the essential feature of the mechanism of glyoxalase II.

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Physical Map of the Ribosomal Ribonucleic Acid Gene from *Tetrahymena pyriformis*†

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ABSTRACT: A physical map of the rRNA gene from the ciliated protozoan *Tetrahymena pyriformis* has been determined. The isolated rDNA palindrome was labeled by nick translation and digested with the sequence-specific endonucleases *Kpn*I, *Bcl*I, *Pvu*II, *Pst*I, *Sst*I, *Ava*I, *Ava*II, *Hpa*I, *Bam*HI, *Bgl*II, or *Hin*dIII, and the molecular weights of the products were determined by gel electrophoresis. The DNA fragments generated by digestion with each endonuclease were ordered into a unique sequence either by analyzing the products produced by concomitant cleavage with one or more additional restriction enzymes or by hybridizing to 17S and 25S rRNA. Hybridization of the 35S rRNA primary transcription product to *Hin*dIII and *Bam*HI restriction fragments localizes the promoter to the left of 16.9% and the terminator to the region between 71.6% and 78% on the physical map.

Tetrahymena is a genus of ciliated protozoa which possesses multiple nuclei: a diploid micronucleus found only in conjugating strains and a polyploid macronucleus present in all strains, which is the site of gene expression during vegetative growth. The rRNA gene is isolated from the macronuclei as an extrachromosomal element of unique size (Gall, 1974; Engberg et al., 1974). The gene is a perfect palindrome which possesses two transcription units for the 35S rRNA precursor (Karrer & Gall, 1976; Engberg et al., 1976) and represents 1%–2% of the total DNA (Yao et al., 1974).

In the micronucleus, the rRNA gene is present in an integrated, nonrepeated linear form (Yao & Gall, 1977). The palindromic structure must be a product of the amplification process which occurs after conjugation, during the production of the new macronucleus. In the nucleoli, the rRNA gene possesses a nucleosomal structure (Mathis & Gorovsky, 1976; Piper et al., 1976). Recent evidence indicates that the nucleosomes in the transcribed region of the rDNA from *T. thermophilia* exist in an altered conformation (Cech & Karrer, 1980). rDNA replication initiates at the center of the palindrome and proceeds bidirectionally to the termini (Truett & Gall, 1977). The termini of the palindrome are heterogeneous in size due to the presence of a variable repeat of the hexanucleotide C_4A_2. The ends of the molecule are unavailable for either 3′ or 5′ enzymatic labeling, suggesting either a hairpin loop or a chemical block (Blackburn & Gall, 1978).

Transcription is initiated near the center of the palindrome and proceeds outward (Gall et al., 1977; Niles, 1978; Engberg & Klenow, 1978), generating a 35S rRNA precursor which is processed to the mature 17S rRNA, 5.8S rRNA, and 25S rRNA (Kumar, 1970; Prescott et al., 1971; Pousada et al., 1975; Eckert et al., 1978; Niles, 1978). The 35S rRNA is a primary transcription product, at least at the 5′ end since high yields of pppAp can be isolated (Niles, 1978).

In *T. pigmentosa* (Wild & Gall, 1979; Din & Engberg, 1979) and *T. thermophila* (Cech & Rio, 1979; Din et al., 1979; Din & Engberg, 1979), an intron is found in the 25S rRNA region. The intron is transcribed, and its removal may be the first step in the processing of the 35S rRNA (Cech & Rio, 1979; Din & Engberg, 1979). Processing of the 35S rRNA proceeds by a cleavage into a pre-17S rRNA and pre-25S rRNA followed by subsequent nucleolytic steps to generate the mature forms. Within the ribosome, the 25S rRNA is clipped, forming two stable species of about 16 S in size (Bostock et al., 1971; Eckert et al., 1978).

The positions of the 5′ and 3′ termini of the 35S rRNA from *T. thermophilia* have been mapped at about 17% and 79%, respectively, by Cech & Rio (1979) and at 30% and 75%, respectively, by Din et al. (1979) from the center of the

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Table I: Molecular Weights of the Endonuclease Digestion Products[a]

	*Bcl*I	*Kpn*I	*Pvu*II	*Pst*I	*Sst*I	*Ava*I	*Ava*II	*Hpa*I	*Bam*HI	*Bgl*II	*Hin*dIII
A	9.0	6.1	5.1	4.3	4.0	3.7	6.4	3.9	7.8	4.8	2.5
B	1.7	0.25	2.3	4.1	3.8	3.1	2.4	2.8	1.3	1.38	2.2
C					0.5	1.4	0.71	0.88	0.95	1.32	1.4
D						0.02	0.05	0.68	0.45	1.08	0.75
E										0.3	0.45
F										0.2	0.29

[a] The molecular weights were calculated by comparing the migration distances of each fragment with the products of digestion of T7 DNA with either *Dpn*II or *Hpa*I (McDonnell et al., 1977).

palindrome. The map position of the 3′ end of the 35S rRNA is similar if not identical with the 3′ ends of the p25S rRNA and mature 25S rRNA in these strains.

Isolated nucleoli from *T. pyriformis* have been employed to synthesize an rRNA precursor identical in size with the 35S rRNA (Gocke et al., 1978). The addition of ammonium sulfate apparently prevents transcription termination and promotes read-through to the end of the palindrome. The suppression of read-through has permitted the identification of a factor which may play a role in transcription termination in vitro (Leer et al., 1979). From size estimates of the in vitro synthesized 35S rRNA, the 5′ and 3′ termini of the 35S rRNA have been located at approximately 5% and 75% from the center of the palindrome.

In this report we present a detailed physical map of the *T. pyriformis* rRNA gene within which the 5′ and 3′ termini of the 35S rRNA are located.

Materials and Methods

Cell Growth. *T. pyriformis*, an amicronucleate strain, was obtained from Dr. Kathleen Karrer. Cells were maintained and grown as described by Gorovsky et al. (1975).

Preparation of Nucleic Acids. Macronuclei were isolated by a modification of the procedure of Higashinakawa et al. (1975), developed by Ronald Aloysius. Cells were harvested by centrifugation at 5000*g* for 5 min at 4 °C in a Sorvall GSA rotor. The cell volume was calculated from the cell number with the correlation factor of 2 mL/10^8 cells. The cells were resuspended at 4 °C in 9 volumes of Me_4Si buffer containing 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.25 M sucrose. To this suspension 0.2 volumes of Me_4Si containing 1.0% v/v NP40 was added with gentle stirring, and lysis occurred within 15 min at 4 °C; 0.815 g of sucrose was added per milliliter of lysate, and the nuclei were collected by centrifugation in a Sorvall GSA rotor 10 000 rpm for 15 min. The nuclear pellet was resuspended in Me_4Si, the sucrose concentration was readjusted, and the nuclei were resedimented as above. Yields of 80%–95% were normally achieved.

DNA was extracted from the macronuclei by the following procedure. The nuclear pellet was lysed by resuspension of 10^8 nuclei in 5 mL of 0.10 M Tris-HCl, pH 8, 40 mM EDTA,[1] and 1% $NaDodSO_4$.[1] After the mixture was heated at 60 °C for 10 min, the lysate was extracted with an equal volume of phenol saturated with 10 mM Tris-HCl, pH 8, and 1 mM EDTA. The phenol layer was reextracted with 10 mM Tris-HCl, pH 8, and 1 mM EDTA, the aqueous phases of the two extractions were combined and reextracted with saturated phenol, and the nucleic acid was precipitated at −20 °C with 2.5 volumes of 95% ethanol. RNA and glycogen were removed, and the rDNA was purified by two $HgCl_2$–Cs_2SO_4 equilibrium gradient sedimentations, as describhed by Karrer & Gall (1976). The rDNA containing fractions were pooled, dialyzed against 0.01 M Tris-HCl, 1 mM EDTA, and 250 mM NaCl, and precipitated at −20 °C by the addition of 2.5 volumes of ethanol.

^{32}P-Labeled 35S, 17S, and 25S rRNA were prepared as previously described (Niles, 1977, 1978).

Restriction endonucleases were purchased from either Bethesda Research Labs or New England Biolabs, and DNA was digested according to the procedures described in their literature. DNA polymerase I and T4 polynucleotide kinase were purchased from P-L Biochemicals. [γ-^{32}P]ATP was prepared as described by Maxam & Gilbert (1977) to a specific activity of about 1000 Ci/mmol. [α-^{32}P]TTP was purchased from Amersham, Inc., at a specific activity of 200–400 Ci/mmol. 5′ end labeling of DNA fragments was carried out as described by Maxam & Gilbert (1977). Nicked translation was carried out according to Maniatis et al. (1975).

DNA fragments were separated by electrophoresis in 20 × 20 × 0.15 cm vertical gels, or 20 × 30 × 0.15 cm horizontal gels, made with 1.0% or 1.6% agarose, at 1 V/cm for 16 h or 20 × 40 × 0.15 cm 5% polyacrylamide gels at 2 V/cm for 16 h in a buffer which contained 50 mM Tris–borate, pH 8.3, and 1 mM EDTA. DNA fragments were isolated from agarose gels by electroelution (Galibert et al., 1974) and were transferred from agarose gels to nitrocellulose paper by the method of Southern (1975). Hybridization of 17S rRNA, 25S rRNA, or 35S rRNA [approximately (1–5) × 10^6 dpm/μg] was carried out in 2 × SSC (0.15 M NaCl and 0.015 M sodium citrate = 1 × SSC) at 65 °C for 16 h. The filters were incubated for 2 h at 37 °C with 10 μg/mL pancreatic RNase (preboiled) and washed 3 times with 2 × SSC at room temperature. The ^{32}P-labeled RNA or DNA was located by autoradiography by using Kodak XR-5 film and DuPont Cronex Lighting Plus screens at −70 °C. The relative levels of hybridization of [^{32}P]rRNA to each DNA fragment was determined by densitometry of the exposed autoradiograph by using a Corning densitometer.

Results

Maps of the Restriction Endonuclease Cleavage Sites. Nick-translated rDNA was digested with several restriction endonucleases, and the products were separated by gel electrophoresis (Figure 1A,B). The molecular weights presented in Table I were determined by comparison of the mobilities of the cleavage products to standards derived from T7 DNA cleaved with either *Dpn*II or *Hpa*I (McDonnell et al., 1977).

Construction of a physical map of the rDNA from the restriction endonuclease digestion products is aided by three aspects of the structure of the rDNA. Since the rDNA is a palindrome, the center fragment must be present in one-half the amount of other products. This low yield is readily apparent in most cases. In addition, the sum of the molecular weights of the fragments must add up to 12.6 × 10^6. Therefore, any fragment whose molecular weight is in excess of 6.3 × 10^6 must be derived from the center of the palindrome.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

Table II: Enzymes Used in the Double Digestions Employed in Determining the Cleavage Maps

*Kpn*I *Eco*RI	*Pvu*II *Eco*RI	*Pst*I *Eco*RI	*Bcl*I *Eco*RI	*Sst*I *Eco*RI	*Ava*I *Eco*RI *Ava*II	*Ava*II *Eco*RI *Ava*I	*Hpa*I *Eco*RI *Ava*I	*Bam*HI *Eco*RI	*Bgl*II *Eco*RI *Hpa*I *Hin*dII	*Hin*dIII *Eco*RI *Kpn*I *Pvu*II *Bam*HI

Table III: Location of Endonuclease Cleavage Sites as Percent of Length from the Center of the Palindrome[a]

site	*Kpn*I	*Pvu*II	*Pst*I	*Bcl*I	*Sst*I	*Ava*I	*Ava*II	*Hpa*I	*Bam*HI	*Bgl*II	*Hin*dIII
1	2.0	18.3	34.4	72.5	29.7	29.2	50.3	31	58.6	35.9	16.9
2					92.2	51.0	51.0	41.7	78.5	38.9	21.5
3						51.6	62.1	55.6	92.5	55.0	60.0
4										74.8	71.6
5										95.6	93

[a] For each digest, the total weight of the rDNA was calculated by summing the molecular weights of the products, taking into account the stoichiometry of each product. The cleavage site was calculated by dividing the molecular weight of each fragment, or groups of fragments, by the molecular weight of the total rDNA. The center and the termini of the palindrome are 0% and 100%, respectively.

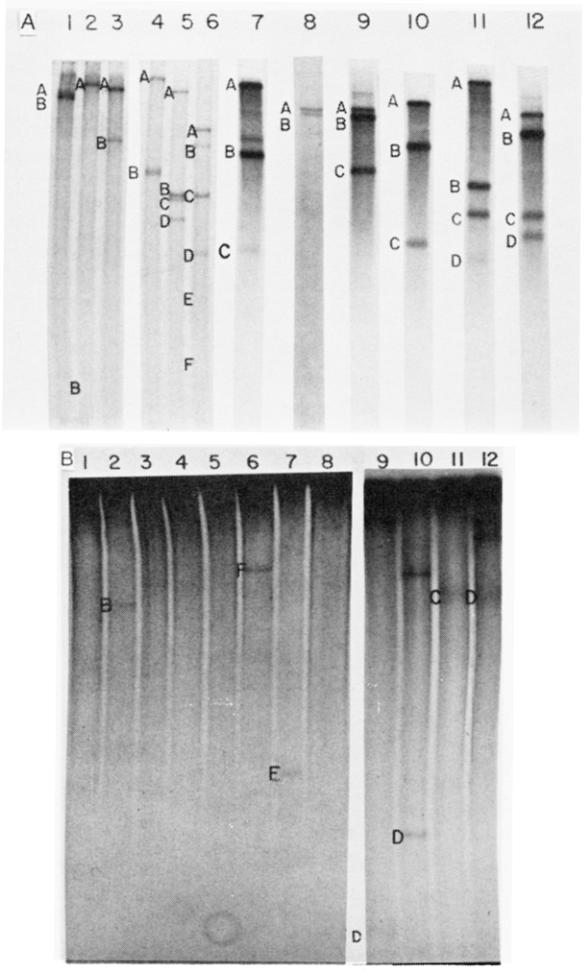

FIGURE 1: Cleavage of rDNA with restriction endonucleases. (A) Autoradiograph of [^{32}P]rDNA cleaved with restriction endonucleases and separated on a 20 × 30 × 0.15 cm horizontal 1% agarose gel. Enzymes: (1) *Pst*I, (2) *Kpn*I, (3) *Pvu*II, (4) *Bcl*I, (5) *Bgl*II, (6) *Hin*dIII, (7) *Eco*RI, (8) *Sst*I, (9) *Ava*I, (10) *Ava*II, (11) *Bam*HI, and (12) *Hpa*I. Samples 1–6 and 7–12 were separated together. (B) Autoradiograph of [^{32}P]rDNA cleaved with restriction endonucleases and separated on a 20 × 40 × 0.15 cm 5% acrylamide gel. Enzymes: (1) *Pst*I, (2) *Kpn*I, (3) *Pvu*II, (4) *Hpa*I, (5) *Bam*HI, (6) *Hin*dIII, (7) *Bgl*II, (8) *Bcl*I, (9) *Ava*I, (10) *Ava*II, (11) *Sst*I, and (12) *Bam*HI. Samples 1–8 were separated on one gel and 9–12 on another.

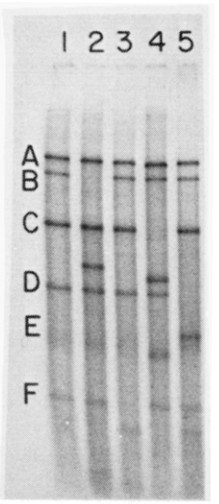

FIGURE 2: Double digestion of rDNA. Samples were separated on a 20 × 30 × 0.15 cm 1% agarose gel. Samples 1–5 were cleaved with *Hin*dIII and (1) no addition, (2) *Kpn*I, (3) *Pvu*II, (4) *Bam*HI, and (5) *Eco*RI.

Finally, the termini are heterogeneous in size, and, if the end fragment is small enough, the heterogeneous fragment can be easily identified by the width of the band in the autoradiograph (Blackburn & Gall, 1978). The *Eco*R1 cleavage sites, mapped by Engberg et al. (1976), served as a reference in these determinations.

The digestion products produced by the 12 restriction endonucleases are presented in Figure 1A,B. The enzyme combinations used in double digestions which permitted the unambiguous ordering of the fragments are listed in Table II.

The derivation of the *Hin*dIII map will be discussed in detail. Six fragments are produced by digestion of rDNA with *Hin*dIII (Figures 1, 2, and 5). Double digestion with *Kpn*I, *Pvu*II, *Bam*HI, and *Eco*RI (Figure 2) removes the *Hin*dIII B, F, C, and D fragments, respectively. Since the cleavage sites for these enzymes are known, the map order of *Hin*dIII B, F, D, and C are determined. *Hin*dIII E is heterogeneous, and therefore terminal. Room for *Hin*dIII A can only be found between the *Hin*dIII F and D fragments, thus yielding the order BFADCE.

The order of the *Hin*dIII products was confirmed by hybridizing [^{32}P]rRNA to restriction fragments transferred to nitrocellulose (Figure 3). ^{32}P-Labeled 25S rRNA hybridizes to *Hin*dIII A, D, and C, confirming the order determined by the double digestions. ^{32}P-Labeled 25S and 17S rRNA hybridizes to F, A, D, and C. Since the 17S rRNA is known to map to the left of the 25S rRNA (Karrer & Gall, 1976; Engberg et al., 1976), the F fragment must lie to the left of *Hin*dIII A.

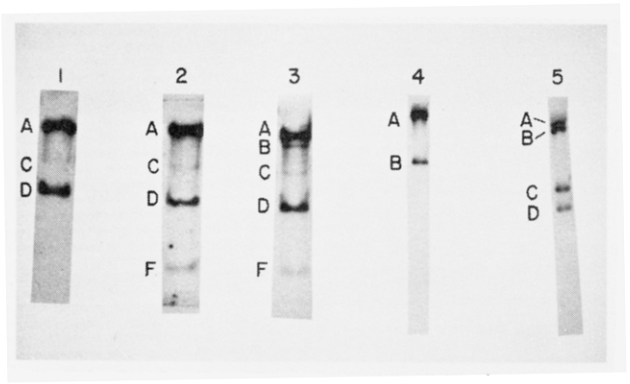

FIGURE 3: Hybridization of [^{32}P]rRNA to restriction enzyme fragments of DNA. *T. pyriformis* DNA was cleaved with a restriction endonuclease, and the products were separated by electrophoresis in a 1.6% agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with either ^{32}P-labeled 25S rRNA, ^{32}P-labeled 25S rRNA plus 17S rRNA, or ^{32}P-labeled 35S rRNA. The ^{32}P hybrid regions were located by autoradiography. The restriction endonuclease used in each sample and the rRNA probe employed in the hybridization are the following: (1) *Hin*dIII, 25S rRNA, (2) *Hin*dIII, 25S rRNA + 17S rRNA, (3) *Hin*dIII, 35S rRNA, (4) *Bam*HI, 35S rRNA, and (5) *Hpa*I, 35S rRNA.

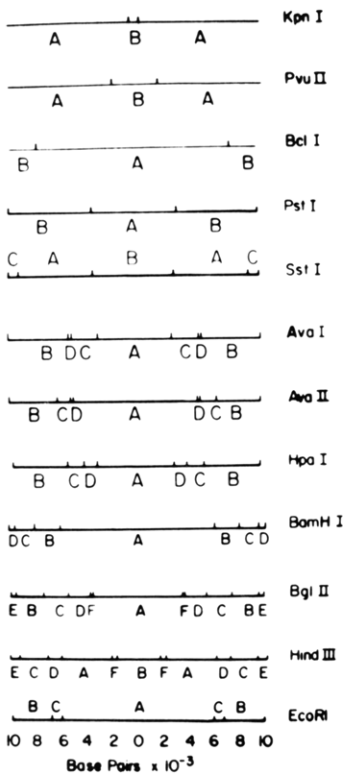

FIGURE 4: Physical map of rDNA. A collection of the restriction endonuclease cleavage maps of the rDNA palindrome is presented. The center of the palindrome is marked 0, and the maps are drawn on the basis of the length of the fragments in base pairs. The *Eco*R1 map was determined by Engberg et al. (1976).

The physical maps derived for these 11 enzymes are presented in Figure 4. The map positions of the cleavage sites for each enzyme are listed in Table III. The total length of the DNA, determined by summing the length of each fragment, varies from 19 200 to 20 400 base pairs, which is in good agreement with previous estimates of the size of the rDNA palindrome (Gall, 1974; Engberg et al., 1976). The variation that is found is due to the error in the determination of the molecular weights of the DNA.

Terminal Fragment. Blackburn & Gall (1978) demonstrated terminal heterogeniety of the rDNA due to a variable-sized repeat of the hexanucleotide C_4A_2. The termini of the intact rDNA cannot be modified by 5′ or 3′ end labeling, resulting in a proposed hairpin loop model for the DNA at the termini. Blackburn & Gall (1978) suggested the possibility that the end of the rDNA may be blocked by a protein such as that found on adenovirus 5 DNA (Rekosh et al., 1977), but since their DNA preparation scheme employed a Pronase digestion step, most of the proposed terminal protein may have been removed, leaving a blocking amino acid or polypeptide.

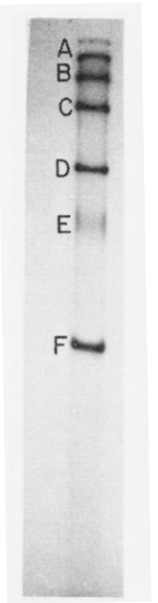

FIGURE 5: rDNA, purified by a method which avoids a Pronase digestion step, was cleaved with *Hin*dIII, and the products were treated with alkaline phosphatase, phenol extracted, and labeled at the 5′ ends with [γ-^{32}P] ATP by T4 polynucleotide kinase. The fragments were separated by electrophoresis through a 20 × 40 × 0.15 cm 1% agarose gel and located by autoradiography. The *Hin*dIII E fragments are heterogeneous in size and derived from the termini.

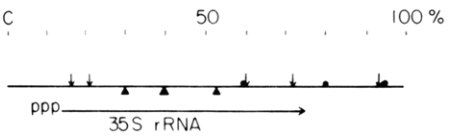

FIGURE 6: Location of the 35S rRNA on the rDNA map. A map of one-half of the rDNA palindrome in which the DNA length is shown as the percent of length from the center (0%) to the termini (100%) is presented. The restriction endonuclease cleavages sites for *Hin*dIII (↓), *Hpa*I (▲), and *Bam*HI (●) are marked. The 35S rRNA is drawn with the 5′ and 3′ ends at 13% and 75% from the center, respectively. These values are a very rough estimate based on an attempt to quantitate the data in Figure 3 and may be in error by ±3%. One percent equals approximately 97 base pairs as calculated by averaging the length of the rDNA determined by summing the lengths of the individual restriction fragments for each restriction endonuclease map in Figure 4.

Terminal heterogeniety can be readily seen in Figure 5 in which rDNA fragments generated by *Hin*dIII digestion were end labeled with [^{32}P]ATP by T4 polynucleotide kinase and separated by gel electrophoresis. The terminal *Hin*dIII E comigrates with end fragments derived from rDNA prepared with a Pronase digestion step. Their mobility is not affected by Pronase treatment of isolated DNA (50 γ/mL, 37 °C, 30 min). The fragments do not stick preferentially to either agarose or acrylamide gels as does the Adenovirus 5 terminal protein (Rekosh et al., 1977). These negative results support the absence of a terminal protein of the Ad5 type on the rDNA.

Location of the 5′ and 3′ Termini of the 35S rRNA. The regions of the rDNA which encode the transcription initiation

and termination sites were identified by hybridizing ^{32}P-labeled 35S rRNA to restriction fragments produced by cleavage with *Hin*dIII, *Bam*HI, and *Hpa*I (Figures 3 and 6). The 35S rRNA hybridizes to the *Hin*dIII B fragment which places the promoter to the left of 16.9% on the physical map. It also hybridizes to the *Hin*dIII C and *Bam*HI B fragments, while failing to hybridize to the *Bam*HI C fragment (Figure 3). This localizes the termination site to the region between 71.6% and 78% on the physical map.

Discussion

The rRNA gene from *T. pyriformis* is a palindrome of approximately 12.6×10^6 daltons or 19400 pairs in length (Gall, 1974; Engberg et al., 1976). The restriction endonuclease cleavage sites for the 11 enzymes presented here generate a detailed physical map of this gene (Figure 4). The map positions of the restriction fragments were determined by double cutting with one or more other restriction enzymes and confirmed by hybridization with 17S rRNA or 25S rRNA. The physical maps derived from *Bam*HI and *Hin*dIII digestion were recently described by Din & Engberg (1979). The *Bam*HI map is identical with ours, but their *Hin*dIII map contains an additional cleavage site in the *Hin*dIII F/A region, producing a small fragment. We have rechecked the 5% acrylamide gel analysis of the *Hin*dIII fragments (Figure 1B) and do not see an additional small fragment when on the same gel; we readily see the *Ava*I D (30 base pairs) and *Ava*II D (70 base pairs) fragments. Possibly we are missing a fragment or there may be a strain difference.

The termini of this gene are heterogeneous due to a variable repeat of the hexanucleotide C_4A_2 and are also unavailable for end labeling (Blackburn & Gall, 1978). The possibility of the presence of a terminal protein on the rDNA, prepared without Pronase treatment, was investigated and proved negative. The likely possibility of a terminal hairpin structure proposed by Blackburn & Gall (1978) suggests a role for the snapback termini in DNA replication. The size difference observed may be to variability in the termination process, which over many generations results in this heterogeniety. It would be interesting to test the degree of heterogeniety of the termini found at different generation times after conjugation when rDNA amplification occurs and a "new" rDNA palindrome is formed. If it is due to variable termination, one would predict that the degree size heterogeniety would increase with increasing number of cell generations.

The sites of transcription initiation and termination of the rRNA gene were located by hybridizing 35S rRNA to rDNA cleaved with *Hin*dIII, *Bam*HI, and *Hpa*I (Figure 3). The promotor was shown to be to the left of 16.9% by demonstrating that 35S rRNA hybridizes to the *Hin*dIII B fragment. The termination region is found between 71.6% and 78% since the 35S rRNA hybridizes to the *Hin*dIII C fragment but not the *Bam*HI C fragment (Figure 3). These values are in close agreement with the mapping data of Cech & Rio (1979) and Din et al., (1979) of 17% and 75%–79% for the *T. thermophilia* gene.

The nucleotide sequence of the *Hin*dIII C DNA and the *Hin*dIII B DNA in the regions of the 3′ and 5′ termini of the 35S rRNA and the ends of the 35S rRNA and the 25S rRNA are being determined. These sequences will yield an exact position for the 5′ and 3′ ends of the 35S rRNA in the physical map of the *T. pyriformis* rDNA.

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