

## Molecular Biology of DNA in *Acanthamoeba*, *Amoeba*, *Entamoeba*, and *Naegleria*

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### I. Introduction

Amoebae have been favorite models for the study of problems in cell biology for years. There have been comparatively few studies, however, on the molecular biology of these organisms. One reason for this lack of interest is that the larger free-living amoebae, which are particularly useful for nuclear transfers and other kinds of micromanipulation, have never been cultured axenically. Consequently, studies of molecular biology in

these organisms potentially are complicated by the presence of food organisms. Many of the smaller amoebae can be cultured axenically, but the absence of evidence for sexual reproduction and, therefore, classical genetic analysis, has dampened the enthusiasm of molecular biologists for studies on these organisms. With the advent of recombinant DNA technology, however, the genomes of asexual organisms have become much more accessible to study. Several laboratories already have begun investigating the structure and function of amoeba genes and others have indicated an interest in starting. There are a number of interesting problems that might be examined in the amoebae. I have tried to summarize here what is known about amoeba DNA in order to encourage others to explore the molecular biology of these organisms.

The four genera of amoebae that have been studied most extensively will be discussed. I will only consider amoebae that remain as single cells, but four distinctly different life cycles are found in these groups. Interested molecular biologists should be aware that there are many additional genera of amoebae that are essentially untouched with respect to any significant biochemistry or molecular biology (Singh, 1981; Levine *et al.*, 1980). Some of these may prove to be very interesting subjects for future studies.

The genus *Amoeba* includes large free-living organisms. Work on the biology of these amoebae was last reviewed by Jeon (1973). The cell cycle is simple; replication is by binary fission and differentiation is unknown. These amoebae are large enough to manipulate by various surgical procedures, and this attribute has been exploited extensively (Jeon, 1973). Cultures typically are grown using the ciliated protozoan *Tetrahymena* as the major food organism.

The genus *Acanthamoeba* includes small amoebae that are ordinarily free-living, but can be opportunistic pathogens of animals and humans (Martinez, 1980, 1983; Kadlec, 1978; Visvesvara, 1980). Such organisms are considered amphizoic (Page, 1974). In a few cases, infection has been lethal, but these organisms are ubiquitous in nature and most humans appear to have good resistance to infections. Unfortunately, the taxonomy of *Acanthamoeba* is confused, and it is unclear whether pathogenicity is restricted to certain species or whether all strains are capable of it. *Acanthamoebae* replicate by binary fission. In addition, they differentiate into dormant cysts (i.e., they encyst) during adverse environmental conditions. A number of reviews of this process are available (Byers, 1979; Weisman, 1976; Griffiths, 1970; Krishna Murti, 1975). Cultures are readily grown axenically in complex or chemically defined media.

The genus *Naegleria* includes small amoebae that can differentiate either into a cyst or a flagellated stage (Fulton, 1970, 1977). In *Naegleria*, it

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clear that certain species are pathogenic and that other species are not (John, 1982; Schuster, 1979). The pathogenic *Naegleria fowleri* is highly virulent and fatal to humans. Human infections are rare, but still, there has been little interest in the molecular biology of this species. The non-pathogenic species *Naegleria gruberi* has been the main organism studied. As in the case of *Acanthamoeba*, cultures of *Naegleria* can be grown axenically in complex or chemically defined media (Fulton *et al.*, 1984).

The genus *Entamoeba* is distinct from the rest because it includes anaerobes and parasites. The life cycle includes a phase of replication by binary fission and encystment, which occurs in response to environmental factors (Martinez-Palomo, 1982; Albach and Booden, 1978). Nuclear division can continue in the cyst in the absence of cytoplasmic division. Species are typically identified according to their hosts. *Entamoeba histolytica*, the human parasite, and *Entamoeba invadens*, a parasite of reptiles, are the two most commonly studied species. Both can be grown axenically under anaerobic conditions. *Entamoeba moshkovskii* is interesting because it appears to be free-living.

My objective is to discuss what is known about amoeba DNA during the vegetative replication cycle and during differentiation. I have attempted to present a reasonably current review of what is known about the structure and the overall organization of the DNA and its metabolism. In the past, such a review probably would have had a reasonable lifetime. Now, however, an increasing number of good molecular biologists are becoming interested in these organisms, and it can be expected that knowledge about amoeba genomes and the molecular biology of the cell cycle and cell differentiation will increase rapidly in the near future.

## II. Characteristics of Nuclear DNA and Gene Structure

### A. NUCLEAR DNA CONTENT AND CHROMOSOME NUMBERS

The nuclear DNA content of growing amoebae varies widely among species for which data are available (Table I). The lowest contents are found in the *E. histolytica*-like Laredo and Huff strains, which have 0.08–0.09 pg/amoeba, and in *E. moshkovskii*, which has 0.06 pg/amoeba. Reported values for *E. invadens* and *E. histolytica* are higher, ranging up to about 2 pg/amoeba. It is unknown whether the large differences in DNA content found in different laboratories for the HK 9 strains of *E. histolytica* are real or due to differences in methodology. The differences would suggest changes in ploidy levels or in gene amplification if substantiated. Two species of *Naegleria* have intermediate values around 0.2–0.3

TABLE I  
CHARACTERISTICS OF AMOEBA NUCLEAR DNA

Species/strain	DNA/amoeba (pg) <sup>a</sup>	GC (%) nuclear main-band DNA <sup>b</sup>	GC (%) nuclear satellite DNA <sup>b</sup>	References
<b>Acanthamoeba</b>				
<i>A. castellanii</i> /Neff	1.28	57-61		Adam <i>et al.</i> (1969); Bohnert and Herrmann (1974); Hettiarachchy and Jones (1974); L. E. King and T. J. Byers, unpublished; Jantzen (1973)
<i>A. astronyxis</i>		50		Band and Mohrlok (1973)
<i>A. palestinensis</i>		62		Adam <i>et al.</i> (1969)
<i>A. polyphaga</i>		57		Adam <i>et al.</i> (1969)
<b>Amoeba</b>				
<i>A. indica</i>		59.8	28.4	Fritz (1982)
<i>A. proteus</i> /F		57.4	23.5	Fritz (1982)
<i>A. proteus</i> /A/D <sub>1</sub> D <sub>2</sub> D <sub>3</sub> D <sub>4</sub>		23.4-29.5	44.7-61.8	Fritz (1982)
<i>A. proteus</i> /Bk		34.0	55	Spear and Prescott (1980)
<i>A. proteus</i>	42.8			Tautvydas (1971)
<b>Amoeba hybrids</b>	34			
<i>A. proteus</i> (nucleus) + <i>A. indica</i> (cytoplasm)		63.5	28.9	Fritz (1982)

<i>A. indica</i> (nucleus) + <i>A. proteus</i> (cytoplasm)				25.3	48.6	Fritz (1982)
<b><i>Entamoeba</i></b>						
<i>E. histolytica</i> /200: NIH/F-22	0.5, 0.6	29.2, 30.6				Gelderman <i>et al.</i> (1971b); Reeves <i>et al.</i> (1971)
<i>E. histolytica</i> /HK9/HK9-1/HK9-2	0.45-2.21	27.6				Gelderman <i>et al.</i> (1971a,b); Reeves <i>et al.</i> (1971); Lopez-Revilla and Gomez (1978)
<i>E. histolytica</i> -like/Laredo	0.09	30.8				Gelderman <i>et al.</i> (1971b); Reeves <i>et al.</i> (1971)
<i>E. histolytica</i> -like/Huff	0.08	30.7				Gelderman <i>et al.</i> (1971b); Reeves <i>et al.</i> (1971)
<i>E. invadens</i> /PZ/IP/165	0.32-0.82	26.1-33.49 ( $T_m$ )				Gelderman <i>et al.</i> (1971b); Lopez-Revilla and Gomez (1978)
<i>E. invadens</i> /IP-1	0.15					Sirijinktarn and Bailey (1980)
<i>E. moshkovskii</i> /FIC	0.06	31.7 ( $T_m$ )				Gelderman <i>et al.</i> (1971b); Lopez-Revilla and Gomez (1978)
<b><i>Naegleria</i></b>						
<i>N. fowleri</i> /Lee	0.2					Weik and John (1978)
<i>N. gruberi</i> /NEG	0.17	34			43	Fulton (1977)
<i>N. gruberi</i> /NB-1	0.34					Fulton (1977)

<sup>a</sup> Includes cytoplasmic DNA in *Acanthamoeba* and *Naegleria*. All values are for mononucleated populations except for *Entamoeba*, where the averages ranged from 1 to 1.2 nuclei per amoeba in different strains (Lopez-Revilla and Gomez, 1978).

<sup>b</sup> Percentage of GC is based on buoyant density except for *E. invadens* and *E. moshkovskii*, where it is based on melting temperature ( $T_m$ ).

pg/amoeba, whereas *Acanthamoeba castellanii* Neff most commonly has 1–2 pg/amoeba. Substantially higher values have been reported for this strain (Marzzoco and Colli, 1974; Coulson and Tyndall, 1978), but these values seem inconsistent with measurements from other laboratories (Byers, 1979). The largest amoeba DNA values are for *Amoeba proteus*, at about 34–43 pg/nucleus.

Entamoebae are anaerobic and lack mitochondria; therefore, the nuclear DNA and whole cell DNA contents are the same. Very little is known about mitochondrial DNA contents of the aerobic amoebae. Our measurements indicate that mitochondrial DNA can be as high as 20% of total cell DNA in early log-phase *A. castellanii* (King and Byers, 1985). Earlier work by Adam *et al.* (1969) indicated that the mitochondrial DNA could be as high as 30% of the total, but that it was variable. In *N. gruberi*, the mitochondrial DNA was 14% of the cell total (Fulton, 1977). No figures are available for *Amoeba*, but in general, amoebae appear to have relatively high proportions of mitochondrial DNA.

Both nuclear and mitochondrial DNA contents vary during culture growth. The nuclear DNA content of unagitated cultures of *A. castellanii* decreases during the log to post-log-phase transition (Byers *et al.*, 1969; King and Byers, 1985). This decrease is associated with preparations for encystment (see Section V,A), but probably is not essential for differentiation, since suspension cultures induced to encyst by  $\text{MgCl}_2$  double their DNA content during encystment (Chagla and Griffiths, 1974). Since these authors have demonstrated elsewhere that  $G_1$  is the predominant cell-cycle phase under their culture conditions (Chagla and Griffiths, 1978), the increase may simply be due to arrest of amoebae in  $G_2$  phase after the DNA has been replicated. Variations in DNA content also were noted in *N. gruberi* where the DNA/nucleus also increased post-log phase (Weik and John, 1977). In contrast, the DNA/nucleus was relatively constant during the log- and post-log-phase transition in *N. fowleri* (Weik and John, 1978). No explanation is currently available for this difference.

Our experience with *A. castellanii* indicates that mitochondrial DNA is extensively degraded during late log- and post-log-growth phases (see Section V,A). No evidence about this matter is available for the other amoeba genera.

Amoeba chromosomes are small, and estimates of chromosome numbers are difficult to obtain. As many as 500–1000 small chromosomes have been observed in *A. proteus* (Andresen, 1973; Ord, 1973). Early authors reported counts of 3–16 chromosomes for *Naegleria*, but Fulton (1977) concluded that the chromosomes of *N. gruberi* were too small and too tightly packed to count. In *Acanthamoeba* also, chromosomes were small and too tightly clustered to permit accurate counts (Pussard, 1964,

1972). The picture is even more confused in *Entamoeba* where chromosomes have yet to be identified (Martinez-Palomo, 1982).

Until recently, the prospect of obtaining information about numbers, sizes, and linkage groups of amoeba chromosomes was bleak. With the development of pulse-gradient electrophoresis, however, the prospect has brightened considerably (Schwartz and Cantor, 1984; Carle and Olson, 1984). With this technique, which has been used successfully to separate small chromosomes from trypanosomes and yeast, it should be possible to study the chromosomes of many lower eukaryotes.

### B. UNIQUE AND REPETITIOUS SEQUENCES

The sequence organization of amoeba nuclear DNA is interesting because of the primitive nature of these organisms. Very limited data are available on the proportions of unique and repetitious nucleotide sequences in amoeba DNA. Estimates of the unique fraction range from 0.74 to 1.0 for *A. castellanii* Neff, but the lower value seems more likely because it has been obtained in more than one laboratory (references in Myers, 1979). The values for three strains of *Entamoeba*, estimated from a published graph (Gelderman *et al.*, 1971a), range from 0.4 to 1.0. Since these data are all from the same laboratory, the variability may be real. The *E. histolytica*-like Laredo strain, which appears to have the least amount of repetitious sequences, has an estimated genome size of 0.01 pg. A variety of fungi, which are the main group of eukaryotic organisms having very little repetitious DNA, also have genomes of less than 0.05 pg (references in Pellegrini *et al.*, 1981). *E. moshkovskii*, which seems to have the largest repetitious fraction, has an estimated genome size of 0.1 pg. Further studies of *Entamoeba* genomes would be particularly significant because they currently are evolving in the absence of any possible interaction with organelle DNA. It would be interesting to determine whether they carry any organelle-like sequences indicative of interactions with symbionts during their previous evolutionary history.

### C. GENOME SIZE AND PLOIDY LEVELS

There is no definitive evidence for sexual reproduction in any of the species examined here, and ploidy levels are unknown. Fulton (1970) suggested that haploid (NEG) and diploid (NB-1) strains of *N. gruberi* occur in the laboratory. The strongest evidence favoring this view is the observation that strains usually contain DNA in relative amounts of 1× or 2× (Table I) and that a 1× strain (NEG) is readily mutagenized, whereas a 2× strain (NB-1) is not. Knowledge about ploidy levels and possible

sexual processes may be available from analyses of interstrain variations in the electrophoretic mobility of selected enzymes. This approach has been applied with some success in studies of the same problems in the kinetoplastida (Tait, 1983). Ward (1985) has briefly reported on variations in the mobility of lactic dehydrogenase with respect to its genetic coding in *Acanthamoeba*, but the data raise more questions than they answer. Nevertheless, a thorough genetic analysis of isozyme variants in each of the four amoeba genera certainly would be worthwhile.

The likelihood of a polyploid state in *A. proteus* is suggested by the very large amounts of nuclear DNA (40–50 times the amount in a human cell) and by evidence that some strains may have 500–1000 chromosomes (Andresen, 1973; Ord, 1973). More direct evidence for polyploidy is available for *Acanthamoeba* and *Entamoeba* from comparisons of total nuclear DNA and estimates of genome size based on measurements of kinetic complexity. The haploid genome of *A. castellanii* Neff is 0.045 pg based on the best estimates of the kinetic complexity of the unique fraction ( $2 \times 10^{10}$  D), the proportion of the total nuclear DNA that is unique (74%), and the proportion of the cell DNA that is nuclear (85%) (Bohnert and Herrman, 1974; Jantzen, 1973; King and Byers, 1985). The average amoeba in a log-phase population has 1.28 pg DNA (King and Byers, 1985) and, therefore, would be around  $25n$ . The Laredo strain of *Entamoeba*, with an estimated haploid genome size of 0.01 pg ( $10^7$  nucleotide pairs) and a nuclear content of 0.09 pg (Gelderman *et al.*, 1971a,b), would be about  $9n$ . *E. moshkovskii*, with an estimated genome size of 0.1 pg ( $10^8$  nucleotide pairs) and an estimated nuclear content of 0.06 pg, would be about  $1n$ . *E. histolytica* HK 9, with a genome size of 0.033 pg ( $10^{7.5}$  nucleotide pairs) and a nuclear content of at least 0.45 pg (Table I), would be  $14n$  or greater. (See also Section V,B for *E. invadens*.) Of course, there is a potentially large error in these calculations. Nevertheless, they provide the best estimates of ploidy levels that we currently have, and they suggest that several strains are likely to be polyploid.

#### D. NUCLEAR SATELLITES

The nuclear DNAs of a number of *Amoeba* strains and at least two strains of *Naegleria* (NEG and NB-1) separate into two bands on CsCl gradients (Fritz, 1981, 1982; C. Fulton, 1977, personal communication), whereas only one band has been reported for *Entamoeba* and *Acanthamoeba* (Gelderman *et al.*, 1971a; Adam *et al.*, 1969). The main band of *A. proteus* typically was 75% or more of the total nuclear DNA and had an average GC content of about 30% in 10 strains (Fritz, 1981, 1982). The heavier satellite band included the balance of the nuclear DNA and had an

verage GC content of 54% in six strains. The heavier band, however, was not always the quantitatively minor band. In the closely related *Amoeba indica* and in *A. proteus* F, the heavy band included the majority of the DNA and, therefore, was referred to as the main band (Table I). Experiments with hybrid amoebae, which contained a nucleus from one strain and cytoplasm from another, suggested that the main and satellite bands could change in relative proportions under the influence of cytoplasmic conditions (Fritz, 1982). For example, in a hybrid including a nucleus from *A. proteus* and cytoplasm from *A. indica*, the GC contents of the main and satellite bands were 63.5% and 28.9%, respectively (Table I). In the reciprocal hybrid, the main band was 25.3% GC and the satellite was 48.6%. In each case, the distribution of DNA between the two bands appeared to be characteristic of the strain that was the source of cytoplasm. Clearly, a study of the regulation of the relative proportions of DNA in the main and satellite bands might contribute to our understanding of roles that cytoplasmic factors can play in differential replication of nuclear DNA. Unfortunately, the present lack of axenic culture conditions weighs against the use of *Amoeba* for these studies, but it should at least be possible to characterize differences between donor strains and reciprocal hybrids in main band and satellite DNA. In addition, the possibility that variations in proportions of main band and satellite DNA might occur under different conditions should be explored with *Naegleria*, which can be grown axenically.

It is unknown whether the nuclear satellites contain unique or repetitive sequences or both. The possibility has been considered that the nuclear satellite in *A. proteus* includes the genes coding for ribosomal DNA (Spear and Prescott, 1980). This proposal is appealing because the amoeba nucleus has an unusually large number of nucleoli (Andresen, 1973) and because DNA associated with the nucleoli replicates late in the cell cycle and, therefore, somewhat independently of the main body of DNA (Minassian and Bell, 1976). Furthermore, starved amoebae in G<sub>2</sub> initiate DNA synthesis in the satellite upon refeeding (Spear and Prescott, 1980). Since rDNA replication could be induced by refeeding of starved *Tetrahymena* (Engberg *et al.*, 1974), it was possible that the same might be true for *A. proteus*. However, satellite DNA failed to hybridize with rRNA from *Tetrahymena*, seemingly ruling out the possibility that satellites are amplified rDNA (Spear and Prescott, 1980).

Microspectrophotometric analysis of fluorescent-stained nuclei indicated that nuclear DNA in *A. proteus* more than doubles during the cell growth-duplication cycle (Makhlin *et al.*, 1979). It would be interesting to know whether the excess DNA is associated preferentially with either of the nuclear DNA bands. It has been suggested that the excess might be DNA (Makhlin *et al.*, 1979), but there is no evidence for this.

### E. STRUCTURE OF ACTIN, MYOSIN, AND RIBOSOMAL RNA GENES IN *Acanthamoeba*

At present, data on nucleotide sequences are available for one gene, for portions of two others, and for two RNA products in *A. castellanii* Neff. Actin is the most abundant polypeptide in *Acanthamoeba*. Two-dimensional electrophoresis revealed at least three different actin molecules (Jantzen, 1981). A gene for one of these molecules, actin I, has been cloned and totally sequenced (Nellen and Gallwitz, 1982). Actin I is a protein of 374 amino acids and, therefore, requires a coding sequence of 1122 bp (base pairs). In addition, the gene has a single intron of 129 bp. Common eukaryotic signal sequences are found in the promoter region. A modified TATA box 5'-CATAAAA-3' begins 30–31 nucleotide pairs upstream from the mRNA start site. The sequence 5'-AACCAAGCG-3', which is similar to the eukaryotic consensus sequence 5'-GGC/TCAATCT-3', begins 70–71 nucleotide pairs upstream from the start site. The exact 3' end of the mRNA has not been identified, but the sequence 5'-AATACA-3' located 44 nucleotide pairs downstream from the translation termination codon may be a modification of the more common polyadenylation signal 5'-AATAAA-3'. The overall amino acid sequence of actin I is closely related to the predominant actins from *Physarum* and *Dictyostelium*, but is very different from yeast actin. The amino acid sequence is unusual in having an N-terminal glycine. The codon usage is very biased; there is a strong preference for C and an avoidance of A in the third position. Nellen and Gallwitz (1982) found evidence for at least three different actin genes, an observation consistent with previous electrophoretic data (Jantzen, 1981).

At the time of this writing, the sequencing of an actin gene of *N. gruberi* is nearly complete (C. Fulton, personal communication). It will be interesting to compare the two amoeba genes with respect to coding biases, signal sequences, and other organizational features.

Three different myosin molecules, myosin IA, IB, and II, have been found in *A. castellanii* Neff (Gadasi *et al.*, 1979). The gene for the heavy chain of myosin II has now been cloned and partially sequenced (Hammer *et al.*, 1984; J. A. Hammer, personal communication). The putative gene was isolated from a genomic library of *Acanthamoeba* DNA in phage  $\lambda$  using a portion of the myosin heavy-chain gene from the nematode *Caenorhabditis elegans* as a probe. Most or all of the gene is contained in a 6.5-kb restriction fragment. The fragment is complementary to a 5300-bp mRNA which codes *in vitro* for a protein of 185 kDa. The protein comigrated in SDS–polyacrylamide gel electrophoresis with purified *Acanthamoeba* myosin II heavy chain and is quantitatively and selec-

vely precipitated by antiserum to the heavy chain. The identity of the gene has been confirmed by antiserum to the heavy chain. The identity of the gene has been confirmed by sequencing of the 3' end. Preliminary results suggest that introns are absent or very short and that there may be more than one myosin II heavy-chain gene in *Acanthamoeba*.

The genes for ribosomal RNA in *Acanthamoeba* include the typical 18–26 S repeat unit plus 5 S genes which are unlinked to the repeat unit. The organization of the 5 S genes is unknown. The repeat unit has been cloned and partially sequenced (D'Allessio *et al.*, 1981; M. R. Paule, personal communication). The number of repeat units is unknown, but all seem to be the same. There is no evidence for introns, but an unusual transcribed spacer occurs within the 26 S gene. Stevens and Pachler (1972) discovered that the 26 S rRNA in the large ribosomal subunit actually consisted of two large fragments held together by noncovalent bonds. Paule and his associates have shown that the coding region for these fragments includes two sequences, one about 2 kb and the other about 2.4 kb, separated by a 200-bp transcribed spacer. An *in vitro* transcription system for the ribosomal repeat unit in which transcription constantly begins at the authentic initiation site has been developed (Paule *et al.*, 1984a). The site is located within a 74-bp sequence and is recognized by purified RNA polymerase I plus a single species of initiation factor, designated TIF-1. Correct initiation is species specific for both the polymerase and the initiation factor (Grummt *et al.*, 1982; M. R. Paule, personal communication).

The promoter used for transcription of the ribosomal genes can be subdivided into three regions, referred to as motifs (Kownin *et al.*, 1985; da Silva *et al.*, 1985). The *start motif* overlaps the initiation start site, defined as nucleotide pair 0; it extends from upstream position –26 to downstream position +8. This region is necessary for transcription of the 18 S rRNA. It is very AT rich, which might facilitate strand separation for initiation, but this probably is not essential, since start motifs in other organisms are not especially AT rich. The *Acanthamoeba* start motif does not bind TIF-1. The second region, *motif A*, extends further upstream from approximately –20 to –31; the boundaries are somewhat uncertain. This motif does bind TIF-1. Together, motif A and the start motif constitute the *core promoter*, extending from –31 to +8, which is essential for faithful *in vitro* translation. The third region of the promoter, *motif B*, extends from –32 to –47. It is not essential for transcription, but the efficiency of the process is greater when motif B is left intact. The region necessary for the formation of a stable preinitiation complex that includes one or more initiation factors plus part of the core promoter sequence. The complex does not include RNA polymerase I. Although

transcription of rRNA is species-specific, motif B contains a sequence ACTTT, extending from -40 to -45, that closely resembles the ATCTTT sequence found in mouse, rat, and human rRNA promoters. Paule's group suggests that the sequence may have a general function in promoting transcriptional efficiency (Kownin *et al.*, 1985).

Ribosomal RNA synthesis is shut down during the encystment of *Acanthamoeba* (Stevens and Pachler, 1973). Paule and his colleagues have determined that the down-regulation of the rRNA genes is due to a modification of RNA polymerase I (Paule *et al.*, 1984b). This is the first example of eukaryotic gene regulation by polymerase modification. The nature of the modification has not been determined, but a phosphorylation, adenylation, or similar modification is suspected.

Indirect information about organization of the 5 and 5.8 S rRNA genes has been obtained from sequencing of the two RNAs (MacKay and Doolittle, 1981). The 5 S RNA consists of 119 nucleotides, and the base sequence fits a secondary structure model that is consistent with other eukaryotic 5 S rRNAs. The nucleotide sequence was compared to 5 S rRNA sequences for two other protozoans, *Tetrahymena* and *Crithidia*, and a variety of other organisms. The protozoan:protozoan comparisons gave sequence homologies averaging 64%. In contrast, 5 S rRNA from the insect *Drosophila melanogaster* is 76% homologous with human 5 S rRNA and 85% homologous with 5 S rRNA from the echinoderm *Lytechinus variegatus*. Furthermore, the *Acanthamoeba* sequences were as divergent from those of other protozoans as from organisms in other kingdoms (i.e., wheat, human, and yeast 5 S rRNAs are all 65% homologous with the amoeba RNA).

The 5.8 S rRNA of *Acanthamoeba* consists of 162 nucleotides, including four pseudouridine and four methylated bases (Mackay and Doolittle, 1981). The sequence only partially fits either of two secondary structure models previously proposed, even though the models were both consistent with 5.8 S rRNA from organisms as diverse as yeast and vertebrates. The indications of extensive molecular diversity in the amoeba 5 and 5.8 S rRNAs are fully consistent with evidence from other sources which indicate an unusually high level of molecular diversity in the protozoans, probably explained by a long evolutionary history (Nanney, 1984; Williams, 1984).

#### F. RIBOSOMAL RNA GENES IN *Amoeba*

No information is available for the nucleotide sequences of any genes in the genus *Amoeba*. However, Murti and Prescott (1978) probably have observed transcriptionally active rRNA genes in *A. proteus* by using elec-

on microscopy and the Miller technique to obtain spreads of nucleolar material. Of the putative rRNA genes observed, 90% occurred as single units (matrix units) averaging  $6.07 \mu\text{m}$  in length and decorated with 100–200 lateral fibers that were 20 nm in diameter and ranged up to an average of  $0.85 \mu\text{m}$  in length. The central axis, presumably DNA plus associated protein, was 4 nm in diameter. The lateral fibers, presumably ribonucleoproteins, increased in length from one end of the unit to the other, but the length gradient was not nearly as great as is typical for transcriptionally active rRNA genes isolated from other organisms. There was no evidence for the typical tandem repeating arrangement of the basic units. Of the units, 10% were seen in aggregates of two or three, but it was impossible to determine whether the central axes of the aggregated units were continuous with each other.

The 25 and 19 S rRNA molecules of *A. proteus* have molecular weights of  $1.5$  and  $0.84 \times 10^6$ , respectively (L. Goldstein, quoted in Murti and Prescott, 1978). Similar molecular-weight values have been reported for *A. discoides* ( $1.55$  and  $0.8 \times 10^6$ ; Hawkins and Hughes, 1973) and *A. castellanii* ( $1.53$  and  $0.89 \times 10^6$ ; Stevens and Pachler, 1972). Approximately  $2.2$ – $2.3 \mu\text{m}$  of DNA is required to code for the two rRNA molecules of *A. proteus*. If the basic ribosomal gene repeat unit in *A. proteus* is typical and includes coding sequences for 29, 19, and 5.8 S rRNA plus transcribed spacers, then a single matrix unit would be large enough to contain one or two repeat units. Information about the size of rRNA precursors might help distinguish between these two possibilities, but so far no precursor has been identified for *A. proteus*. However, the single gradient of lateral fiber lengths suggests that the matrix unit is transcribed as a single unit rather than as two units.

### III. Mitochondrial DNA Characteristics

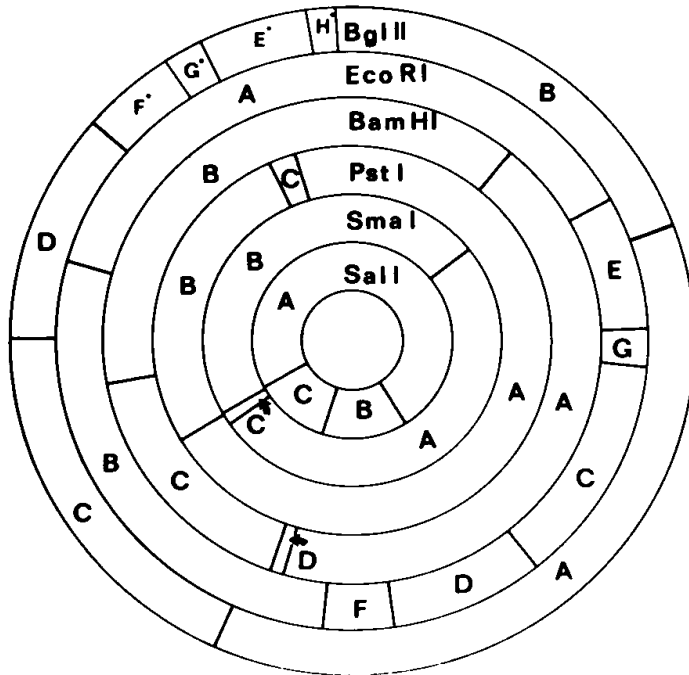
*Acanthamoeba*, *Amoeba*, and *Naegleria* are genera of obligate aerobes and, consequently, all possess mitochondria. Entamoebae are anaerobes and do not have mitochondria or cytochromes. With the exception of *Acanthamoeba*, very little is known about amoeba mitochondrial DNA. Several laboratories are currently investigating the mitochondrial DNA of *Naegleria*, but the studies are only in preliminary stages. It is only known that the mitochondrial DNA of *N. gruberi* NEG has a buoyant density of 1.683 (23% GC), compared to the main-band nuclear DNA density of 1.693 (34% GC), and is 14% of the total DNA (Fulton, 1970, 1977).

Mitochondrial DNA has been isolated from *Acanthamoeba* in a number of laboratories (reviewed in Byers, 1979). In our own laboratory, DNA

from more than 20 strains has been examined (Bogler *et al.*, 1983; T. J. Byers, S. A. Bogler, V. Stewart, and E. Hugo, unpublished). The mitochondrial genome occurs as a circular molecule (Bohnert, 1973; Bogler *et al.*, 1983). The average size that we have measured for 19 strains is  $41.5 \pm 1.5$  (SD) kb. Sizes ranged from 38.7 to 44.0 kb. A twentieth strain, HOV.6, has a significantly larger genome of 49 kb. The G + C compositions have been determined from CsCl buoyant density measurements by Adam and Blewett (1974) for *A. castellanii* Neff (34%), *A. castellanii* Reich (*A. palestinensis*) (37%), *A. polyphaga* (43%), and *A. astronyxis* Ray (30%). Clearly, as in *Naegleria*, all mtDNAs are rich in A + T. A more extensive effort to study sequence heterogeneity in *A. castellanii* Neff examined the following: the buoyant density, the differential melting profile, the elution of mtDNA from hydroxyapatite by a gradient of sodium phosphate, and the percentage GC of fragments retarded on Sephadex G-25 in samples progressively degraded by micrococcal nuclease (Mery-Dugeon *et al.*, 1981). The overall percentage GC was 32.9, essentially the same as for the fungus *Ustilago cyanodontis*, which was examined in the same study. Although both organisms were equally rich in A + T, the fungal DNA appeared to have long nonalternating A–T regions, such as previously discovered in *Saccharomyces*, whereas the amoeba DNA did not.

We have digested mtDNA obtained from 20 strains of *Acanthamoeba* with 5–17 different restriction endonucleases (see Section VI,B) and are currently constructing physical (restriction site) maps from these data. In addition, we have begun to identify putative gene locations by use of heterologous gene probes from *Neurospora* (R. A. Akins, S. A. Bogler, E. Hugo, V. Stewart, and T. J. Byers, unpublished). The first physical map of the mitochondrial genome was constructed for *A. castellanii* Neff by H. J. Bohnert and A. von Gabain (Fig. 1, unpublished results). The techniques used to order the DNA fragments included double digestions using combinations of enzymes (restriction fragment sizes have been published by Mery-Dugeon *et al.*, 1981, and Bogler *et al.*, 1983), partial digestions, redigestion of isolated DNA fragments with other restriction endonucleases, and DNA–DNA hybridization. Bohnert and von Gabain also localized the rRNA genes. The large ribosomal RNAs are very similar in size to the 16 and 23 S rRNAs of *Escherichia coli*. One set of rRNA genes was found in *Acanthamoeba*, and the two genes were separated by about 3.5 kb.

Log-phase amoebae of the Neff strain contained an average of about 0.15 pg of mitochondrial DNA per amoeba (King and Byers, 1985). With a genome size of  $\sim 2.7 \times 10^7$  Da, there should be  $\sim 3300$  mitochondrial DNA molecules per cell. During encystment, King and Byers found mitochon-



1. Restriction fragment map of mitochondrial DNA from *Acanthamoeba castellanii*. The map is from unpublished results of H. J. Bohnert and A. von Gabain (used with permission). These authors located the 16 S rRNA gene on *SalI* fragment C at the end of fragment A. The 23 S rRNA gene was located on *BamHI* fragment B overlapping sites of *EcoRI* fragments A and B, but mostly B. The order of *BglII* fragments E, F, G, is uncertain. Fragment sizes for *EcoRI*, *BamHI*, *PstI*, *SalI*, and their double digests have been published (Mery-Drugeon *et al.*, 1981). Unpublished fragment sizes for *SmaI* are 13.9, 13.1, and 0.25 mDa and 9.40, 5.7, 4.9, 3.0, 2.4, 1.0, 0.52, and 0.48 mDa, respectively.

DNA levels dropping to between 0.01 and 0.02 pg per amoeba; this should be enough for 223–446 molecules per cyst. No rigorous estimates of mitochondrial numbers are available, but rough estimates can be made from data available for suspension cultures of the Neff strain (Bowers and Korn, 1969). According to these workers, cyst volumes averaged about  $1 \mu\text{m}^3$ , and the mitochondrial volume was about 6.8% of the total cell volume. The cyst mitochondria in their pictures appeared to be roughly spherical with an average diameter of about  $0.5 \mu\text{m}$ ; thus, the average volume for a mitochondrion was about  $0.065 \mu\text{m}^3$ . Cysts would have 2300 mitochondria of this size. This number is much larger than the estimated number of mitochondria based on our data. However, for amoebae grown in monolayer cultures in our laboratory, the average volume of trophozoites was only about 29% of the trophozoite volume in suspension cultures of Bowers and Korn (Byers *et al.*, 1969). Assuming constant mitochondrial size and fraction of total cell volume, the

smaller cell might have about 670 mitochondria per cyst ( $0.29 \times 2300$ ). Because of the uncertainties in this estimate, this number probably is not significantly different from the number of molecules of DNA estimated from King's data. Unfortunately, we do not have any reasonable estimate of the numbers of mitochondria in trophozoites. However, in the work of Bowers and Korn, the total mitochondrial volume of log-phase cells is 5.3 times that of cysts. If this factor is used for the monolayer cultures and mitochondrial sizes remain unchanged, the trophozoites should have about 3600 mitochondria ( $5.3 \times 670$ ); this agrees well with the estimate of 3300 mitochondrial DNA molecules. Thus, it seems likely that the number of mtDNA molecules per mitochondrion is relatively small and may even be one.

#### IV. DNA Replication during the Growth-Duplication Cycle

##### A. DNA POLYMERASES OF *Amoeba*

Very little is known about the enzymatic machinery of DNA replication in amoebae. To date, only the DNA polymerases of *Amoeba discoides* have been examined (Abbott and Hawkins, 1980). These studies were stimulated by the observation that certain phenotypic traits, e.g., streptomycin resistance, can be heritably transmitted by microinjection of fractions containing small-molecular-weight RNA (Hawkins, 1973; Abbott and Hawkins, 1980). The authors wondered whether this phenomenon might be explained by the presence of an RNA-dependent DNA polymerase capable of synthesizing DNA from the injected RNA. Consequently, template preferences for thymidine triphosphate ( $[^3\text{H}]\text{TTP}$ ) incorporation into acid-insoluble material were examined using a variety of deoxyribonucleotide and ribonucleotide templates. The DNA polymerase activities from whole cell homogenates were pooled after passage through Sephadex G-200 and had maximum activity when either heat-denatured calf thymus DNA or synthetic poly[d(A-T)] were used as templates. They also had respectable activity with *E. coli* rRNA and *A. discoides* microsomal RNA as templates. The template activity of amoeba RNA was reduced 81% by treatment with RNase. Three peaks of nuclear polymerase activity were identified using nicked double-stranded calf thymus DNA as a template. Two of these activities also were able to utilize heat-denatured calf thymus DNA as a template. When short columns were used for separations, the void volume activity was able to efficiently utilize RNA templates and poly(A)·d(pT)<sub>10</sub>, a synthetic riboadenylic polymer base paired to decadeoxythymidylate. The evidence is consistent

with the possibility that an RNA-dependent DNA polymerase is present. When longer columns were used, the ability to utilize the synthetic polymer disappeared; possibly a necessary cofactor was removed under these conditions. DNA polymerase activity able to utilize nicked double-stranded calf thymus DNA was found in the cytosol, but was not characterized further. The synthetic polymer poly(A·d(pT))<sub>10</sub> could not be used by enzymes present in the cytosol. Relationships between the DNA polymerases of *A. discoides* and the higher eukaryotes are unclear. However, the low-molecular-weight polymerase  $\beta$  seems to be missing, as in a number of other lower eukaryotes (Abbott and Hawkins, 1978).

### B. THE CELL CYCLE IN *Acanthamoeba*

The DNA replication cycle of *Acanthamoeba* has been reviewed previously (Byers, 1979). The review should be consulted for additional information. Based on [<sup>3</sup>H]thymidine pulse labeling, Neff (1971) concluded that the cell cycle of *A. castellanii* Neff was 2% M, 10% G<sub>1</sub>, 3% S, and 85% G<sub>2</sub>. We reexamined this problem in the Neff strain, combining [<sup>3</sup>H]thymidine pulse labeling with microspectrophotometric measurements of nuclear DNA content (King and Byers, 1985). The DNA measurements revealed a unimodal distribution characteristic of a population primarily in one phase of the cell cycle. Autoradiographs of the same cells indicated that the amoebae with the lowest DNA contents were in S phase. Thus, we agree that G<sub>2</sub> is the predominant phase, but find no evidence for G<sub>1</sub>. We conclude that the cycle under our conditions is 2% M, 9% S, and 89% G<sub>2</sub>. Evidence for the absence of G<sub>1</sub> also was obtained for *A. castellanii* HR (*A. rhysodes*; Band and Mohrlok, 1973). Colchicine blocked the cell cycle and the nuclear, but not cytoplasmic, incorporation of [<sup>3</sup>H]thymidine. Incorporation resumed following cell division after removal of the drug, but before complete reformation of nuclei in the daughter cells. Thus, G<sub>1</sub> would have been very brief if it existed at all. A third approach to analyzing the cycle utilized synchronous cultures of the Neff strain (Chagla and Griffiths, 1978). In this study, [<sup>3</sup>H]thymidine incorporation occurred just before cell division, suggesting that G<sub>1</sub> was both present and the predominant phase. It seems likely that the difference was due to differences in culture methods. It appears that G<sub>1</sub> can be present in the Neff strain, but it may not be essential.

### C. THE CELL CYCLE IN *Amoeba*

The DNA replication cycle of *Amoeba* was most recently reviewed by Prescott (1973). The large size of *A. proteus*, *A. discoides*, and *Amoeba*

*indica* has made it possible to obtain small synchronous populations by using micropipettes to select mitotic cells (Prescott and Goldstein, 1967; Ord, 1968; Rao and Chatterjee, 1974). Studies of [ $^3\text{H}$ ]thymidine incorporation into nuclear DNA in these cultures generally have demonstrated an S phase beginning immediately after cell division, as in *Acanthamoeba*. The  $G_1$  phase is either very brief or absent. One report of evidence for a  $G_1$  phase could not be substantiated by Prescott and Lauth (quoted in Prescott, 1973). Microspectrophotometric measurements revealed a unimodal distribution of nuclear DNA contents (Makhlin *et al.*, 1979), suggesting that amoebae were mostly in a single phase, presumably  $G_2$ . In *A. indica*, S phase lasted 3 hours out of a total generation time of 24 hours (Rao and Chatterjee, 1974). In *A. proteus* the S phase typically was 5–6 hours out of a cycle time of 36–48 hours (Prescott and Goldstein, 1967; Ord, 1968; Rao and Chatterjee, 1974). Ord demonstrated that the S period in *A. proteus* is biphasic, and Minassian and Bell (1976) demonstrated that the second burst of synthesis was associated with nucleoli and presumably included replication of DNA coding for ribosomal RNA. It would be interesting to know when the nuclear satellite DNA (see Section II,D) replicates, especially since one suggestion is that it might include rDNA (Spear and Prescott, 1980), but no evidence seems to be available on this point. Mitochondrial DNA continues to replicate throughout  $G_2$  in *A. proteus*, but Rao and Chatterjee (1974) found very little [ $^3\text{H}$ ]thymidine incorporation in the  $G_2$  phase of *A. indica*.

#### D. THE CELL CYCLE IN *Naegleria*

It has been difficult to determine the durations of cell-cycle phases in *Naegleria*; it has not been possible to obtain synchronous populations by selection of mitotic cells, the uptake rate of [ $^3\text{H}$ ]thymidine is low and internal pools appear to saturate slowly, the stages of mitosis are difficult to discern, and effective inhibitors of mitosis have not been identified (Fulton, 1977). In spite of these difficulties, estimates of cell-cycle phase durations for axenic cultures have been obtained (Fulton, 1977). The duration of mitosis was determined using estimates of the mitotic index. The duration of S phase was determined from the accumulation of labeled nuclei and from the percentage of labeled mitoses during continuous labeling of asynchronous cultures with [ $^3\text{H}$ ]thymidine. The latter method also was used to determine the duration of  $G_2$ . The best estimates of the cell-cycle parameters were as follows: M, 28 minutes (6%);  $G_1$ , 180 minutes (38%); S, 180 minutes (38%); and  $G_2$ , 90 minutes (19%), for a total cell-cycle time of 8 hours. Somewhat shorter estimates of S were obtained for monoxenic cultures. No published distributions of nuclear DNA con-

tent are available to confirm the conclusions based on labeling studies, but these should be easy to obtain using fluorescence-activated cell analysis (Coulson and Tyndall, 1978). There also is no evidence on the timing of replication of the nuclear satellite relative to the main band.

#### E. NUCLEOCYTOPLASMIC INTERACTIONS AND THE REGULATION OF DNA SYNTHESIS

Regulation of DNA synthesis has only been studied in *Amoeba* where the large size of these organisms has facilitated nuclear transplantation operations. Much of this work has been reviewed by Prescott (1973). The most obvious question to ask was what would happen to nuclear activities if nuclei from one phase of the cell cycle were transferred to cytoplasm at another phase. Consequently, S phase nuclei were transferred into G<sub>2</sub> phase cytoplasm and G<sub>2</sub> nuclei were placed into S cytoplasm. The results from short-term experiments are conflicting. In *A. proteus*, Goldstein and Prescott (1967) (Prescott, 1973) found that the [<sup>3</sup>H]thymidine incorporation decreased in S nuclei transplanted into G<sub>2</sub> cytoplasm and increased in G<sub>2</sub> nuclei transplanted into S cytoplasm. Thus, they proposed that the cytoplasm had immediate control of nuclear DNA synthesis. In contrast, following an extensive series of careful experiments, Ord (1969) concluded that the nuclear synthetic activity was unchanged in both types of transfers and, therefore, that DNA synthesis was not under the immediate control of cytoplasmic factors. This conflict remains unresolved. However, in a related experiment, Rao and Chatterjee (1974) found that S phase nuclei from *A. proteus* continued to incorporate labeled thymidine at an undiminished rate when transferred to G<sub>2</sub> cytoplasm of *A. indica*. These results may be consistent with those of Ord, but, of course, they also could indicate that cytoplasmic regulatory factors are species-specific. Rao and Chatterjee (1974) and Chatterjee and Rao (1974) clearly demonstrated, however, that the cytoplasm controlled nuclear diameter and the duration of S in the progeny of cells that survived nuclear implants and produced viable clones. When the smaller nuclei of *A. indica* were placed in *A. proteus* cytoplasm, they increased in size prior to the first cell division. However, when the larger nuclei of *A. proteus* were placed in cytoplasm of *A. indica*, the size adjustment did not occur until after the first division. It was technically impossible to follow changes in S phase in the same way; therefore, it is unknown when changes might have occurred. It is even possible that the S period did not change. This could be true if survival of hybrid cells only occurred when nuclei with S phase lengths at the appropriate extreme of the normal range, i.e., nuclei of *A. proteus* with the shortest S phases and nuclei of *A. indica* with the longest

S phases, were transferred into foreign cytoplasm. This would be consistent with the observation that only 22% of the hybrids with an *A. proteus* nucleus and *A. indica* cytoplasm and 3% of the reciprocal hybrids produced clones. Even if this explanation is correct, however, it is clear that the cytoplasm controls the duration of S phase in the long term. Goldstein and Ron (1969) suggested that nuclear proteins that are released to the cytoplasm at mitosis might include the factors controlling the duration of S. Their conclusions were based on the observation that S phase was prolonged in the daughters of cells from which 30–50% of the cytoplasm had been amputated during mitosis. It was presumed that the amputations resulted in daughter nuclei that were deficient in these particular proteins. As these authors recognized, however, other cytoplasmic factors that might be important also would be removed by the amputations.

## V. DNA Metabolism during Differentiation

### A. ENCYSTMENT IN *Acanthamoeba castellanii*

Several inhibitors of nucleic acid synthesis induce cyst formation in *Acanthamoeba* (reviewed in Byers, 1979). Since this fact was first discovered by Neff and Neff (1969, 1972), there has been considerable effort to understand why this phenomenon occurs. One approach has been to examine changes in DNA content and metabolism. The DNA content of log-phase amoebae is quite variable. The reason is unknown, but might include the possibility of a polyploid state (see Section II,C). Whole cell DNA content decreases during post-log phase or during starvation-induced encystment (Neff and Neff, 1969; Byers *et al.*, 1969; King and Byers, 1985). On the average, cysts, which have single nuclei, have about 50% as much nuclear DNA as early log-phase trophozoites, which also are mononucleated. The decrease would be explained if encystment medium stimulated a final cell division and daughter cells arrested without entering S (Rudick, 1971). Two observations make this unlikely. First, all available data indicate that cell multiplication ceases as soon as amoebae are transferred to encystment medium (King and Byers, 1985). In contrast, a final division does seem to occur when *Entamoeba* is incubated in encystment medium. Second, the decrease in DNA content during post-log phase probably is associated with preparations for encystment (Byers *et al.*, 1969) and, therefore, may occur for the same reasons that a decrease occurs in encystment medium. If the post-log amoebae arrest prior to S phase after dividing, then cultures diluted into fresh medium should exhibit a lag of nearly 8 hours before resuming multiplication. This does

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occur. Rather, King and Byers observed a small initial burst of division that was more consistent with a cell-cycle arrest near the end of the log phase. This result would be consistent with a reduction division in which amoebae divided and then continued to a late stage in the cycle without replicating DNA. The likelihood that acanthamoebae are polyploid is consistent with this possibility. Alternatively, amoebae might divide and complete S phase, but simultaneously or subsequently degrade approximately half of the total DNA; again, this would be more likely if the cells are polyploid. Either of these models would produce nuclei with about half the log-phase amount of DNA. Measurements of nuclear cross-sectional area indicate that the cyst nuclear volume averages 43% of the log-phase trophozoite nuclear volume (King and Byers, 1985). This decrease is consistent with the decreased DNA content, but does not help choose between the two models.

King and Byers have obtained evidence for nuclear and mitochondrial DNA degradation during post-log phase and starvation-induced encystment. Log-phase cultures were labeled with [ $^3\text{H}$ ]thymidine, and then the loss of label was observed by examining the DNA on CsCl gradients during encystment. Label was lost from both nuclear and mitochondrial DNA. When unlabeled amoebae were incubated in encystment medium with labeled thymidine, only the mitochondrial DNA became labeled. The simplest explanation for all the data is that net losses of both nuclear and mitochondrial DNA occur during encystment, nuclear loss occurring by degradation in the absence of synthesis and mitochondrial loss occurring by turnover. The nuclear DNA loss must be around 50%, but the mitochondrial loss can be 90% or greater (King and Byers, 1985).

#### B. ENCYSTMENT IN *Entamoeba invadens*

Little work has been done on the molecular biology of encystment in *Entamoeba*. The earliest work on [ $^3\text{H}$ ]thymidine incorporation was complicated due to the use of monoxenic cultures (Albach *et al.*, 1966). The first work with axenic cultures that has been reported explored the relationship between DNA synthesis, nuclear division, and cell division in *invadens* (Sirijintakarn and Bailey, 1980). Cultures growing in a nutrient-rich medium were induced to encyst in a hypoosmotic encystment medium supplemented with labeled thymidine or were prelabeled with thymidine and then induced to encyst in unlabeled medium. Data on changes in DNA content per amoeba and per nucleus as well as observed increases in levels of incorporated thymidine suggested the following. Binucleate amoebae with 0.151-pg DNA divided once upon transfer to encystment medium, presumably reducing the nuclear DNA content in

half. DNA synthesis and cyst wall formation were initiated and duplication of the DNA most likely occurred. One or two nuclear divisions followed, resulting in cyst populations with approximately one-half binucleates and one-half tetranucleates. The average DNA content was then 0.155 pg per amoeba. If the binucleates and the tetranucleates had the same whole cell DNA content, as seems likely, then the tetranucleate nucleus contained 0.039 pg. This nucleus, which was visibly smaller than the binucleate or mononucleate nuclei, resulted from three nuclear divisions, the first associated with cell division followed by DNA duplication and the latter two without cell division or DNA synthesis. If this interpretation of the results is correct, then the amoebae must have had nuclei that were at least  $4n$  at the time of transfer to encystment medium. However, a few octanucleate cysts were observed, and amoebae became octanucleate when first induced to excyst. If the octanucleates were formed without DNA synthesis, then the trophozoites initially induced to encyst could have been  $8n$ . This value agrees well with estimates of ploidy levels for other species of *Entamoeba* (see Section II,C). Sirijintakarn and Bailey observed that the total [ $^3\text{H}$ ]thymidine content of prelabeled cells decreased more than 50% during the course of differentiation from trophozoite to mature cyst. A 50% decrease would be expected if there was only one cell division following transfer of trophozoites to encystment medium. The authors attributed the additional loss to continued cell multiplication by some cells. However, considering the observations for *Acanthamoeba* (see Section IV,A), nuclear DNA turnover should be considered a good alternative possibility.

### C. FLAGELLATION IN *Naegleria gruberi*

Essentially nothing has been reported on DNA metabolism during encystment in *Naegleria*. Interest has focused on the amoeba-flagellate transformation that is induced by replacing growth medium with a nutrient-free transformation buffer (Fulton, 1970, 1977). Flagellation is accompanied by a decrease in DNA synthesis, and the factors that regulate this decrease have been the subjects of several studies. Under conditions used by Yuyama and Corff (1978), flagellum formation begins about 60 minutes after incubation in transformation buffer. These authors utilized CsCl gradients to examine the accompanying changes in [ $^3\text{H}$ ]thymidine incorporation and observed that there was a marked decrease in the incorporation into nuclear DNA. Mitochondrial DNA synthesis continued and became increasingly dominant as differentiation progressed. Some label continued to be incorporated into nuclear DNA, but this might be explained by the fact that a few amoebae failed to become flagellated.

The decrease in thymidine incorporation was accompanied by a drop in

thymidine uptake and in the activity of thymidine kinase (Bols *et al.*, 1977). The extent of the decrease in enzyme activity was strongly correlated with the proportion of the culture forming flagella. In contrast to thymidine kinase, the activity of nucleotide phosphotransferase, which phosphorylates thymidine to dTMP, remained essentially unchanged throughout differentiation. The thymidine kinase activity in equal mixtures of extracts from log phase and differentiating amoebae was the sum of the two activities mixed; thus, the differences are not due to activators or inhibitors. The decrease in activity during differentiation was only slightly slowed by concentrations of cycloheximide and actinomycin D that block protein and RNA synthesis. Therefore, the lost activity does not result from the concomitant synthesis of a degradative enzyme.

Most cell types arrest in  $G_1$  or sometimes  $G_2$  phase when differentiation is initiated. It was concluded, however, that *N. gruberi* arrests in mid-S phase during flagellum formation (Corff and Yuyama, 1976; Yuyama and Corff, 1978). Since the amoebae are in an asynchronously multiplying culture when first induced to differentiate, it would take about 3 hours for the amoebae in the earliest stages of S phase to complete DNA synthesis ( $S = 3$  hours). Since nearly 100% differentiation occurs in 2 hours, some amoebae must initiate differentiation while in mid-S phase. However, that proportion of the population that was in the first hour of  $G_1$  ( $G_1 = 3$  hours) would not reach S phase within the 2-hour period of differentiation and, therefore, must initiate flagellation from  $G_1$ . In the expected age-frequency distribution of a growing asynchronous population, the number of cells having any particular age decreases continuously from the youngest to the oldest groups (James and Cook, 1958; Steel, 1973). In *N. gruberi*, where the duration of  $G_1$  equals the duration of S, substantially more cells should be in  $G_1$  than in S. Thus, a relatively large proportion of the population could be in  $G_1$  when they differentiate. Additional amoebae must differentiate from  $G_2$  phase ( $G_2 = 1.5$  hours) unless nuclear division occurs and they enter  $G_1$  during the induction period. Thus, the rapid nature of the flagellation process makes it unlikely that differentiating cells could be blocked at a common point with respect to DNA replication. It would be interesting to determine whether the same conclusion could be drawn for encystment.

## VI. DNA and Phylogeny

### A. INTERSTRAIN VARIATION IN NUCLEAR DNA BASE COMPOSITION

Variations in base composition have been used to examine interstrain relationships in *Amoeba*, *Acanthamoeba*, and *Entamoeba*. In all cases,

the compositions have been determined indirectly from buoyant densities or melting temperatures. Reeves *et al.* (1971) attempted to distinguish strains of *E. histolytica* that were tolerant of high temperatures from *E. histolytica*-like strains that were intolerant of these temperatures. Although some data indicate that these strains differ very significantly in DNA content and in the presence or absence of repetitious DNA sequences (Gelderman *et al.*, 1971a,b), the estimated percentage of G + C was very similar for both types:  $28.79 \pm 0.98\%$  for 10 high-temperature strains and  $29.86 \pm 0.97\%$  for 5 low-temperature strains. There are some statistically significant pairwise differences among strains, but the overall intraspecific diversity is relatively low. For example, the variations among strains are less than the variations of repetitive measurements on DNA from a single strain of *Amoeba* (Fritz, 1981). In two other species, *E. moshkovskii* and *E. invadens*, the G + C values were 31.7 and 31.0%, respectively. Thus, diversity is relatively low even at the interspecific level.

Intraspecific diversity in overall base composition also is low in *Acanthamoeba*. Seven strains of *A. castellanii* all were 61% G + C (Adam and Blewett, 1974). *Acanthamoeba palestinensis*, which some classify as a strain of *A. castellanii*, was 62% G + C. The values for these species were clearly distinct, however, from *Acanthamoeba polyphaga*, *Acanthamoeba culbertsoni*, and *Acanthamoeba astronyxis*, which were 57, 56, and 50% G + C, respectively. The *Acanthamoeba* relationships were tested further by DNA–DNA hybridization with the same conclusion that intraspecific diversity was substantially less than interspecific diversity (Adams and Blewett, 1974). At the extreme, *A. astronyxis* Ray showed no hybridization with *A. castellanii* Neff. The significance of the hybridization studies is lessened, however, by the fact that the comparisons were not restricted to unique sequences.

Intraspecific diversity was higher in *Amoeba* where 10 strains of *A. proteus* averaged  $34.4 \pm 5.6\%$  G + C (Fritz, 1981). However, these values are for the main-band DNA and do not take into account the large nuclear satellite. The evidence suggesting that the main band and the satellite might vary in relative proportions (see Section II,D) creates a problem. This is most apparent in a comparison of *A. proteus* with *A. indica* (Table I) where it is unclear whether one should compare main bands, bands of similar density, or total nuclear DNA. If the variation in relative amounts means that differential replication of selected subfractions of nuclear DNA occurs, then the higher diversity indicated by the calculated main-band base compositions may only reflect physiological differences. As Fritz (1981) has recognized, comparisons of base composition have only limited value in phylogenetic studies. Properly executed DNA–DNA hy-

bridization studies (Ahlquist and Sibley, 1983) or studies of ribosomal RNA sequences (Pace *et al.*, 1985) have more promise for the elucidation of phylogenetic relationships.

#### B. INTERSTRAIN VARIATION IN MITOCHONDRIAL DNA

There have been several reports on the possible use of variations in mitochondrial DNA to study the taxonomy of *Acanthamoeba* (Byers *et al.*, 1983; Bogler *et al.*, 1983; Costas *et al.*, 1983). The initial approach has been to compare electrophoretic patterns for mitochondrial DNA fragments obtained by digestion with restriction endonucleases. In our laboratory, 21 strains representing several different species have been examined with at least five different restriction enzymes each. Sixteen different phenotypes have been identified by electrophoresis. The most striking observation is that intraspecific and interspecific differences both are relatively large. The largest difference is between *A. astronyxis* and the other strains. This is consistent with differences in nuclear DNA base composition and DNA-DNA hybridization results (see Section VI,A). Evidence also was obtained for one cluster of strains in which no polymorphisms were detected with up to 19 different restriction enzymes. These strains may form clusters of closely related organisms, but it has been difficult to rule out the possibility that the similarity may have arisen historically through problems with cross contamination.

Although we have attempted to quantitate interstrain relationships based on the differences in electrophoretic patterns (Bogler *et al.*, 1983), it is not known whether the basic assumptions underlying the model used for the calculations are valid for *Acanthamoeba*. Thus, we have begun to examine sequence differences more directly by comparing restriction site and gene distribution maps. The latter are being located using heterologous probes from *Neurospora* (R. A. Akins and T. J. Byers, unpublished results).

### VII. Bacterial Endosymbionts and Viruses

Both bacterium-like endosymbionts and viruses are found in various strains of amoebae. These are important because of influences that their presence may have on studies of nuclear and organelle DNA function. In addition, the existence of viruses offers the possibility of developing useful vectors for recombinant DNA approaches to the study of gene structure and function. Unfortunately, relatively little is known about the nucleic acids of these entities.

Evidence for rhabdovirus-like RNA viruses (Bird and McCaul, 1976) and filamentous and polyhedral DNA viruses (Mattern *et al.*, 1972; Hruska *et al.*, 1973) has been obtained for *Entamoeba*. Particles resembling rhabdoviruses were observed in several strains of *E. histolytica*, in a Laredo strain, and in a strain of *E. invadens* (Bird and McCaul, 1976). Filamentous and polyhedral types of DNA viruses were produced when uninfected cultures of *E. histolytica* were treated with media from infected cultures, but these viruses were not ordinarily seen in healthy axenic cultures. Therefore, it was suggested that the viral DNA might be integrated into the amoeba genome (Diamond and Mattern, 1976). Virus-like particles also have been described for *N. gruberi* EGs (Schuster and Dunnebacke, 1971, 1976). These elements developed in the nucleus, passed into the cytoplasm, and, eventually, into the medium. Particles were not ordinarily seen in axenic cultures, but could be induced by bacteria in the medium or by BUdR (Schuster and Clemente, 1977). Thus, these particles also may have been present in a latent form. It is unknown, however, whether nucleic acids are associated with the particles. I am unaware of any evidence for virus particles in either *Amoeba* or *Acanthamoeba*, although there have been reports of tritiated thymidine incorporation into the cytoplasm of *A. castellanii* in regions other than over mitochondria (Ito *et al.*, 1969; McIntosh and Chang, 1971). Since no particles could be found associated with the incorporation, it was suggested that defective viruses might be present. I am inclined to believe that the incorporation was more likely not into DNA (Byers and King, 1985), but the author's conclusions cannot be ruled out.

Bacterium-like endosymbionts have been described for three of the amoeba genera. An intracellular diptheroid form was observed in *N. fowleri* CJ (Phillips, 1974). The organism could be eliminated by antibiotic treatment, but amoebae tended to grow better in its presence, thus suggesting a symbiotic relationship. Proca-Ciobanu *et al.* (1975) described a bacterium-like symbiont in axenic cultures of *A. castellanii* SN. This organism, which was present free in the cytoplasm, possibly was an obligate symbiont because it never was possible to culture bacteria from the medium during several years of axenic growth. A bacterial parasite which originally was isolated from the soil was able to infect *A. castellanii* and to multiply internally (Drozanski, 1956; Drozanski and Chmielewski, 1979). The amoebae eventually were destroyed. Although the bacterium was able to survive for several months outside of the amoebae, it could not replicate under these conditions. Another bacterium-like parasite was described in one strain of *A. astronyxis*, but details were not discussed (Page, 1967). There have been several reports of *Acanthamoeba* harboring mycobacteria (e.g., Jadin, 1976) and legionella (Holden *et al.*, 1984).

Although it is clear that a few strains of *Naegleria* and *Acanthamoeba* can harbor bacteria, most strains show no signs of any infectious agent. In contrast, numerous strains of *A. proteus* and *A. discoides* contain bacteria or self-replicating DNA-containing bodies (Andresen, 1973; Hawkins, 1973). In some cases, these particles number in the thousands. I am unaware, however, of any studies of the DNA in these symbionts, except for X bacteria described by Jeon (1983) and his colleagues. The X bacteria are obligate symbionts that initially arose from a spontaneous infection of *A. proteus* (Jeon and Lorch, 1967). In the course of time, the amoebae became dependent on the symbionts. It is now possible to deliberately infect cultures and the symbiotic dependency develops over about 200 cell generations. Currently, amoebae carry an average load of about 42,000 symbionts per cell (Jeon, 1983). The X bacteria have circular plasmids of 11 and 39 million Da. To date, nothing is known about the functions or organization of the plasmids or of relationships between bacterial and amoeba DNA.

### VIII. Conclusions

We have only begun to explore the molecular biology of amoeba DNA, and a number of very interesting problems await the attention they deserve. Some of the more interesting questions are the following. Are the nuclear genes polyploid? If so, how is gene expression regulated? Are there any natural mechanisms for gene recombination? How does the overall organization of coding and noncoding DNA sequences in these primitive organisms compare with that of other organisms? Are there really some species of *Entamoeba* that have very little repetitious nuclear DNA and other species with very much? If so, what are the significant differences? What are the nuclear satellites in *Amoeba* and *Naegleria*? How do they relate to the main-band DNAs and how are the relative proportions of the main-band and satellite DNAs regulated? Do amoebae have transposable elements? Are there any viruses in *Amoeba* or *Acanthamoeba*? Do the nuclear genomes of *Entamoeba* species that have no mitochondria differ in any significant way from the genomes of the aerobic amoebae? If so, what is the evolutionary significance of the differences?

In addition to the aforementioned problems in basic molecular biology, there are two practical problems that deserve more attention. Taxonomic studies, especially of *Acanthamoeba* and *Entamoeba*, could benefit from the use of additional molecular approaches to help elucidate the relationships between pathogenic and nonpathogenic or between parasitic and

nonparasitic strains. A beginning has been made with studies of mitochondrial DNA in *Acanthamoeba*, but interstrain variability is high, and more conservative DNA sequences need to be examined. Also, this approach will not be useful for *Entamoeba* where mitochondria are absent. Another practical problem is the development of a library of specific probes that readily could be used to identify living amoebae isolated from human infections or organisms in tissue sections.

A few years ago, a number of the problems cited would have been beyond reach. Today, they are mostly well within reach and only need to attract the attention and interest of creative molecular biologists. I hope that this review will help to stimulate the interest that these organisms deserve.

#### ACKNOWLEDGMENTS

The author thanks Drs. H. J. Bohnert, C. Fulton, J. A. Hammer III, L. E. King, and M. R. Paule for permission to discuss their unpublished work. The research work of the author and his associates that is described in this article was partially supported by grants from the National Institutes of Health and the Ohio State University.

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