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rRNA Genes of *Naegleria gruberi* Are Carried Exclusively on a 14-Kilobase-Pair Plasmid

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An extrachromosomal DNA was discovered in *Naegleria gruberi*. The 3,000 to 5,000 copies per cell of this 14-kilobase-pair circular plasmid carry all the 18S, 28S, and 5.8S rRNA genes. The presence of the ribosomal DNA of an organism exclusively on a circular extrachromosomal element is without precedent, and *Naegleria* is only the third eucaryotic genus in which a nuclear plasmid DNA has been found.

Naegleria is a genus of free-living soil amoebae that includes species known to be opportunistically pathogenic in mammals. *Naegleria fowleri* is the etiological agent of primary amoebic meningoencephalitis in humans (16). All members of the genus can undergo rapid transformation from amoeboid cells to transient, nonfeeding, nondividing, free-swimming flagellated forms. In the laboratory this change can be induced synchronously and reproducibly in a population of cells by various stress factors, such as suspension in water (reviewed by Fulton [9]). *Naegleria* is being studied as a model system for the control of differentiation (8–10). The transformation requires de novo synthesis of both RNA and protein (12). New mRNAs produced coordinately include those coding for both α- and β-tubulin and for two forms of calmodulin (11, 18, 25). mRNAs encoding all the other proteins necessary for the construction of the typically eucaryotic flagella are presumably also synthesized. During transformation the level of translatable actin mRNA decreases dramatically (32).

In the early stage of our investigations, a DNA clone was isolated that encoded an RNA which, while not differentially regulated, was present at a high level in the cell. Southern blot analysis showed that the gene comigrated with repetitive DNA bands in restriction digests and with what appeared to be extrachromosomal bands when undigested DNA was analyzed. Further analysis revealed that this extrachromosomal element was circular and carried the rRNA genes (ribosomal DNA [rDNA]) of the organism, and since no chromosomal copy was detected, we concluded that this rDNA plasmid must be self-replicating.

MATERIALS AND METHODS

Cells. *Naegleria gruberi* NEG-M (ATCC 30224) was obtained from the American Type Culture Collection (Rockville, Md.). Cell cultures were maintained as migrations on petri plates of PM agar (8) spread with a lawn of *Escherichia coli* DH1 and grown inverted at room temperature. For preparative isolation of nucleic acids, 85-mm PM plates were gently spread with a mixture of 0.5×10^5 to 1×10^5 *N. gruberi* cells and 200 μl of a stationary-phase *E. coli* culture, and the cells were grown inverted at 32°C for 16 to 20 h until the lawn was 80 to 90% cleared. Plates were harvested by suspending the cells in 5 ml of 2 mM Tris hydrochloride (pH 7.4) with a spreader and repeatedly pelleting at 400 × *g* for 60 s each time to remove most of the unconsumed bacteria (8). By this method, around 10^7 cells per plate could be obtained.

Nucleic acids. RNA was isolated by guanidinium thiocyanate-sodium dodecyl sulfate lysis, and poly(A)$^+$ RNA was purified as described previously (4). The lysis solution was added directly to the cell pellet, which was then gently resuspended. DNA was isolated by treatment with sodium dodecyl sulfate and proteinase K as described previously (1) followed by the addition of solid CsCl (0.905 g/ml of lysis mixture; $r = 1.3875$) and 10 μg of ethidium bromide per ml to the preparation and ultracentrifugation. Separation of the *Naegleria* rDNA plasmid from chromosomal DNA was achieved essentially as described by Hirt (15). Cells were suspended at 10^7/ml in 10 mM Tris hydrochloride (pH 8)–10 mM EDTA (TE10), and 10% sodium dodecyl sulfate was added dropwise with gentle swirling to a final concentration of 1%. NaCl (5 M) was then added in a similar manner to a final concentration of 1 M. The chromosomal DNA was allowed to precipitate overnight at 4°C and then pelleted at 6,000 × *g* for 45 min. The supernatant was decanted, and the chromosomal DNA pellet was suspended at 65°C in TE10. Self-digested pronase was added to both the pellet and supernatant to a concentration of 0.5 mg/ml and incubated at 37°C for 2 h. The supernatant was gently extracted several times with phenol-$CHCl_3$ and ethanol precipitated, while the pellet was dialyzed extensively against 10 mM Tris hydrochloride (pH 8)–1 mM EDTA. Total cell DNA for copy number estimation was isolated in the same manner as the Hirt DNAs except that the NaCl precipitation was omitted and that several phenol-$CHCl_3$ extractions were performed before dialysis. Size selection of partially digested DNA and purification of the rDNA plasmid used linear 10 to 40% sucrose gradients (24).

Restriction endonuclease digestion of DNA and agarose gels for RNA and DNA were as described previously (4). Recombinant plasmids were isolated from *E. coli* by the alkali lysis method (24). Restriction fragments of DNA were purified by electroelution or by NA45 paper elution (20). DNA labeling was by the primer extension method of Feinberg and Vogelstein (7). Labeled poly(A)$^+$ RNA for screening colonies was prepared by partial hydrolysis with NaOH (50 mM, 4°C, 1 h) and treatment of the fragments with [8-^{32}P]dATP and T4 polynucleotide kinase. DNA fragments were cloned into the vector pUC12 by using T4 ligase, transformed into the *E. coli* host DH1 (14), and screened by the method of Grunstein and Hogness (13). The cDNA clone pcNgex1 was isolated from a cDNA recombinant library constructed by the standard hairpin loop method and in-

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serted into the *Pst*I site of the vector pUC9 by G-C tailing (24). Both RNA and DNA nitrocellulose filters were prehybridized and hybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–50 mM Tris hydrochloride (pH 8.0)–50 mg of denatured, sheared salmon sperm DNA per ml–100 μg of yeast tRNA per ml–10 μg of poly(A) per ml–50% deionized formamide at 42°C. Slot blots were performed according to the recommended protocol of the manufacturer (Schleicher & Schuell Inc., Keene, N.H.).

RESULTS

An incomplete library of CsCl-purified *N. gruberi* total DNA was constructed in pUC12 by using size-fractionated, partially *Sau*3AI-digested DNA. While screening for recombinant plasmids carrying differentially expressed genes, we detected a number of colonies that, while not showing differential hybridization, contained sequences that were represented at high levels in cellular RNA. Restriction digests of 25 such recombinant plasmids showed many common fragments. A 0.6-kilobase-pair (kb) *Hin*dIII fragment that was present in each recombinant was used to screen recombinants in a pUC9 cDNA library and to analyze Southern and Northern (RNA) blots.

A single cDNA clone carrying a 1.4-kb insert was isolated and had a restriction map identical to the region of the *Sau*3AI clone from which the probe was derived. On Northern blot analysis the probe detected an RNA that comigrated with the presumptive large subunit rRNA that was visible upon staining the formaldehyde-agarose gel. On Southern blot analysis, the probe detected fragments that comigrated with major repetitive DNA bands visible upon ethidium bromide staining of the gel. Of greatest interest was the fact that when undigested DNA was analyzed by this method, it was clear that the hybridization was not to the main, presumptive chromosomal DNA band but to less intensely staining bands migrating above and below the major DNA band on 0.8% agarose gels.

To confirm the identity of the RNA sequence encoded by the cDNA, we generated a small amount of sequence data from the cDNA clone. Comparison of the sequences with the GenBank data base revealed similarity to the large subunit rRNAs from a number of organisms in the region covering domains IV to VI (22), with the greatest degree of similarity being to eucaryotic nuclear-encoded rRNAs.

Hirt fractionation of cellular DNA (15) allowed enrichment of the extrachromosomal element. As expected of a circular DNA, the migration of the rDNA plasmid relative to linear DNA markers depended on the percentage of agarose that was present in the gel (17). The difference was particularly striking when migration in 0.6% and 0.8% agarose was compared (Fig. 1). In 0.6% agarose, the major component of the plasmid (presumptive form II, open circular DNA) migrated in front of the contaminating chromosomal DNA, while in 0.8% agarose it migrated well behind this chromosomal DNA. Similarly, the fastest-migrating component (presumptive form I, supercoiled DNA) migrated ahead of the 9.7-kb lambda DNA marker in 0.6% gels, while it migrated behind this marker on 0.8% gels. In both gel systems the small amount of linear (form III) DNA migrated close to a 13.3-kb linear DNA marker.

Two additional bands were detectable by ethidium bromide staining (1 and 2 in Fig. 1). Occasionally, other, more slowly migrating bands were faintly visible, but only by autoradiography. These minor components must be present in very small quantities relative to the other rDNA forms. A diffuse ethidium bromide-stained band was seen in some Hirt preparations migrating behind components 1 and 2. It did not hybridize to the probe and is possibly mitochondrial DNA.

Hirt fractionation-enriched rDNA plasmid was further purified on a sucrose gradient. Under the conditions used, the high-molecular-weight contaminating chromosomal DNA pelleted. While the different plasmid forms could not be cleanly separated from each other, chromosomal DNA contamination was minimal. The slowest-sedimenting plasmid-containing fraction of the gradient was digested with *Bam*HI and cloned into the vector pUC12. A partial restriction map of one rDNA clone, pNgex27, is shown in Fig. 2. The total size estimated from restriction digests is 14 kb. The sizes of fragments obtained by restriction digestion of gradient-purified rDNA were totally consistent with those predicted by the restriction map of the cloned example, as well as confirming the circularity of the rDNA (see also Fig. 5).

Hybridization of labeled pNgex27 to blots of total *Naegleria* RNA detected the 18S, 5.8S, 28S, and the presumptive precursor rRNAs (Fig. 3). Hybridization of labeled pNgex27 to blots of the small rRNAs confirmed that the 5.8S rRNA was encoded with the 18S and 28S rRNAs on the plasmid but showed that the 5S rRNA was not—a situation consistent with the organization of the rRNA genes in most other eucaryotes (data not shown). Locations of the rRNA genes were mapped by hybridization of pNgex27 restriction fragments to Northern blots and are indicated on Fig. 2. The 28S rRNA (ca. 3.3 kb) is entirely located within the 4.1-kb *Cla*I-*Cla*I fragment, while the 18S rRNA (ca. 1.9 kb) is within the 3.0-kb *Cla*I-*Bam*HI fragment. The 0.5-kb *Eco*RI fragment spanning this delimiting *Cla*I site contains the 3′ end of the 18S rRNA, the 5.8S rRNA, and a small amount of

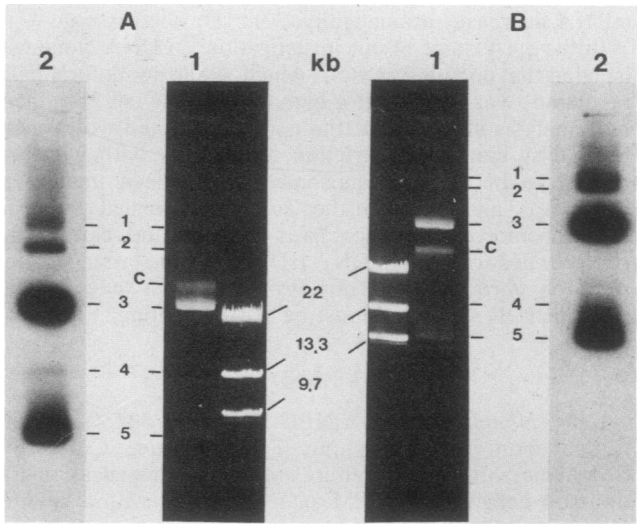

FIG. 1. Variable migration of the rDNA plasmid forms in agarose gels. Undigested Hirt supernatant DNA was loaded onto a divided gel in either 0.6% (A) or 0.8% (B) agarose and run for 18 h in TAE buffer (24) at 2 V/cm. After staining with ethidium bromide and photographing (lane 1), the gels were blotted onto nitrocellulose and hybridized to labeled H6 probe (Fig. 2) before autoradiography (lane 2). rDNA plasmid components 1 and 2 remain uncharacterized; components 3, 4, and 5 are the presumptive forms II (open circular), III (linear), and I (supercoiled), respectively. The material labeled C is visible only in the stained gel and is presumed to be contaminating chromosomal DNA. The size marker is *Bgl*II-digested bacteriophage lambda DNA.

the 5′ end of the 28S rRNA (as judged by the relative intensity of hybridization of the probe to the three rRNAs). The 5′ end of the 18S rRNA is within the 1.0-kb *Pst*I-*Bam*HI fragment. No transcripts were detected with the 6.7-kb *Sst*I-*Bam*HI fragment nor with the 0.2-kb *Bgl*II-*Cla*I fragment from the 3′ end of the 28S rRNA gene. This indicates that there are no non-rRNA transcription units on the plasmid.

The copy number of the plasmid in the cell was determined by slot blot comparison of specific numbers of pNgex27 with total DNA isolated from specific numbers of cells (Fig. 4). Any contaminating rRNA in the total DNA preparation was destroyed by alkaline hydrolysis before diluting. Three experiments gave values of 3,000 to 5,000 plasmids per cell, which is a relatively high number of rDNA cistrons for a protozoan, but well within the range known to exist in eucaryotes (21).

To test whether there is a copy of the rDNA plasmid integrated into chromosomal DNA, we used pellet DNA from the Hirt fractionation procedure. DNA from 10^6 cells was digested to completion with *Hin*dIII or *Bam*HI and electrophoresed on an agarose gel with 10^6 copies of *Bam*HI-digested pNgex27 as a control for detection of single-copy sequences. If a chromosomally integrated copy of the rDNA existed, at least one band other than that expected from digestion of residual rDNA plasmid would be visible at an intensity similar to that of the 10^6 copies of pNgex27 and at a size greater than half that of the plasmid, in each of the two chromosomal DNA lanes. These bands would arise from cleavage within and outside the integrated copy. No such bands were seen (Fig. 5), and we conclude that there is less than one chromosomally integrated copy of the rDNA per cell and therefore that the plasmid is self-replicating.

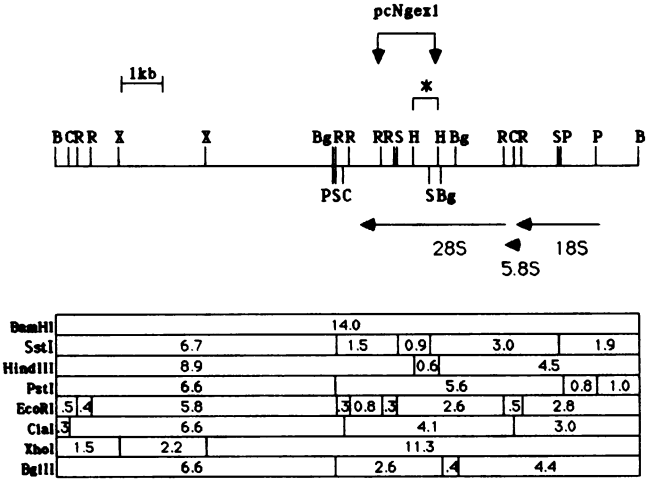

FIG. 2. Restriction endonuclease map of rDNA plasmid clone pNgex27. pNgex27 was analyzed by a combination of single and double restriction digests, and the resulting restriction map is shown here linearized at the single *Bam*HI site. The locations of the rRNA sequences contained in cDNA clone pcNgex1 and of the 5.8S, 18S, and 28S rRNAs are indicated. The direction of transcription was determined by limited sequencing of pcNgex1. The asterisk indicates the location of fragment H6, which was used to probe nitrocellulose blots. B, *Bam*HI; P, *Pst*I; S, *Sst*I; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; R, *Eco*RI. In the lower panel, the approximate sizes of restriction fragments obtained in digestions of *Bam*HI-linearized rDNA plasmid are shown. The presence of a unique *Bam*HI site in the rDNA plasmid was proven by using a recombinant that covers this region. The 3.3-kb *Cla*I fragment that covers this region was partially digested with *Bam*HI; no indication of additional *Bam*HI sites was found under conditions that would have allowed discrimination of restriction sites less than 10 nucleotides apart.

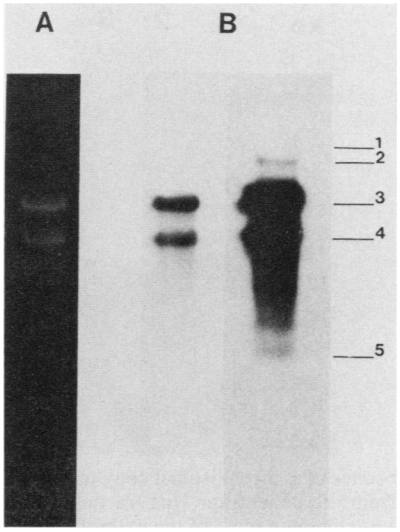

FIG. 3. Detection of RNAs encoded on pNgex27. A 5-μg sample of total *Naegleria* RNA was electrophoresed on a 1% agarose-formaldehyde gel, stained with ethidium bromide (A), blotted to nitrocellulose, and hybridized to total labeled pNgex27 (B). The RNAs detected are: 1 and 2, presumed precursor rRNAs; 3, 28S rRNA; 4, 18S rRNA; 5, 5.8S rRNA. Two exposures of the same hybridization are shown in panel B.

DISCUSSION

We demonstrated that all the 5.8S, 18S, and 28S rRNA genes of *N. gruberi* NEG-M (ATCC 30224) are located on a 14-kb circular extrachromosomal DNA element. This organization for the rRNA genes of an organism is without

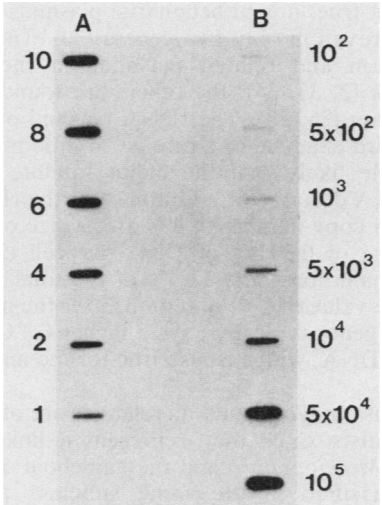

FIG. 4. Determination of rDNA plasmid copy number. Total *Naegleria* DNA (A) and *Bam*HI-restricted pNgex27 (B) were digested to completion with *Hin*dIII and treated with 0.1 M NaOH (100°C, 5 min) to remove contaminating RNA. After neutralization and dilution, the samples were denatured, transferred to nitrocellulose with a slot blot apparatus, and then hybridized to probe H6. (A) Number of cells × 10^4; (B) number of plasmids × 10^4.

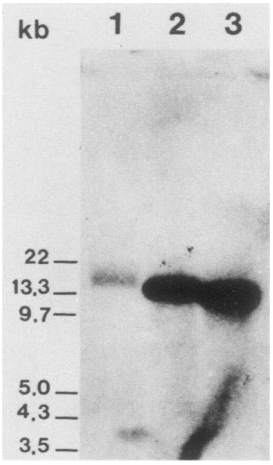

FIG. 5. Absence of a chromosomal copy of rDNA in *N. gruberi*. Using DNA from the pellet after Hirt fractionation (see Materials and Methods) to eliminate most of the extrachromosomal copies of the rDNA plasmid, an amount of DNA equivalent to that obtained from 10^6 cells was digested to completion with either *Bam*HI (lane 2) or *Hin*dIII (lane 3) and loaded on a 0.8% agarose gel with 10^6 copies of *Bam*HI-restricted pNgex27 (lane 1) to provide a control level of hybridization for single-copy genes. The DNA was electrophoresed at 10 V/cm for 4 h, transferred to nitrocellulose, and probed with the 1.9-kb *Bam*HI-*Sst*I fragment (Fig. 2). The size markers are from phage lambda DNA digested with *Bgl*II and with *Eco*RI plus *Hin*dIII.

precedent. The rRNA genes of *Physarum* and *Dictyostelium* species are extrachromosomal (3, 27, 35), but in these organisms the DNA is linear. Amplified, extrachromosomal rRNA genes have been described in several organisms where the germ line rDNA location is intrachromosomal (for example, *Tetrahymena* macronucleus and *Xenopus* oocyte; reviewed in reference 23), but in no other case is the entire rDNA complement circular and extrachromosomal.

Although many cases are known of extrachromosomal circles being associated with DNA amplification events, the existence of true, nonmitochondrial plasmids in eucaryotes has been proven in only two genera (30). The best studied are the 2μm and related plasmids in the genus *Saccharomyces* (2, 33, 34); the others are found in the genus *Dictyostelium* (26, 28). The rDNA plasmid of *Naegleria* is thus the third example of a rare eucaryotic phenomenon.

It is quite likely that the major satellite of *Naegleria* nuclear DNA described by Fulton (9) is the rDNA plasmid. Assuming a copy number of 4×10^3, a size of 14 kb, and a genome size of 0.34 pg of DNA per cell (9), the rDNA plasmid would constitute 17.5% of the total DNA. This is close to the value of 17% determined for the major satellite. Ribosomal genes typically have a higher G+C content than main band DNA, which is also true for the major *Naegleria* satellite.

The genus *Naegleria* and its relatives are of great interest to systematists since they represent a link between the flagellated *Mastigophora* and the amoeboid *Sarcodina*. Although classified in the same subclass as the genus *Acanthamoeba* (19), several differences between the rRNA genes of these two species can already be seen. The 28S rRNA of *Acanthamoeba* has a centrally located gap (31), while that of *Naegleria* does not (Fig. 3). Additionally, the restriction maps are significantly different; none of the restriction sites for *Bam*HI, *Pst*I, and *Hin*dIII appear to be in common between the two (compare Fig. 2 with reference 5). An extrachromosomal location for the rRNA genes in *Acanthamoeba* has not been absolutely ruled out, but their organization is certainly not the same as in *Naegleria* (5). In both species, however, the 5S rRNA genes are unlinked to the major transcription unit.

The gene for the 28S rRNA of certain protozoa is interrupted by one or more introns (*Physarum* [29]; *Tetrahymena* [6, 36]). All these introns have been localized to a relatively short segment of the 28S gene close to the 3′ end. The fortuitous isolation of a cDNA clone that covers this region of the 28S rRNA sequence meant that the presence of an intron in this region of the *Naegleria* gene could be easily tested. Since the restriction maps of the cDNA and rDNA plasmid clones are identical, the presence of an intron, in this region at least, can be ruled out.

The relative amounts of the different rDNA plasmid structures varied slightly between preparations, mostly in the proportion of linear and supercoiled forms. The major component was always that which was interpreted to be form II DNA. This may be the form in which the plasmid is transcriptionally active, although whether this represents relaxed or nicked circular DNA has not been determined. In addition to the putative form I, II, and III DNAs, at least two other bands that migrate very slowly are always seen. Their structure has not been analyzed, but they may represent replication intermediates, multimeric or catenated circles.

No chromosomally located copy of the rRNA transcription unit was detected in this study. This implies that the rDNA plasmid carries an origin of replication that is recognized by the *Naegleria* DNA replication machinery. The long-term aim of this project is to study the regulation of the phenotypic transformation event in *Naegleria*. The availability of a homologous, autonomously replicating plasmid has obvious utility for the development of a DNA transformation system in *Naegleria*.

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