ORGANIZATION OF RIBOSOMAL GENES IN *PARAMECIUM TETRAURELIA*

R. CRAIG FINDLY and JOSEPH G. GALL

From the Department of Biology, Yale University, New Haven, Connecticut 06520. Dr. Findly's present address is the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545.

ABSTRACT

The macronuclear ribosomal DNA (rDNA) of the ciliated protozoan *Paramecium tetraurelia* (stock 51) was analyzed by digestion with restriction endonucleases. The fragments which contained ribosomal RNA (rRNA) coding sequences and spacer sequences were identified. The spacer sequences exhibited some heterogeneity in size. The genes coding for 5.8S RNA, but not for 5S RNA, are linked to the 17S and 25S rRNA genes.

Complementary RNA, synthesized from rDNA of stock 51, was hybridized with restriction digests of whole cell DNA from six other allopatric stocks of this species. The restriction patterns of the rDNA from these seven stocks were, in general, very similar, and the sizes of the coding sequences were identical in all seven stocks. Only the restriction pattern of rDNA from stock 127 differed significantly from that of stock 51. The rDNA from stock 127 was isolated and characterized, and with the exception of the restriction pattern of its spacer, it resembled the rDNA from stock 51. It is concluded that the rDNA repeat in *Paramecium*, including the spacer, has, in general, been conserved during the course of evolution. It is suggested that in some species, even in the absence of genetic exchange among geographically separated populations, selection pressure may act to conserve spacers of tandemly repeated rDNA. The conservation may be related to the number of rDNA copies in the germinal nucleus.

KEY WORDS isolated rDNA · restriction endonuclease digestion · spacer DNA · evolution · protozoa

The genes coding for ribosomal RNA (rDNA) or 5S RNA (5S DNA) are generally organized in eukaryotes as tandem repeats which are longer than the coding regions alone. The coding regions are separated by DNA sequences termed "spacer." Our current knowledge of the organization of rDNA and 5S DNA has come mostly from studies of these genes in *Xenopus* and *Drosophila*. This work has raised intriguing questions about the concurrent evolution of coding and spacer sequences in families of repetitive genes, and has demonstrated that for two species of *Xenopus* the coding sequences of rDNA or 5S DNA have been conserved during evolution, but that spacer sequences have not (reviewed in reference 17). In contrast, in the *Drosophila melanogaster* subgroup the rDNA spacer has been highly conserved during evolution (44). Although it is now apparent that spacers are present in other tandemly repeated genes, their function remains unclear.

The macronuclear rDNA of the ciliated protozoan *Paramecium tetraurelia* exists as relatively small extrachromosomal molecules with both linear and circular forms. *Paramecium* rDNA is arranged as nonpalindromic, tandem repeats with an average repeat size of 5.5×10^6 daltons, unlike other lower eukaryotes such as *Tetrahymena* (16, 26), *Physarum* (32, 46), or *Dictyostelium* (11 and footnote 1), in which the rDNA is a palindrome. Limited heterogeneity of repeat lengths in *Paramecium* rDNA was found both by electron microscopy and by restriction endonuclease analysis (20).

P. tetraurelia has a world-wide distribution of presumably allopatric populations (38). We have taken advantage of this distribution to investigate the evolution of rDNA in a single species and have examined the organization of rDNA in seven stocks of *P. tetraurelia*. The stocks are the descendants of single animals collected at locations on five continents. Although the stocks belong to the same species and will mate with each other, by virtue of their geographic origins they have undoubtedly been isolated from one another for considerable periods of time. They have diverged from each other to some degree, because, for instance, none of them possesses the same range of serotypes and they exhibit difference in their karyotypes (14, 38). Our results indicate that both the gene and spacer regions of the rDNA have remained relatively constant in size and sequence during the evolution of these *Paramecium* stocks.

MATERIALS AND METHODS

Cell Stocks and Culture Conditions

Stocks of *P. tetraurelia* (endosymbiont free) were kindly supplied by T. Sonneborn (Indiana University) and are listed in Table I. These stocks are the descendants of single, wild cells, collected at the geographic locations listed in Table I (38). This species was previously referred to as syngen 4 of *P. aurelia* (39). The stocks were maintained at room temperature in Cerophyl infusion (Cerophyl Corp., Kansas City, Mo.) inoculated with *Klebsiella aerogenes* as described by Sonneborn (37).

Paramecia were grown at 27°C in 2-liter diphtheria toxin bottles containing 1 liter of Cerophyl infusion. When the cultures were inoculated, and again 24 h later, each 1 liter was fed with *Klebsiella* pelleted from 100 ml of a shaker culture grown overnight in 1% Bacto-tryptone (Difco Laboratories, Detroit, Mich.) and 0.5% NaCl at 30°C. The cultures of paramecia became very turbid when fed, but the paramecia "cleared" them in <24 h. Paramecia were harvested from cleared cultures after 40–48 h of growth, when the cells were in late logarithmic to early stationary phase (43). They were filtered through several layers of cheesecloth, and concentrated in a modified DeLaval cream separator. Packed cells were collected by centrifugation at 1,800 rpm for 1 min in 100-ml pear-shaped oil-testing centrifuge tubes in an IEC HN-S centrifuge (Damon/IEC Div., Needham Heights, Mass.). The cell pellet was washed in Dryl's solution (15) and repelleted. A pellet of 10–15 ml of packed cells (1 to 1.5×10^8 cells) was routinely obtained from 30 liter of culture.

[1] Grainger, R., and N. Maizels. Personal communication.

DNA Extraction and Isolation of Ribosomal DNA

Cell pellets were resuspended in a small volume of Dryl's solution and DNA was extracted under conditions of high salt and temperature (27) as previously described (23). rDNA was isolated from stock 127 whole cell DNA by equilibrium centrifugation in actinomycin D/CsCl and CsCl gradients as described for stock 51 (20). rRNA was hybridized to denatured DNA attached to nitrocellulose filters (25) in 40% formamide, 0.1 M Tris-HCl, pH 7.6, and 4 × SSC (1 × SSC is 0.15 M NaCl, 15 mM Na_3 citrate) at 37°C for 18–24 h. *Paramecium* rDNA was melted as previously described (26).

Preparation of Ribosomal RNA

RNA was prepared from whole cell lysates in the presence of polyvinyl sulfate and diethyl pyrocarbonate (42). rRNA was purified by centrifugation in a 30-ml sucrose (RNAse free; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) gradient (10–30% wt/vol in 0.1 M NaCl, 1 mM ethylenediaminetetraacetate, 10 mM Tris-HCl, pH 7.4, and 0.5% SDS) in a Beckman SW 25.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 22,000 rpm, 15°C for 18 h, or in a 3.6-ml sucrose gradient (5–20%, wt/vol) in a Beckman SW 56 rotor at 46,000 rpm for 1.75 h at 20°C. The 17S and 25S peaks from these gradients were pooled separately and stored as a precipitate in 70% ethanol at −20°C.

Restriction Endonuclease Digestion and Gel Electrophoresis

Digestions of DNA with the restriction endonuclease EcoR1 (a generous gift of T. Barnett), Bgl II (supplied by H. Erba), or Hind III (New England Biolabs, Beverly, Mass.) were carried out in an appropriate buffer at 37°C. For double digestions the DNA was first digested with Hind III or Bgl II, which was then inactivated by heating at 65°C for 5 min. The buffer was adjusted appropriately, and the DNA redigested with EcoR1.

The conditions of agarose gel electrophoresis have been described (51). DNA in the gels was denatured and neutralized (6) and eluted onto nitrocellulose filters (40). The denatured DNA, bound to the nitrocellulose filters, was hybridized with ^{32}P-labeled RNA (at least 1×10^5 cpm/ml) in 4 × SSC, 40% formamide, 0.1 M Tris-HCl, pH 7.6, at 37°C for 18–24 h. The filters were then washed in 2 × SSC, incubated in 2 × SSC containing 20 μg/ml pancreatic RNAse for 2 h at 37°C, and rinsed extensively in 2 × SSC and then in 70% ethanol. After the filters were dried, they were overlaid with Kodak NS-54T x-ray film for autoradiography.

End-labeling of RNA

RNA was end-labeled at 5′ termini with γ-[^{32}P]ATP (1 to 2×10^3 Ci/mmol) (31). Specific activities of the end-labeled RNAs ranged from 2×10^5 cpm/μg (5.8S), 8×10^5 cpm/μg (5S) to 1.3

TABLE I

Paramecium tetraurelia Stocks and Their Geographic Sources

Stock	Serotypes*	Karyotypes‡	Source
47	A,B,C,D,E,G,J,N	49 (± 2)	California
51	A,B,C,D,E,G,H,I,J,N,Q,U	43 (± 2)	Indiana
127	A,C,D,E,G,N,R		Florida
148	A,B,C,D,E,F,G,J,M		Lake Kogawara, Japan
172	A,B,C,D,G,H,I,J,M,N,P		Macchu Picchu, Peru
203	D,J		Sydney, Australia
316	C,D,F		Amsterdam, Holland

* Reference 38.

‡ Minute, dotlike chromosomes are also present in stock 51, but not in stocks 47 or 172. (14).

to 5×10^6 cpm/μg for 17S and 25S. The 5S RNA and 5.8S RNA were isolated from *Tetrahymena thermophila* and were purified by polyacrylamide gel electrophoresis. They were kindly provided by E. Stephenson.

Preparation of RNA Complementary to Ribosomal DNA

Complementary RNA (cRNA) to *Paramecium* rDNA (isolated from stock 51) was prepared using *Escherichia coli* RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (24). The cRNA was radioactively labeled using α-[^{32}P]-UTP (120 Ci/mmol; New England Nuclear, Boston, Mass.) as one of the four nucleoside triphosphate precursors.

Electron Microscopy

DNA samples were prepared for electron microscopy using the formamide procedure of Davis et al. (13). The RF II form of φX 174 (a generous gift of N. Godson) was included as an internal mass standard. Its mol wt was assumed to be 3.4×10^6 (13). Molecules were examined in a Philips EM 200 electron microscope. Photomicrographs were projected with a photographic enlarger, the projected images of the molecules traced, and the tracings measured with a Dietzgen map measurer (Dietzgen, Switzerland).

RESULTS

Paramecium rDNA from stock 51 was purified as previously described (20). The rDNA was digested with the restriction endonucleases EcoR1 or Hind III, and after electrophoresis in agarose gels, was eluted onto nitrocellulose filters (40). *Paramecium* whole cell DNA was restricted and eluted in a similar manner. The restricted fragments which contained rRNA coding regions were identified by hybridization with ^{32}P-labeled 17S and 25S rRNA (Fig. 1), and the molecular weights determined. The hybridization patterns of the rDNA and whole cell DNA were similar, suggesting that gradient-purified rDNA is representative of the genomic rDNA. The repeat size obtained by summing the molecular weights of the products of restriction endonuclease digestion of the rDNA agreed closely with that from electron microscopy (20).

The construction of a restriction map of the rDNA (Fig. 2) was initiated by the assignment of 17S and 25S rRNA coding functions to specific restriction fragments. Although we do not know the direction of transcription, we have arbitrarily placed the 17S coding region to the left of the 25S and thus the 1.37×10^6 EcoR1 fragment is placed to the left of the 1.62 and 0.57×10^6 fragments. Similarly, the 2.80×10^6 Hind III fragment is left of 0.69×10^6 fragment.

The complete map was established by double digestion of rDNA with Hind III and EcoR1 followed by hybridization of ^{32}P-labeled 17S and 25S rRNA to the resulting restriction fragments. As shown in Fig. 1, the largest Hind III fragment (2.8×10^6) contains coding sequences for both 17S and 25S rRNA. After double digestion, the two largest prominent fragments had mol wt of 1.62 and 1.09×10^6 daltons. The 1.62×10^6 fragment hybridized with 25S rRNA and thus the largest EcoR1 fragment must be wholly contained in the 2.8×10^6 Hind III fragment. The 1.09×10^6 fragment hybridized with 17S rRNA and must be derived from both the large Hind III fragment and the 1.37×10^6 EcoR1 fragment. The 1.37×10^6 EcoR1 fragment is, however, only partially contained in the 2.8×10^6 Hind III fragment. Fragments with mol wt of 0.45 and 0.28×10^6 hybridized with 25S rRNA and must be derived from the 0.69×10^6 Hind III and 0.57×10^6 EcoR1 fragments.

The probable positions of the 1.14 and 0.76×10^6 EcoR1 fragments were determined by partial digestions of the rDNA with EcoR1 (not shown) and a presumptive EcoR1 map was established. As there are only four Hind III fragments and the relative positions of the two containing coding sequences have been established, there are only

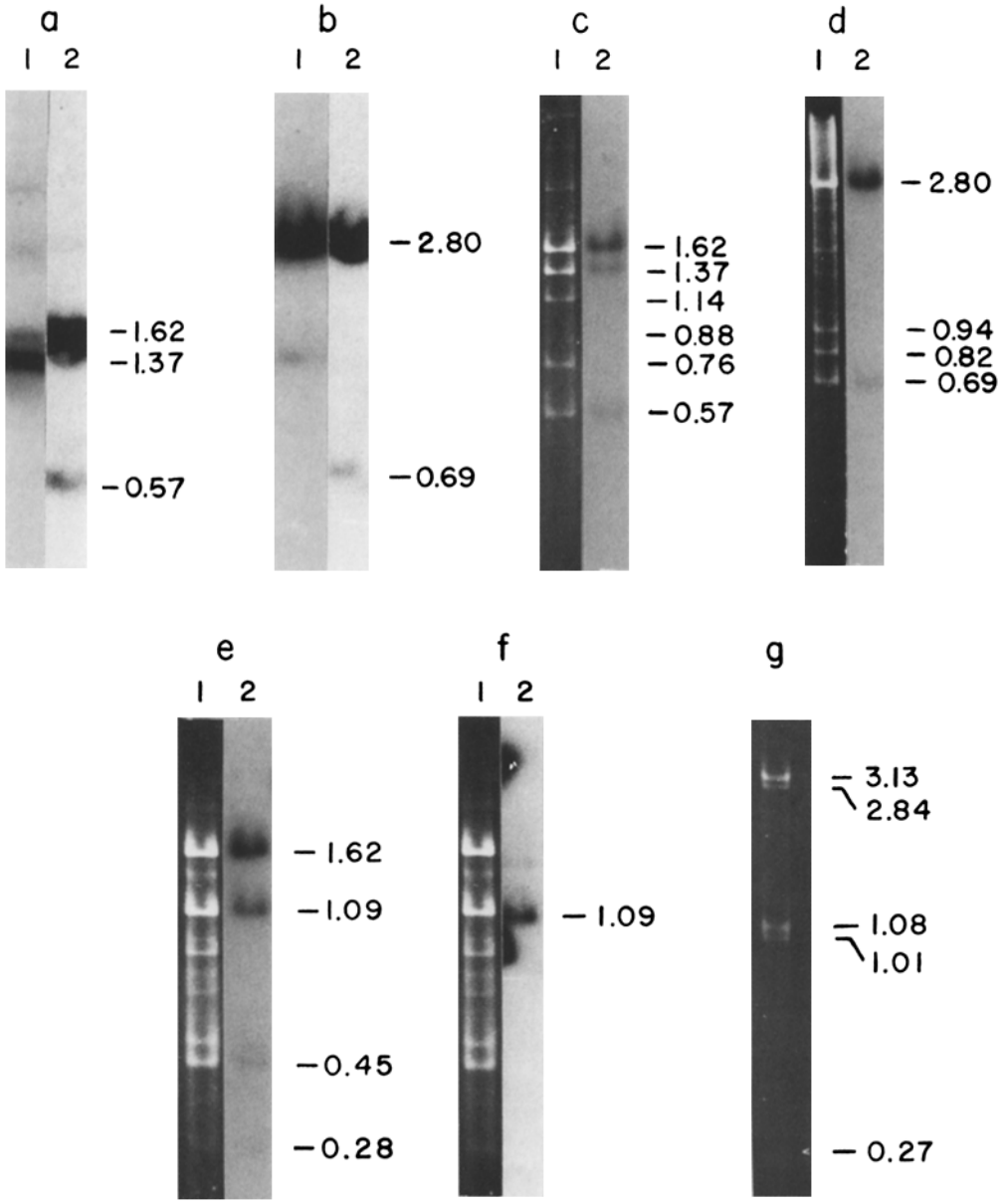

FIGURE 1 Restriction endonuclease digestion and gel hybridization by the Southern procedure of *Paramecium* DNA from stock 51. Digested DNA fragments were separated in agarose gels, eluted onto nitrocellulose filters, and hybridized with *Paramecium* 17S or 25S rRNA end-labeled with ^{32}P. Molecular weights ($\times 10^{-6}$) are indicated. (*a* and *b*) Autoradiographs of whole cell DNA digested with EcoR1 (*a*) or Hind III (*b*) and hybridized with ^{32}P-labeled 17S (*a1* and *b1*) or 25S (*a2* and *b2*) rRNA. The hybridizations to EcoR1 digested DNA indicated that some cross-contamination of 17S and 25S rRNAs was present in this preparation, but do show that the 1.37 $\times$ 10^6 EcoR1 fragment hybridizes primarily with 17S rRNA and that the 1.62 and 0.57 $\times$ 10^6 fragments hybridize with 25S rRNA. (*c* and *d*) Purified rDNA digested with EcoR1 (*c1*) or Hind III (*d1*). (*c2* and *d2*) Autoradiographs of lanes *c1* and *d1*, respectively, after hybridization with ^{32}P-labeled 17S and 25S rRNA. (*e* and *f*) Purified rDNA double-digested with Hind III and EcoR1 (*e1* and *f1*). (*e2* and *f2*) Autoradiographs of lanes *e1* and *f1*, respectively, after hybridization with ^{32}P-labeled 17S and 25S (*e2*) or only 17S (*f2*) rRNA. (*g*) Purified rDNA digested with Bgl II. The 3.13 and 2.84 $\times$ 10^6 dalton fragments hybridize with 17S rRNA and the 1.08 and 1.01 $\times$ 10^6 fragments hybridize with 25S rRNA (not shown). Arrowhead indicates 0.27 $\times$ 10^6 dalton fragment. Minor high molecular weight bands in *c1* and *g* probably represent some mtDNA contamination of these rDNA preparations.

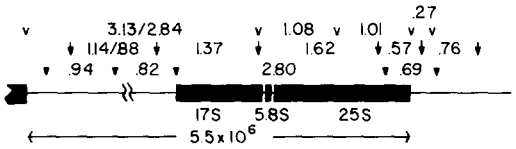

FIGURE 2 Restriction map of the rDNA repeat in *Paramecium*. The restriction sites of Bgl II (*v*), EcoR1 (↓), and Hind III (▼) are indicated. Molecular weights ($\times 10^{-6}$) of the restriction fragments are also shown. Solid blocks represent rRNA coding regions and single lines represent spacer regions. The fragments which are heterogeneous in size are shown, and this is indicated (≷) in the map.

two alternative arrangements for the other two Hind III fragments. Comparison of the predicted double digestion fragment sizes obtained by aligning the presumptive EcoR1 map and either of the two possible Hind III maps with those actually observed generated the map shown in Fig. 2. If the noncoding EcoR1 fragments were arranged in the alternative possibility, the predicted sizes of the double-digestion fragments did not agree with the observed sizes. Confirmation of the map was obtained in an analogous manner by a second double digestion of the rDNA using EcoR1 and Bgl II (not shown). There is no evidence for an intervening sequence in either the 17S or 25S coding regions, although the presence of a small insert cannot be excluded.

Paramecium rDNA digested with either Hind III or EcoR1 was also hybridized with ^{32}P-labeled 5.8S RNA (Fig. 3). The 5.8S RNA hybridized with the 2.8×10^6 Hind III fragment, which also reacts with 17S and 25S rRNA, and with the 1.62×10^6 EcoR1 fragment which, in addition, hybridizes with 25S rRNA. Thus the 5.8S RNA coding sequence is located in the rDNA between the 17S and 25S rRNA coding regions as in other eukaryotes (2, 3, 5, 22, 41, 47).

The rDNA fragments which did not hybridize are presumed to represent spacer sequences. Of the major restriction fragments, two EcoR1 (1.14 and 0.76×10^6 daltons) and two Hind III (0.94 and 0.82×10^6 daltons) fragments did not hybridize. The 1.14 EcoR1 fragment and the two Hind III fragments are present in less than molar yield as judged by the lower intensities of their ethidium bromide staining patterns relative to the other major fragments (confirmed by densitometer scans of photographs of the gels, not shown). In addition to these prominent bands, minor fragments in both the EcoR1 (0.88×10^6) and Hind III (1.75, 1.60, and 1.37×10^6) digestions also did not hybridize. These results suggest that length heterogeneity in the rDNA repeats is located in spacer sequences as indicated in the map. However, the spacer heterogeneity is of a limited nature, as only two prominent repeat lengths differing by 0.3×10^6 daltons are resolved by Bgl II or EcoR1 digestions. The 1.14 and 0.88×10^6 EcoR1 fragments or the 3.13 and 2.84×10^6 Bgl II fragments (Fig. 2) represent alternate forms of the same region of the repeat, and are not present in stoichiometric amounts. The ethidium bromide staining patterns of the digested rDNA suggests that the smaller repeat is also the minor form, as both the 0.88×10^6 EcoR1 and the 2.84×10^6 Bgl II fragments stain less intensely than their larger counterparts (Fig. 1). As a consequence of the heterogeneity, the 1.14×10^6 EcoR1 or 3.13×10^6 Bgl II fragments are also present in less than molar yield. In contrast, the Hind III digestion pattern does not reveal a basic 0.3×10^6 dalton difference in repeat length, although the minor Hind III fragments are indicative of length heterogeneity. The map positions of the minor Hind III fragments were not determined.

The DNA coding for 5S RNA is not linked to

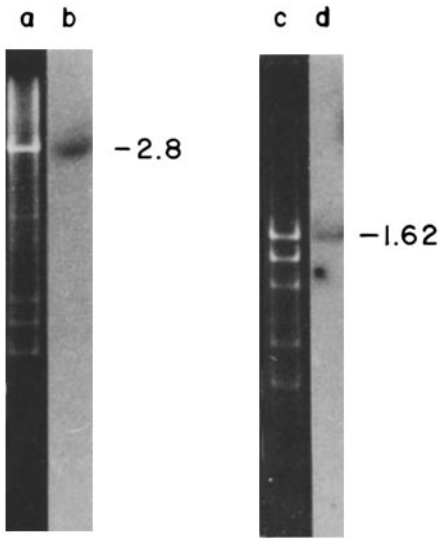

FIGURE 3 5.8S RNA hybridization with *Paramecium* rDNA from stock 51. (*a*) rDNA digested with Hind III. (*b*) Autoradiograph of lane *a* after hybridization with *Tetrahymena* 5.8S RNA end-labeled with ^{32}P. (*c*) rDNA digested with EcoR1. (*d*) Autoradiograph of lane *c* after hybridization with ^{32}P-labeled 5.8S RNA. Molecular weights ($\times 10^{-6}$) are indicated.

the other ribosomal RNA genes. *Paramecium* whole cell DNA was centrifuged in an actinomycin D/CsCl gradient, which separates *Paramecium* rDNA from main peak DNA (20), and an aliquot of each fraction was loaded onto a nitrocellulose filter. The filters were cut in half, and one half was hybridized with ^{3}H-labeled 17S and 25S rRNA and the other half was hybridized with ^{32}P-labeled 5S RNA. The hybridization results show clearly that the 5S genes are not associated with the other rRNA genes (Fig. 4). The 17S and 25S rRNAs hybridized on the light side of the main peak DNA, but the 5S RNA hybridization closely followed the absorbance profile of the main peak DNA. Thus, like the 5S DNA in *Tetrahymena* (28), *Oxytricha* (34), and higher eukaryotes, but unlike that in yeast (3, 35) and *Dictyostelium* (31), the 5S DNA in *Paramecium* is not linked to the other ribosomal RNA cistrons.

Organization of rDNA in Different Stocks

Whole cell DNA was isolated from seven stocks of *P. tetraurelia*, which are listed in Table I. The DNA was digested with EcoR1 or Hind III and the fragments separated by gel electrophoresis. After staining with ethidium bromide, the DNA in the gels appeared as a broad smear, and no bands corresponding in size to the subsequently determined rDNA fragments could be distinguished. However, fragments corresponding in size to the known molecular weights of restriction fragments of purified mtDNA[2] could be distinguished in the ethidium bromide stained gels (results not shown). These fragments served as an internal control and indicated that the DNA was completely digested in these gels. The DNA was eluted onto nitrocellulose filters and the filters were hybridized with ^{32}P-labeled cRNA made from stock 51 rDNA using *E. coli* RNA polymerase. By using cRNA rather than 17S and 25S rRNA as a hybridization probe, one can detect fragments containing only spacer sequences as well as fragments containing coding sequences.

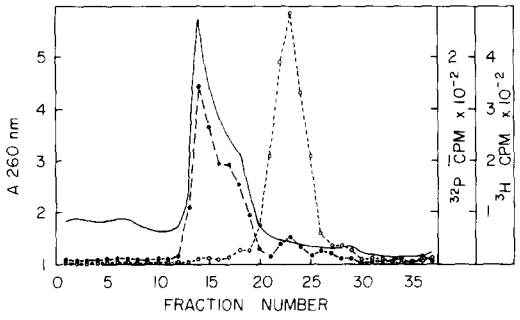

FIGURE 4 *Paramecium* DNA (stock 51) after centrifugation in actinomycin D/CsCl for 26 h at 42,000 rpm, 18°C in the Beckman 50 rotor (~280 μg/DNA per gradient). Fractions (0.15 ml) were collected from the bottom and an aliquot of each was loaded onto a nitrocellulose filter. The filters were cut in half, and one half was hybridized with *Paramecium* ^{3}H-labeled 17S and 25S rRNA and the other half with *Tetrahymena* ^{32}P-labeled 5S RNA. Main peak DNA and 5S DNA were located in fractions 13–19, rDNA in fractions 21–25, and mtDNA in fractions 28–30. The high background absorbance was caused by the actinomycin D. –, A_{260}; ○, ^{3}H cpm (rDNA); ●, ^{32}P cpm (5S DNA).

As shown in Fig. 5, the cRNA hybridization patterns of the different stocks are remarkably similar. After EcoR1 digestion only stock 127 exhibited a significantly different pattern; a unique band with a mol wt of 2.0×10^6 appeared, and the bands at 0.88 and 0.76×10^6 were absent. One or both of these latter two bands were present in EcoR1 digests of all the other stocks. The other four bands (mol wt of 1.62, 1.37, 1.14, and 0.57×10^6) were identical in size among the seven stocks. The sizes of the bands seen after hybridization of Hind III digested DNA also showed little variability. No bands of unique size appeared in any of the stocks, although the bands at 1.75 and 1.60×10^6 were absent in stock 127. One or both were present in Hind III digests of the other six stocks.

The molecular weights of the bands which appeared in the autoradiographs of digested whole cell DNA from stock 51 after hybridization with [^{32}P]cRNA were similar to the sizes obtained using purified rDNA from stock 51 (Fig. 1). However, the relative intensities of the bands in the autoradiographs, especially the lower molecular weight bands, do not correspond to the relative intensities of the equivalent bands in ethidium bromide stained gels of purified rDNA. This is seen by comparison of the autoradiographs of stock 51 DNA (Fig. 5) with purified rDNA from stock 51 (Figs. 1 and 7). In particular, in the autoradiographs as contrasted with the ethidium bromide staining pattern, the Hind III bands at 0.94, 0.82, and 0.69×10^6 are not well resolved and are too light in comparison to the fragments at 1.75 and 1.60×10^6. This lack of correspondence is probably a consequence of the failure of the lower molecular

[2] Findly, R. C., and J. G. Gall. Manuscript in preparation.

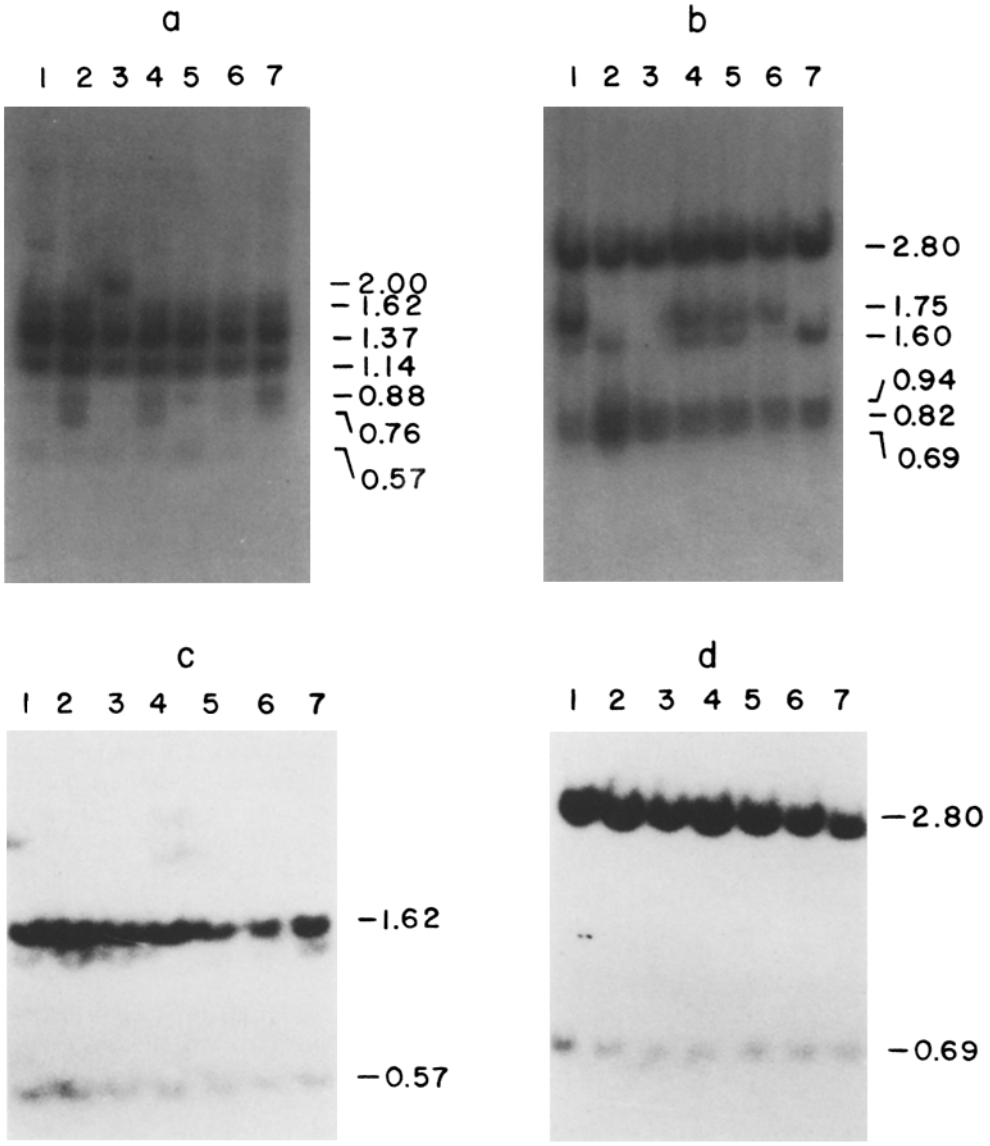

FIGURE 5 Restriction endonuclease digestion and gel hybridization by the Southern method of whole cell DNA from seven stocks of *Paramecium*. Only autoradiographs of the hybridization patterns are shown. In each example (*a–d*) lanes *1*, *2*, *3*, *4*, *5*, *6*, and *7* represent stocks 47, 51, 127, 148, 172, 203, and 316, respectively. The DNA fragments were separated by electrophoresis in 0.7% agarose gels and eluted onto nitrocellulose filters. The filters were hybridized with ^{32}P-labeled cRNA made from stock 51 rDNA or with 25S rRNA end-labeled with ^{32}P. Hybridization with [^{32}P]cRNA of DNA digested with EcoR1 (*a*) or Hind III (*b*). Hybridization with ^{32}P-labeled 25S rRNA of DNA digested with EcoR1 (*c*) or Hind III (*d*). The faint high molecular weight bands in *a* represent mtDNA, and indicate that a very minor amount of mtDNA contaminated the rDNA preparation used to prepare the cRNA. The hybridization to the 1.37 $\times 10^6$ fragment in *c* represents contamination of 25S rRNA with 17S rRNA. Molecular weights ($\times 10^{-6}$) are indicated.

weight fragments to bind fully to the nitrocellulose filters.

In similar experiments the filters were hybridized with 17S or 25S rRNA end-labeled with ^{32}P instead of cRNA. As shown in Fig. 5, the hybridization pattern of 25S rRNA is similar in all seven

stocks. After EcoR1 digestion two bands with mol wt of 1.62 and 0.57 × 10^6 are visible in autoradiographs of the filters. A faint band of hybridization with a mol wt of 1.37 × 10^6 is also present, which represents contamination of the 25S rRNA with 17S rRNA. The 17S rRNA hybridized with the 1.37 × 10^6 band (see Fig. 1) and like the 25S rRNA, the hybridization pattern of the seven stocks was the same (not shown). The hybridization pattern of 25S (Fig. 5) or 17S (not shown) rRNA to Hind III digested DNA was also the same among these seven stocks. These results demonstrate that the coding regions of the rDNA repeat are the same size in the different stocks.

The bands which were present after hybridization with cRNA, but which were not visible in the rRNA hybridization patterns represent spacer sequences. Two significant points concerning the organization of the rDNA spacer in these seven stocks were revealed by the hybridizations: (*a*) the spacer fragments have similar sequences, (*b*) they show only limited size heterogeneity. As judged by the intensities of the bands seen in the autoradiographs, the cRNA appears to hybridize about as well with the other six stocks as back to stock 51. Thus the sequences of the spacers must be similar in all seven stocks. This conclusion is further supported by the observation that the restriction sites located in the spacer sequences are generally similar with the single exception of stock 127. In particular, a 1.14 × 10^6 EcoR1 band and a 0.94 × 10^6 Hind III band are present in the rDNA restriction patterns of all seven stocks. Assuming that all of the stocks have the same map as stock 51 (with the possible exception of stock 127), then there are restriction fragments contained in the rDNA from all of these stocks which have the same size and which contain only spacer sequences. The stocks do vary in the presence or absence of certain bands—the EcoR1 bands with mol wt of 0.88 and 0.76 × 10^6 and the 1.75 and 1.60 × 10^6 Hind III bands—and in the relative intensities of these bands. This presumably indicates that not all of the spacer fragments are present in each repeat, but that they may exist as alternate possibilities.

The gels clearly show that only a very limited number of restriction fragments containing spacer sequences are present in these stocks. A multimeric pattern of restriction fragments or a broad hybridization pattern in one region of the gel indicating a continuum of sizes was not observed. Rather, with one exception, the sizes of the spacer fragments are the same in all of the stocks, although not all of the spacer fragments are present in each stock. The cRNA hybridization patterns seen in Fig. 5 were confirmed by comparison with the ethidium bromide staining pattern of restricted rDNA purified from several stocks (stock 51 [Figs. 1 and 7], stock 127 [Fig. 7], and stocks 172 and 203 [not shown]). It is apparent that in none of the stocks has the sequence or organization of the rDNA repeat been substantially altered. Taken together, these studies suggest that the rDNA repeat in *Paramecium*, including spacer regions, has been relatively conserved in the course of evolution.

Characterization of rDNA from Stock 127

Because of its unique EcoR1 digestion pattern, we picked the rDNA of stock 127 for further study and comparison with stock 51 rDNA. Both stocks 51 and 127 had similar saturation hybridization values with rRNA (not shown). Approximately 0.2% of total DNA hybridized with ^{3}H-labeled 17S and 25S rRNA, in agreement with the value reported for *P. primaurelia* (12).

The rDNA from stock 127 was isolated by equilibrium centrifugation in actinomycin D/CsCl and CsCl gradients. As shown in Fig. 6, the purified rDNA banded in neutral CsCl in the Beckman model E Analytical Ultracentrifuge as a single, essentially homogeneous peak with a density of 1.699 g/cm^3, corresponding to a G + C content of 39.8% (36). This is the same density as that of stock 51 rDNA (20).

The EcoR1 and Hind III restriction patterns of rDNA isolated from stock 51 and 127 were compared by simultaneous gel electrophoresis (Fig. 7), and concurred with the cRNA hybridization studies. As predicted, hybridization with 17S and 25S ^{32}P-labeled rRNA demonstrated that the coding regions of stock 127 were identical in size with those of stock 51 (not shown). This verified that the differences in the EcoR1 digestion patterns of these stocks are confined to the spacer sequences. The lower intensity of the ethidium bromide staining of the 2.00 and 1.14 × 10^6 fragments (spacer sequences) relative to the 1.62 and 1.37 × 10^6 fragments (coding sequences) suggests that the former are present in less than molar yield, and implies that both of these spacer fragments are not present in a single repeat. This again demonstrates that the heterogeneity in the rDNA repeat is located in the spacer.

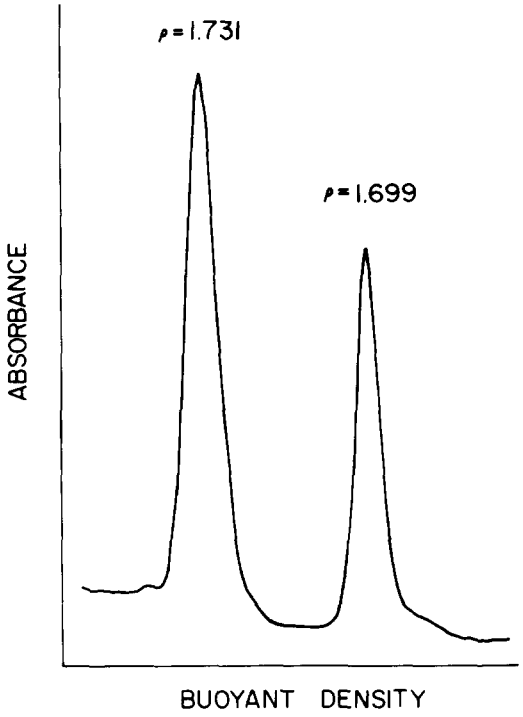

FIGURE 6 *Paramecium* rDNA from stock 127 centrifuged to equilibrium in CsCl in the Beckman model E Analytical Ultracentrifuge at 44,770 rpm, 25°C, for 20 h. The density standard was *Micrococcus lysodeikticus* DNA (ρ = 1.731 g/cm^3). The rDNA has a buoyant density of 1.699 g/cm^3.

The melting temperature (T_m) of the rDNA was determined by melting in 0.1 × SSC (not shown). The T_m of 68.5°C predicts a buoyant density in CsCl of 1.695 g/cm^3 which is slightly <1.699 g/cm^3 actually observed. The reason for the difference is not known, but similar differences have been reported for the ciliates *Stylonychia mytilus* (1) and *Oxytricha fallax* (34). The G + C content of the rDNA predicted from its buoyant density or its T_m is less than the 44% G + C content of *Paramecium* rRNA determined by base composition analysis (19). Consequently, the spacer sequences must be A + T rich relative to the coding sequences.

After melting, the rDNA was cooled quickly under conditions which did not favor bimolecular reassociations. The rDNA had a high double-stranded molecular weight as determined by electrophoresis in 1% agarose gels, but the amount of nicking was not examined. Upon cooling, the rDNA showed little decrease in its hyperchromicity. The melting behavior demonstrates, in agreement with previous observations (20), that *Paramecium* rDNA is not a palindrome.

Stock 127 rDNA was spread for electron microscopy under partially denaturing conditions in 84% formamide, and showed the same partial

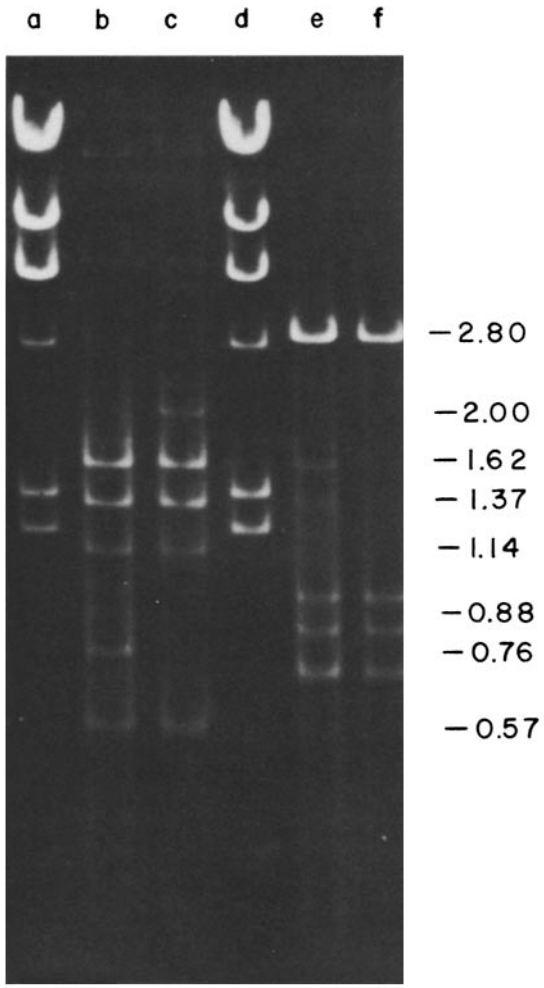

FIGURE 7 Restriction endonuclease digestion of rDNA from stocks 51 and 127. Electrophoresis was in a 1% agarose gel. (*a*) Lambda DNA digested with Hind III. (*b*) Stock 51 rDNA digested with EcoR1. (*c*) Stock 127 rDNA digested with EcoR1. Note that the 2.00 × 10^6 band is absent in stock 51, and that bands at 0.88 and 0.76 × 10^6, present in stock 51, are absent in stock 127. (*d*) Lambda DNA digested with Hind III. (*e*) Stock 51 rDNA digested with Hind III. Minor fragments at 1.60 and 1.37 × 10^6 are visible. (*f*) Stock 127 rDNA digested with Hind III. Minor fragments are not readily visible. Undigested rDNA from stock 51 or 127 ran at the limit mobility of the gel (not shown). Molecular weights (× 10^{-6}) are shown.

denaturation pattern as stock 51 rDNA (20). Denaturation bubbles occurred at regular intervals in a repeating pattern with one native and one denatured region per repeating unit (Fig. 8). The denaturation bubbles, which are A + T rich relative to the double-stranded regions, probably contain spacer sequences. The mean center-to-center spacing of the denaturation bubbles was 5.53 × 10^6 daltons (SD ± 0.5 × 10^6 daltons; n = 152), determined by comparison with double-stranded ϕX 174 in the same sample. As is the case with stock 51, linear and circular molecules had the same partial denaturation pattern, the circles represented a very low percentage of the molecules on the grids, and no monomer circles were observed. The largest linear and circular molecules had mol wt of 51.7 × 10^6 and 51.9 × 10^6, respectively. Examination of individual molecules in Fig. 8 shows that size heterogeneity in adjacent repeats occurs, although the repeats along most molecules are equal in length. Thus, like stock 51, stock 127 rDNA shows limited intramolecular heterogeneity. A representative partially denatured molecule is shown in Fig. 9. These studies indicate that with the exception of the restriction patterns, the rDNAs of stocks 51 and 127 are very similar.

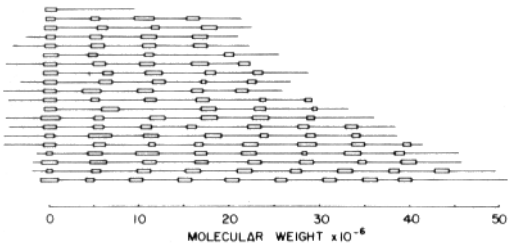

FIGURE 8 Partial denaturation pattern of linear and circular rDNA molecules from stock 127 spread in 84% formamide. Native regions are indicated by a single line, denatured by double lines. The molecules were arranged arbitrarily by aligning a denatured region near the left ends of the molecules. The seven molecules with a denatured region at their extreme left end were circular.

DISCUSSION

Nuclear Organization of Paramecium rDNA

Like other ciliated protozoans, *Paramecium* has two different nuclei; a small, metabolically inactive, diploid micronucleus which is responsible for the genetic continuity of the organism, and a very large, transcriptionally active, polyploid macronucleus. After conjugation or autogamy, the old macronucleus breaks down, and a new one is formed from the micronucleus. The extrachromosomal macronuclear rDNA presumably arises

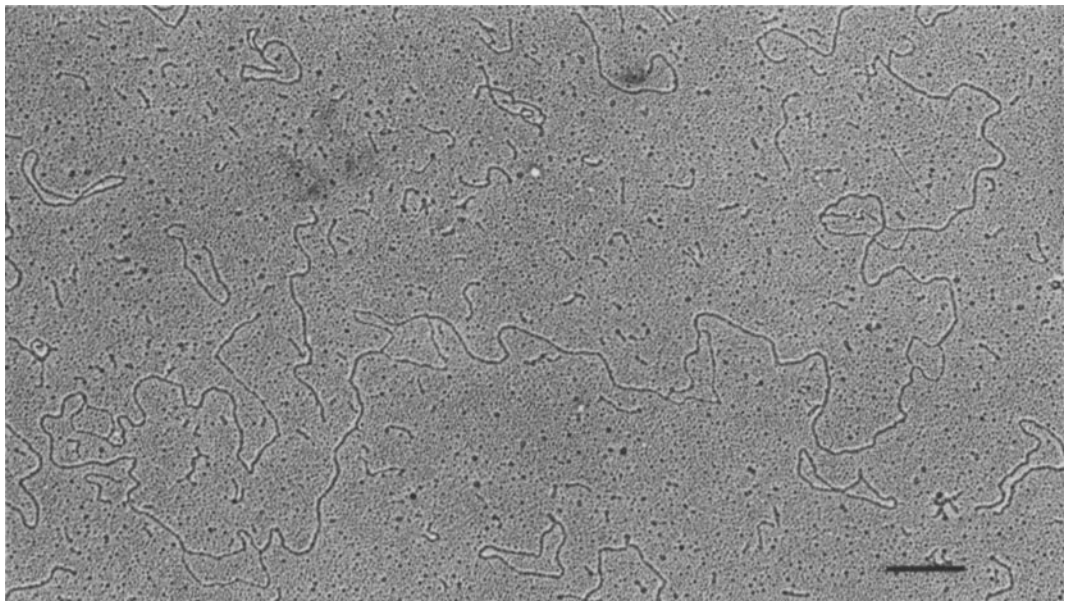

FIGURE 9 Partially denatured rDNA molecule spread in 84% formamide, which has a mol wt of 41.1 × 10^6 and contains six denaturation bubbles. The small circular molecules are ϕX 174. Bar, 0.5 μm. × 21,000.

from micronuclear rDNA after conjugation, and is probably amplified during polyploidization of the new macronucleus. As intramolecular heterogeneity in the tandem repeats of the extrachromosomal rDNA is observed, more than one copy of the rDNA must exist in the micronucleus. Most likely the micronucleus contains integrated tandem copies of rDNA of variable unit lengths. Thus, the organization of *Paramecium* rDNA is distinctly different from that of another ciliate, *Tetrahymena.* In *Tetrahymena*, only a single integrated copy of the rDNA is present in the micronucleus (51, 52), and the extrachromosomal macronuclear rDNA is a palindrome (16, 26). *Paramecium* rDNA is apparently organized in a manner similar to that of *Xenopus* chromosomal rDNA in which adjacent repeats can differ in length (49). However, the number and arrangement of the different classes of repeats in *Paramecium* rDNA is not presently known.

Conservation of Spacers

The size and sequence of the rDNA spacer has in general been conserved among the seven stocks of *P. tetraurelia* investigated here. As cRNA made from rDNA of stock 51 hybridizes equally well with the rDNA restriction fragments of all seven stocks, the sequences in *Paramecium* rDNA, including the spacer, must be similar in all seven stocks. These hybridization studies further indicated that the EcoR1 or Hind III restriction patterns were similar in all the stocks, with the single exception of the EcoR1 pattern of stock 127. Inspection of the restriction map in Fig. 2 shows that an EcoR1 fragment (1.14×10^6) and a Hind III fragment (0.94×10^6) both contain only spacer sequences. These two fragments are present in the restriction patterns of all seven stocks. Thus, not only do the spacer sequences cross hybridize among these seven stocks, but some of the same restriction sites have been retained in the spacers. It is apparent that among these seven stocks the size of the rDNA repeat ($\sim 5.5 \times 10^6$) is very similar and that they have all retained a similar organization of the spacer, including both sequence and size of the restriction fragments. The differences in the sizes of the spacer fragments which do exist among these stocks are of a limited nature and are more like the situation found in human rDNA (30) than in *Xenopus* rDNA in which a continuous variation in size is seen (10). None of these *Paramecium* stocks has opted for a radical departure in size or sequence of the spacer regions.

The structural organization and sequence of spacer DNA in both rDNA and 5S DNA has been studied in most detail in the two closely related species *Xenopus laevis* and *X. borealis.* It is primarily from these investigations that our understanding of the organization and evolution of spacer sequences has come (reviewed in reference 17). Briefly, in these toads the coding regions of the rDNA are identical, and although the rDNA spacers are similar in size, they show substantial differences in sequence (9, 21, 48). Likewise, the coding regions for oocyte 5S RNA in these two species are similar, but their spacers are greatly different in size, and the sequences of the spacers have diverged to such an extent that they will no longer cross hybridize (7, 8, 18, 29). The general conclusion drawn from these studies has been that spacers are a rapidly evolving set of sequences of unknown function under little selection pressure. This conclusion does not preclude the conservation of some short sequences as, for example, those directly influencing gene expression (7, 29). The mechanisms responsible for the rapid evolution of spacer have not been determined. However, sequence analysis of the rDNA spacer in *X. laevis* has led to the suggestion that saltation of a short segment of spacer DNA may be involved (4). In contrast, it has been shown that the rDNA spacers of the six species of the *D. melanogaster* subgroup have been highly conserved (44). These latter studies suggest that selection pressure may play a significant role in preserving the similar organization of rDNA spacers among these sibling species (44), and also between the X and Y chromosomes (45).

The seven stocks of *P. tetraurelia* used in these experiments are classified as one species (39). However, they have evolved from each other to some extent for they show differences in their karyotypes and serotypes (Table I), and the viability of the progeny from crosses between the stocks is low, probably reflecting the karyotype differences (14). The restriction patterns of mtDNA isolated from certain of these stocks also differ in some instances.[3] It is, of course, impossible to determine the actual evolution of these stocks. However, the present distribution of *Paramecium*

[3] Findly, R. C., and J. G. Gall. Manuscript in preparation.

species is probably best explained by wide geographic dispersal of their ancestral stocks before the continental land masses separated (33). Dispersal after this event is viewed as less likely because *Paramecium* does not encyst and cannot survive drying or seawater. According to this interpretation, the seven stocks of *P. tetraurelia* used here are probably the descendants of a single widely dispersed ancestor, and several of the stocks have presumably been geographically and sexually isolated from each other for about 100 million years.

It is possible that the evolutionary history of *Paramecium* is different from the presently accepted view, and that these stocks have either separated only recently or that they have maintained some genetic contact. We feel it is more probable that the stocks are of ancient origin. If this is true, and the stocks have in fact been separated for a considerable time, then the rDNA spacers of these stocks have undergone little divergence. Why rDNA spacer sequences evolve rapidly in *Xenopus*, but not in *Paramecium* or *Drosophila* is unknown.

One way ciliates may differ from higher eukaryotes is in the number of copies of rDNA in the germinal nuclei. Most higher eukaryotes have several hundred copies of rDNA per haploid genome. *Tetrahymena*, on the other hand, has only a single integrated rDNA copy in the micronucleus (51, 52), and in the micronucleus of *Paramecium* there are probably only a few integrated tandem copies of the rDNA. For unknown reasons, fewer germinal copies of rDNA may be related to the homogeneity observed in the rDNA among the different stocks of *Paramecium* and *Tetrahymena* (50). However, the results of the *Drosophila* studies indicate that factors other than just the number of rDNA copies may be involved in the conservation of spacer sequences.

The results presented in this paper demonstrate that the evolution of spacer sequences in rDNA is more conservative in *Paramecium* than in *Xenopus*. They suggest that in some organisms, even in the absence of genetic exchange among geographically separated populations, selection pressure may act to conserve the general structure and sequence of spacer regions in tandemly repeated rDNA.

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