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THE MITOCHONDRIAL DNA OF *HYDRA ATTENUATA* AND *HYDRA LITTORALIS* CONSISTS OF TWO LINEAR MOLECULES

BY

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ABSTRACT

Gel electrophoresis of native DNA prepared from *Hydra attenuata* and *Hydra littoralis* shows the presence of two prominent 8 kilobase bands in addition to the expected chromosomal DNA at the limit of mobility. Partial purification followed by electron microscopy, restriction enzyme digestion, and nucleic acid hybridization demonstrate that the bands consist of linear DNA molecules that contain the hydra mitochondrial genome.

Key words: *Hydra* — mitochondrial DNA — telomeres.

INTRODUCTION

The mitochondrial DNA of all multicellular animals so far studied consists of a circular molecule 15-18 kb in size (Wallace, 1982). In DNA preparations from the simple metazoan *Hydra attenuata* we have demonstrated the presence of two linear DNA molecules each approximately 8.0 kb long. We suggest that these molecules contain the mitochondrial genome of the animal—a highly unusual arrangement.

MATERIALS AND METHODS

H. attenuata was obtained from Charles David (Munich) and Hans Bode (U.C. Irvine). *H. littoralis* was obtained from Carolina Biological Supply Company. They were maintained according to standard techniques (Lenhoff, 1971) and fed on freshly hatched brine shrimp (*Artemia*) also obtained from Carolina Biological Supply Company.

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DNA Preparation: Cultures were starved for 48 hours prior to harvesting. The animals were then scraped off the bottoms of the culture dishes and collected in a fine mesh net. They were washed with culture medium and then transferred to a tissue homogenizer having a moderately tight fitting glass ball plunger. Excess medium was aspirated off and replaced with homogenization buffer (0.2 M raffinose, 0.25% bovine serum albumin, 1 mM KH_2PO_4 pH 6.3) (Suyama and Preer, 1965). All succeeding steps were performed at 4°C. After 40 strokes the homogenate was transferred to 30 ml Corex tubes and centrifuged in a swinging bucket rotor (Sorvall HB-4) for 5 minutes at 2 krpm. The reddish pellet was retained for the isolation of nuclear DNA. The cloudy supernatant was decanted into another tube and spun for 20 minutes at 12 krpm. The supernatant was discarded and the pinkish pellet was examined under the microscope for the presence of intact cells. The pellet was then resuspended in HET-SDS buffer (0.5 M EDTA, 10 mM tris, 0.5% sodium lauryl sulfate, pH 9.5) and the suspension incubated at 65°C for an hour (Kavenoff and Zimm, 1973). Proteinase K was added to a final concentration of 100 μg/ml and the suspension incubated at 37°C for at least 4 hours. The suspension was then extracted three times with phenol-chloroform prior to precipitation with two volumes of 95% ethanol. The DNA pellet was washed with 70% ethanol, dried, and resuspended in a minimal volume of 10 mM tris 1 mM EDTA, pH 8. Saturated CsCl solution was added until the refractive index of the solution was 1.400. The solution was transferred to tubes and centrifuged in a Beckman Ti 50.1 rotor at 42 krpm for 48 hours. Fractions were collected and those containing DNA were pooled.

Southern Blots: DNA was electrophoresed on agarose gels and transferred to nitrocellulose paper as described by Southern (1975). Nick translated probes were prepared as described in Rigby *et al.* (1977). Hybridizations were carried out in 4X SSC (0.6 M NaCl, 0.06 M Na citrate), 40% formamide, 0.1% SDS at 42°C for 12 hours and the filters were washed at 65°C in 0.2X SSC, 0.1% SDS (homologous probes) or in 2X SSC, 0.1% SDS (heterologous probes). Purified hydra doublet DNA was obtained by electrophoresis of DNA from a 'mitochondrial preparation' on a 1% low gelling temperature agarose gel (Seakem). The gels were stained with ethidium bromide and the DNA visualized by illumination with long wavelength ultraviolet light. The region of the gel containing the doublets was excised and heated at 65°C to melt the gel. The DNA was then extracted three times with phenol and precipitated with two volumes of 95% ethanol.

Restriction enzyme digests were carried out as specified by the suppliers (New England Biolabs and Bethesda Research Laboratory).

RESULTS

When DNA prepared from *H. attenuata* was electrophoresed on an agarose gel, two sharp bands were visible running close together at approximately 8.0 kb (Figure 1). These bands were enriched in DNA preparations made from a 'mitochondrial fraction' of both *H. attenuata* and *H. littoralis* (Figure 2). DNA in these bands was deduced to be linear on the basis of two criteria, electron microscopy and restriction endonuclease digestion.

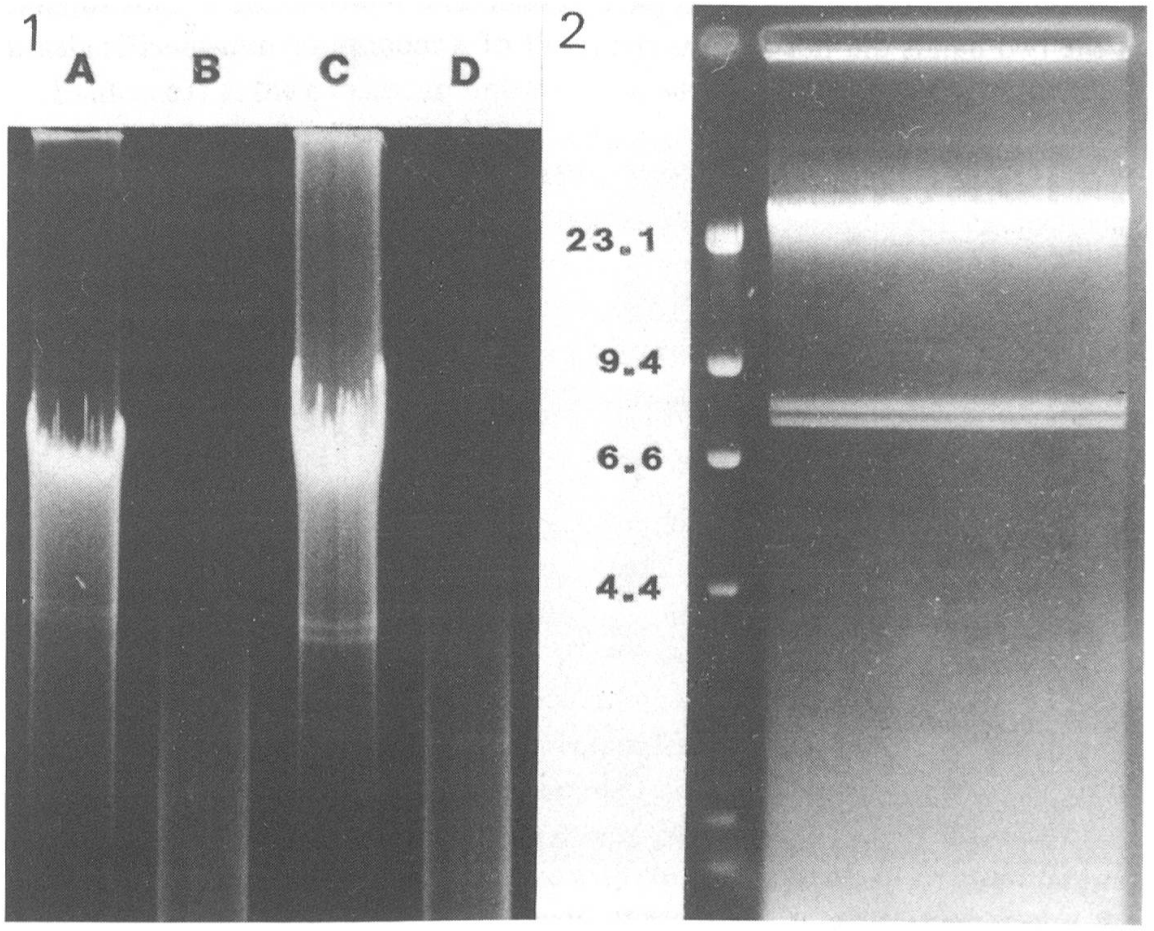

FIG. 1. — *H. attenuata* and *H. littoralis* DNA electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lanes A and C, undigested total DNA; lanes B and D, DNA after digestion with the restriction endonuclease Taq I.

FIG. 2. — DNA extracted from a 'mitochondrial preparation' of *H. attenuata,* electrophoresed on a 1% agarose gel and stained with ethidium bromide. Phage λ DNA cleaved with Hind III run as a marker. Fragment sizes are in kilobases.

Total DNA from *H. attenuata* was electrophoresed on a preparative low gelling temperature agarose gel. After the gel was stained with ethidium bromide, the region containing the doublets was excised. The DNA extracted from the gel slice was spread for electron microscopy (Davis *et al.,* 1971), with circular molecules of phage ØX174 DNA (length 5.39 kb) as internal standards. The lengths of the linear molecules were measured from projected images of the electron micrographs. The mean length of 38 linear molecules was approximately 8.0 kb.

Restriction enzyme digestions of the DNA were carried out and the following facts were deduced (Figure 3). First, the distribution of restriction sites on the two fragments was different, suggesting that they were two distinct molecules and not variants or a precursor and product. For example, the larger of the two molecules, band I, is cut three times by Eco RI while band II is not cut at all by this enzyme. Band II, on the other hand, has one site for Bam HI, while band I has no sites. Second, the products of a restriction digest were defined and reproduceable. This suggested that the two bands did not arise as the result of a random or non-specific cleavage

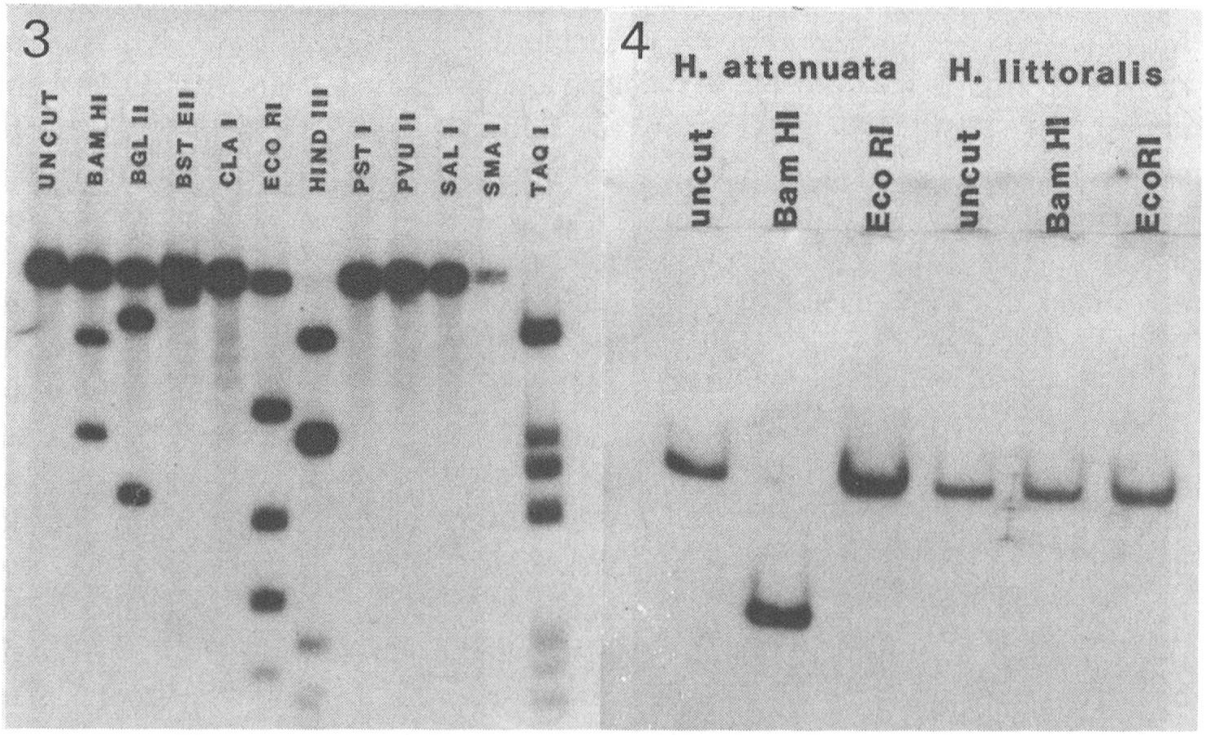

FIG. 3. — Restriction endonuclease digests of *H. attenuata* DNA. DNA from a 'mitochondrial preparation' was digested with the indicated restriction enzymes. The resulting fragments were separated on a 1% agarose gel and transferred to nitrocellulose filter paper. The filter was hybridized with a nick translated probe prepared from gel purified hydra mitochondrial DNA and was then exposed to film.

FIG. 4. — Hybridization of cloned mitochondrial DNA from *Drosophila yakuba* to *Hydra* DNA. DNA from *H. attenuata* and *H. littoralis* 'mitochondrial preparations' was digested with restriction enzymes and electrophoresed on a 1% agarose gel. The DNA was transferred to nitrocellulose paper and hybridized with nick translated DNA prepared from a clone of the *D. yakuba* Hind III B fragment.

of a larger linear or circular precursor. Third, the sum of the molecular weights of the fragments from a restriction digest was equal to the sum of the molecular weights of the two bands, ruling out the presence of palindromes or large tracts of repeated sequence. Fourth, simultaneous digestion of the DNA with two restriction enzymes yielded fragments with molecular weights consistent with the presence of two linear molecules. Thus digestion of band II with Bgl II produced fragments of approximately 5.4 and 2.0 kb. Digestion with Bam HI produced a 4.7 kb and a 2.7 kb fragment. Digestion with both enzymes produced fragments 4.7 kb, 0.6 kb and 2.1 kb in length, the expected result from the cleavage of a linear molecule.

In related experiments DNA from a hydra 'mitochondrial preparation' was treated for increasing lengths of time with the exonuclease Bal 31. This enzyme digests linear DNA sequentially starting from the termini. The Bal 31 treated DNA was then digested with various restriction enzymes. In each case the predicted terminal fragments showed a decrease in size with increasing exposure to Bal 31, confirming the linear nature of the molecules.

Evidence that the two linear molecules encode at least part of the mitochondrial genome was obtained from cross hybridization experiments using cloned mitochondrial DNA probes from *Drosophila yakuba.* Both the Hind III B and the Eco RI B fragments (Clary *et al.,* 1984) hybridized to specific fragments of the linear hydra molecules under conditions of moderate stringency (Figure 4). The Hind III B fragment encodes cytochrome oxidase III, ATPase 6, URFA6L and a portion of cytochrome oxidase II, while the EcoR I B fragment codes for URF1, URF6, URF4L, a portion of URF4 and cytochrome b (Clary *et al.,* 1984).

DISCUSSION

Several lines of evidence suggest that the mitochondrial genomes of *Hydra attenuata* and *Hydra littoralis* consist of two linear molecules. The two molecules we have described are of an appropriate size to contain the mitochondrial genome. They are enriched in DNA preparations from subcellular fractions from which nuclei have been pelleted. No molecules corresponding in size to a conventional 15-18 kb circular mitochondrial DNA were detected, either on gels or on Southern blots. In addition restriction fragments of the molecules hybridize with cloned segments of *Drosophila yakuba* mitochondrial DNA under conditions of moderate stringency. Conclusive proof, however, would require the isolation and cloning of the molecules and the direct demonstration that they encode proteins and RNAs that are known to be encoded on other mitochondrial DNAs. A mitochondrial genome consisting of two linear molecules has not been seen in any other member of the Metazoa, although linear mitochondrial DNA has been demonstrated in the ciliated protozoans *Tetrahymena thermophila* and *Paramecium aurelia,* the yeast *Hansensula mrakii,* and in higher

plants (reviewed in Wallace 1982). Recently the mitochondrial genome of the yeast *Candida rhagii* (Kovac *et al.,* 1984), has been shown to be a 30 kb linear molecule.

The presence of a bipartite mitochondrial genome raises a number of questions. For example, the arrangement of coding sequences along and between the two linear molecules, their transcription, the control of their copy number and replication are all problems that deserve further study.

In addition, if the DNAs originate and are maintained as linear molecules, they may be useful in answering questions about the structure of chromosomal termini. The telomeres of all eukaryotic chromosomes examined so far have certain common structural motifs (reviewed in Blackburn and Szostak, 1984). The immediate subterminal regions often consist of middle repetitive sequences each individual component of which may be up to several kilobases in length. The terminal portions of the chromosomes are made up of a small number of repeats of a simple satellite-like sequence and may contain specific single stranded gaps. One strand of the satellite sequence is rich in C and A residues, the other in G and T. For example, the termini of the extrachromosomal ribosomal DNA of the holotrichous ciliate *Tetrahymena thermophila* consists of about 50 repeats of the sequence CCCCAA with several single nucleotide gaps at specific positions within the cluster of repeats (Blackburn and Gall, 1978). Similar terminal sequences have been demonstrated in *Dictyostelium* rDNA (C_1-$_8$T), *Physarum* rDNA (C_3A^n), *Stylonychia* and *Oxytricha* macronuclear DNA (C_4A_4), and chromosomal DNA from *Trypanosoma* (C_3TA_2) and *Saccharomyces* (C_2-$_3A(CA)_1$-$_3$) (reviewed in Blackburn, 1984). It would be interesting to see if analogous sequences are present at the termini of the linear mitochondrial DNA of hydra.

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