtained. In addition to high molecular weight material, which runs at the origin of the second dimension, a specific series of oligonucleotides was seen, which ran in the same region as oligonucleotides of the sequence $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$. Results of nearest-neighbor analyses and sizing of these labeled oligonucleotides by polyacrylamide gel electrophoresis under denaturing conditions were most consistent with preferred labeling of oligonucleotides comprising two, three and four repeats of the hexanucleotide C-C-C-C-A-A.

(2) rDNA was treated with bacterial alkaline phosphatase followed by labeling of 5′ termini by polynucleotide kinase. The denatured DNA was fingerprinted as described in (1). Again a family of oligonucleotides, with mobilities corresponding closely to runs of $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$, was labeled. Depurination analysis of the 5′-labeled DNA showed that the sequence at the 5′ termini of these oligonucleotides was $pCpCpCp\begin{matrix}A\\G\end{matrix}$. The depurination product $C_4$ would be expected to result from labeling by kinase if the discontinuities were nicks. However, since $C_3$ was the labeled pyrimidine tract found, and because there were no single-stranded gaps that could be effectively utilized by T4 DNA polymerase, it was concluded that there are specific discontinuities in the form of one-nucleotide gaps within the repeating hexanucleotide sequence (Fig. 8).

Taken together, all the results so far indicate that the structure shown in Figure 8 occurs, probably more than once, at or near the termini of the purified rDNA molecules. The number of specific discontinuities has not been accurately determined. An upper estimate of the number of hexanucleotide repeats is about 70, since this number would be accommodated in the smallest terminal restriction fragment of the rDNA identified so far (from *Alu*I digestion). The larger endonuclease IV digestion products contain at least ten labeled repeats. If discontinuities occur on average

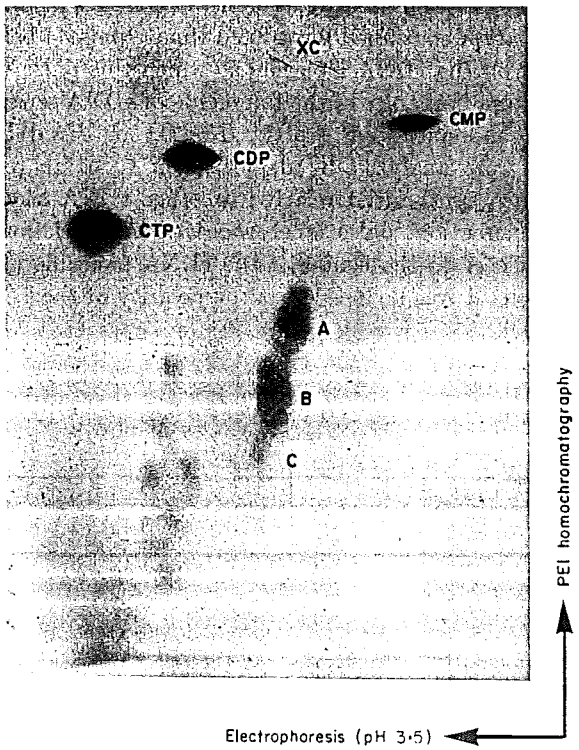


FIG. 7. Fingerprint of denatured fragments of rDNA labeled by DNA polymerase I with [$\alpha$-$^{32}$P]dCTP alone. 3 $\mu$g rDNA were labeled with [$\alpha$-$^{32}$P]dCTP by DNA polymerase I for 15 min at 15°C. The rDNA was denatured and fingerprinted in 2 dimensions as described. The predominant oligonucleotides indicated as A, B and C are found reproducibly in the positions expected for runs of C-C-C-C-A-A repeats. The other spots in the fingerprint were variably seen in experiments such as the one shown here, and have not been further characterized. XC marks the position of the xylene cyanol FF marker dye.

every three repeats, then from three to 20 single-stranded breaks could be present on the C-C-C-C-A-A strand.

### (e) *Strand orientation of the repeating sequence*

The orientation, with respect to the whole rDNA molecule, of the strand carrying the discontinuities in the $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$ sequence was determined by the following experiments. rDNA was incubated with DNA polymerase I and all four deoxynucleoside triphosphates, for different times (0 to 4 h) at 15°C (preincubation). Previous experiments showed that the majority of incorporation by polymerase I under these conditions takes place on the $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$ strand. Then one labeled

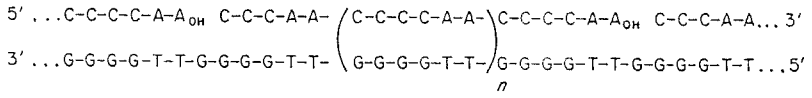


FIG. 8. A portion of the terminal repeated sequence of the rDNA molecule, showing positions of the specific one-nucleotide gaps. $n = 0$ to 2.

dNTP was added to the reaction mixture, still in the presence of the other unlabeled dNTPs, and the incubation continued for a fixed time ("pulse") before stopping the reaction. The location on the rDNA molecule at which this pulse was incorporated was determined by autoradiography of the fragments of rDNA separated after digestion with restriction endonucleases *Eco*RI, *Hin*dIII or *Hin*dII + III. Labeled restriction fragments were analyzed by depurination to compare the amount of hexanucleotide sequence synthesized relative to other rDNA sequences. With increasing time of preincubation, pulse label appeared in restriction fragments that mapped successively closer to the center of the rDNA molecule (data not shown). From these experiments, it was deduced that the polarity of the DNA strands must be as shown in Figure 9.

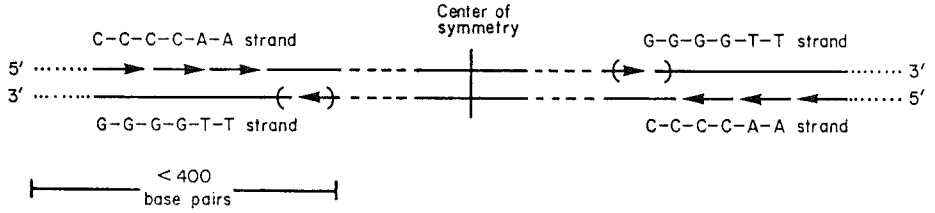


FIG. 9. Schematic representation of the polarity of the rDNA strands carrying the repeating hexanucleotide sequence. Arrowheads indicate specific discontinuities bounded by a 3′ hydroxyl group. Parentheses indicate uncertainty of the position and exact nature of the one or more discontinuities on the G-G-G-G-T-T strand. The extreme termini of the molecule are shown by dotted lines and 5′ → 3′ orientation of the strands is indicated.

It was noted that nearly one hour of preincubation was required before detection of pulse synthesis in restriction fragments that mapped further than about 600 base-pairs from the ends of the molecule. This implies a very slow *average* movement of the sites of elongation by polymerases along the molecule, compared with the rate of strand elongation expected under these conditions. This suggests that the DNA polymerase, which is present in excess over DNA, is acting in a highly unsynchronized manner, consistent with the idea that on the C-C-C-C-A-A strand there are several sites for initiation of synthesis within the repeating sequence, whose spacing may be too small (Kornberg, 1969) to allow all possible sites to be utilized simultaneously by polymerase molecules.

## 4. Discussion

The repeating hexanucleotide sequence that has been characterized in extrachromosomal rDNA occurs at least a thousand base-pairs away from the region coding for rRNA precursor (Karrer, 1976) and is therefore unlikely to be involved in transcription. Internally repetitious sequences have also been found in the non-transcribed spacers of the rDNA (Wellauer *et al.*, 1976*a*) and the 5 S RNA genes (Brownlee *et al.*, 1974) of *Xenopus*. However in *Xenopus* both the chromosomal and the amplified rDNA occur as tandem repeats, in contrast to the rRNA genes of *Tetrahymena*. The presence of the repeating sequence at the termini of *Tetrahymena* rDNA molecules suggests an explanation for the observed heterogeneity of the restriction fragment containing the ends of the molecules. Different molecules, and

possibly the two ends of the same molecule, could have different numbers of repeats. In *Xenopus*, differences in the numbers of the repeated spacer sequences have been observed in the chromosomal rRNA genes; however all the genes in a given amplified rDNA molecule have the same spacer lengths (Wellauer *et al.*, 1976*b*). Whether the heterogeneity of *Tetrahymena* rDNA molecules arises during amplification or during subsequent replication is not known.

The unusual template properties of *Tetrahymena* rDNA for DNA polymerase suggest that the structures shown in Figures 8 and 9 are found in the terminal regions of at least a high proportion of the molecules. However, the nature of the extreme ends of the rDNA molecules is still not clear, all the experiments described here giving no positive indication about their structure. No free single-stranded cohesive ends such as those found in bacteriophage λ DNA have been detected, nor can any sequence be unambiguously identified as the extreme end of the molecule by labeling 3′ or 5′ termini by polymerase or polynucleotide kinase. A possibility that is not eliminated is that a protein (or one or more amino acids left after the Pronase treatment used in purifying the rDNA) could be covalently attached to the 5′ terminus of the rDNA, as has been found for adenovirus DNA (Rekosh *et al.*, 1977). One hypothetical structure for the ends of the rDNA molecules, the simplest consistent with all the experimental data, is a hairpin loop, possibly composed of the repeating hexanucleotide sequence described above. Such a structure for the ends of the linear monomeric rDNA molecule, and for the ends associated in the circular or concatameric forms of the rDNA, is shown in Figure 10. This structure accounts for a number of the observed features of the rDNA. Unfolding of the hairpin loops at the ends of the molecule gives rise to palindromic cohesive ends of the type postulated by Karrer & Gall (1976) to account for the interconversion of circles to linear molecules seen by electron microscopy when the rDNA was subjected to partially denaturing conditions. When the ends of the molecule were associated, as shown in Figure 10(b), the whole terminal region made up of the hexanucleotide repeats would have dyad symmetry, which could be involved in protein recognition of this region.

The origin of the discontinuities within the repeating hexanucleotide sequence is not known. The postulated one-nucleotide gaps could be formed by removal of an unligated 5′-terminal ribonucleotide residue remaining after removal of RNA primers

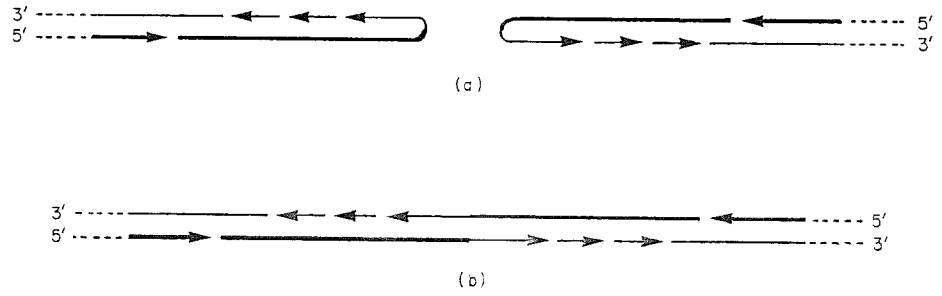


FIG. 10. Model proposed for the structure of the termini of extrachromosomal rDNA molecules. The specific discontinuities are indicated by arrowheads as in Fig. 9. (a) Two termini in the linear form of the rDNA molecule; each terminus has a hairpin loop formed by a self-complementary sequence. (b) The hairpin loops are opened out and the termini are joined together by hydrogen bonding in the circular or concatameric forms of the rDNA. The DNA strands carrying repeated G-G-G-G-T-T and repeated C-C-C-C-A-A sequences are shown by thick and thin lines, respectively.

when replication of rDNA is terminated. This could occur *in vivo* or during purification of the rDNA, since the purification procedure included digestion with RNAase A, which would remove the predicted 5′ rC residue, leaving a one-nucleotide gap. A further question to be resolved is the position and nature of the one or more discontinuities on the repeating G-G-G-G-T-T strand. Experiments analyzing the time-course of sequences labeled by nick-translation showed that this sequence was the first to be synthesized by polymerase I on rDNA template molecules, even in the presence of all four dNTPs, before the accumulation of labeled repeating C-C-C-C-A-A, which occurs on longer times of synthesis. This strongly suggests that there is a discontinuity within the repeated sequence on the G-G-G-G-T-T strand, and this is indicated in Figures 9 and 10. The finding that in the presence of only labeled dGTP and TTP about ten labeled repeats per end are synthesized by polymerase I suggests that the discontinuity might be at least this distance from the ends of the molecule.

Some indication of the functional significance of the specific discontinuities and the repeating hexanucleotide sequence comes from two types of observations. The same repeated sequence is found in the rDNA of the amicronucleate strain *Tetrahymena pyriformis* (formerly classified as *Tetrahymena pyriformis* strain GL). Such evolutionary conservation suggests that the sequence might be important in rDNA replication in vegetatively growing cells, since the original amplification of the rDNA must have taken place from a micronucleus now lost from this strain. Further evidence that the hexanucleotide repeat is required for processes other than amplification of rDNA comes from the finding that the same repeating sequence is present at many sites elsewhere in the chromosomal DNA of *Tetrahymena* (Blackburn and Yao, manuscript in preparation).

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