The Amplified Ribosomal DNA of Dytiscid Beetles

[DNA replication/Dytiscidae (Coleoptera)/gene amplification/repetitive DNA/electron microscopy of DNA]

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ABSTRACT During oogenesis in many animals there is a massive extrachromosomal synthesis of the genes for ribosomal RNA. In Dytiscid beetles, as in the toad Xenopus, the amplified ribosomal DNA occurs as circular molecules of different sizes. The circles fall into size classes that are integral multiples of a unit circle. It is probable that the unit circle contains the coding sequence for one precursor ribosomal RNA molecule plus accompanying spacer sequences.

In many animals the genes coding for ribosomal RNA (rRNA) undergo an extensive extrachromosomal replication or amplification during oogenesis (1, 2). The amplified ribosomal DNA (rDNA) is usually associated with multiple nucleoli whose size, shape, and disposition within the nucleus vary from species to species. Because the nucleoli in some amphibian oocytes occur as circular necklaces, easily broken by treatment with DNase (3, 4), it has been assumed for some years that the amplified rDNA molecules exist as covalently-closed circles. Direct evidence for the circularity of the amplified rDNA molecules in the toad, Xenopus, was provided by the recent electron microscopical studies of Hourcade, Dressler, and Wolison (5, 6). These workers showed that rDNA circles fall into integral size classes whose unit is about $7.5 \times 10^6$ daltons, equivalent to the coding unit for one 18S and one 28S rRNA molecule plus accompanying spacer sequences. We show here that the amplified rDNA in two species of Dytiscid water beetles likewise contains circular molecules of defined circumference.

MATERIALS AND METHODS

Amplified rDNA was obtained from the ovaries of two Dytiscid water beetles, Colymbetes fuscus and Dytiscus marginalis. Ovaries were removed from recently killed animals and placed in 70% ethanol for storage. Most of the DNA we have examined came from ovaries held 2 years at $-10^\circ$. The tips of the ovarioles, which contain a high proportion of amplified rDNA (7), were dissected in 70% ethanol and then lyzed in a solution containing 50 mM Tris (pH 8.4), 0.1 M EDTA, 0.5% Sarkosyl (Geigy), and 100 $\mu$g/ml of self-digested Pronase. The Dytiscus DNA was extracted with an equal volume of water-saturated phenol, precipitated from the aqueous phase with two volumes of ethanol, redissolved in 15 mM NaCl-1.5 mM Na$_2$ citrate (pH 7) and digested with 100 $\mu$g/ml each of pancreatic and T1 RNase. It was then centrifuged to equilibrium on a neutral CsCl gradient having an initial density of about 1.70 g/cm$^3$ (Spinco Ti 50 rotor, 35,000 rpm, 65 hr, 18$^\circ$). The Colymbetes ovary tips were lysed in the same solution, but were then mixed directly with CsCl without further purification. DNA from the CsCl gradient was precipitated with three volumes of 70% ethanol, washed with 70% ethanol, and redissolved in 0.1 ml of 15 mM NaCl-1.5 mM Na$_2$ citrate (pH 7.0).

The DNA was spread for electron microscopy in one of several ways. For measurements of contour length the DNA was prepared either by the "aqueous" or "formamide" modifications of the Kleinschmidt method (8). Spreading in 50% formamide gave more fully extended molecules of somewhat longer contour length. As an internal molecular weight standard, double-stranded $\phi$X174 DNA was included in most preparations. For denaturation mapping, the DNA molecules were spread in high concentrations of formamide (over 90%) in 40–80 mM CO$_2^-$, pH 9.3. Molecules were picked up on parlodion-coated grids, treated briefly with 50 $\mu$M aqueous uranyl acetate, and then dried. The grids were rotary shadowed with palladium–platinum (1:4) at an angle of about 8$^\circ$. Micrographs were taken at an initial magnification of 5,000–12,000 $\times$ in the Philips 200 or 300 electron microscope. The plates were projected by a photographic enlarger onto tracing paper, and the contours of individual molecules were drawn at a magnification of 1 to 3 $\times$ 10$^4$$\times$. Lengths were measured on the drawings with a "map measurer." Lengths were converted to daltons on the assumption that the $\phi$X174 molecular mass is 3.40 $\times$ 10$^6$.

RESULTS

The ovary tips of Dytiscid beetles contain several dozen pachytene oocytes, each with a cap of extrachromosomal rDNA (Fig. 1). These oocytes are morphologically similar to pachytene cells in the toad, Xenopus, except that the beetle oocytes contain more rDNA (up to 43 pg in Colymbetes fuscus and 67 pg in Dytiscus marginalis). When the DNA from ovary tips is centrifuged in a CsCl gradient, two peaks are seen (Fig. 2). The heavier of these contains the amplified rDNA (7). In Colymbetes the heavier peak ($\rho = 1.721$ g/cm$^3$) consists entirely of rDNA, but in Dytiscus approximately half of the heavier peak is a highly repetitive satellite DNA that happens to have nearly the same buoyant density as the rDNA ($\rho = 1.717$ g/cm$^3$). The satellite DNA in the ovary-tip preparations is derived primarily from the nurse cells and follicle cells, which accompany the oocytes. Since the satellite DNA molecules are linear, they do not interfere with the observations on circular rDNA molecules in the same sample.

When the amplified rDNA of Colymbetes was examined in the electron microscope, approximately 13.5% (n = 282) of

Abbreviations: rRNA, ribosomal RNA; rDNA, DNA that codes for rRNA.
the molecules were found to be circular, the remainder being linear (Fig. 3). The smallest circles made up a homogeneous population with mean molecular mass of about 10 × 10^6 daltons. Larger circular molecules were integral multiples of this smallest size, i.e., 20, 30, 40 × 10^6 daltons. A few circular molecules larger than 40 × 10^6 daltons were seen. The average

The heavy peak of *Dytiscus* DNA also contained circular molecules, accounting for approximately 19% (n = 526) of the total. In contrast to *Colymbetes*, the smallest circles of *Dytiscus* had an average molecular mass of about 20 × 10^6 daltons. Larger circular molecules fell into size classes with means of 40 and 60 × 10^6 daltons, and a few molecules of about 80 × 10^6 daltons were found. The average unit mass for all molecules was 19.17 × 10^6 daltons (Figs. 5 and 6). Clearly, therefore, the unit circle size in *Dytiscus* is about twice that of *Colymbetes*.

Partially denatured molecules were prepared by spreading the DNA in formamide concentrations above 90%. Such a high formamide concentration was necessary because of the generally high (G + C) content of the rDNA.

In *Dytiscus* each of the 20 × 10^6-dalton circles contained one major region of denaturation extending for less than 1/4 of the circumference of the circle. The 40, 60, and 80 × 10^6-dalton molecules had 2, 3, and 4 such regions, respectively (Fig. 7). A denaturation map was constructed by aligning all molecules by eye such that apparently homologous regions were in register. The percent of molecules showing denaturation in a given region was plotted for each region of the repeat unit (Fig. 8).

Some partially denatured molecules of *Colymbetes* rDNA were observed, but insufficient material was available to prepare a denaturation map for this species.

**DISCUSSION**

The first suggestion that amplified rDNA might occur as circular molecules came from light microscopic observations.
on the necklace-like nucleoli found in some amphibian oocytes. Miller (3) and Keser (4) showed that the continuity of such nucleoli is disrupted by DNase. Somewhat similar circular nucleoli were found by Kunz (9) in oocytes of the cricket, Acheta. Miller and Beatty (10) examined amphibian oocytes in the electron microscope after partial dissolution in water. Their micrographs demonstrated the newly synthesized rRNA precursor molecules still attached to the rDNA. Because free ends of molecules were essentially absent in their micrographs, Miller and Beatty assumed that the rDNA molecules occurred as circles. Direct evidence for circular rDNA molecules in Xenopus oocytes was recently provided by Hourcade, Dressler, and Wolfson (5, 6), using the Klein- schmidt method of spreading purified DNA. In addition to showing the circular form of some of the molecules, these authors made two important observations: (i) the rDNA circles contained 1, 2, 3, 4, or more repeats of the basic rDNA repeat length (about \(7.5 \times 10^6\) daltons). This was shown both by the total length measurements and by partial denaturation. (ii) One-sixth of the circular rDNA molecules had "tails," implying that they might be undergoing replication by the rolling circle mechanism (11). Their observations have been confirmed by Bird, Rochaix, and Bakken (12), who have also provided autoradiographic evidence that the tailed forms are, in fact, replicating molecules.

Despite fairly extensive searching we have not seen more than one or two tailed forms in the samples of beetle amplified rDNA. We have also failed to see "theta" forms characteristic of replicating bacterial (13) or mitochondrial (14) DNA circles. The absence of apparent replication forms is consistent with what is known about the growth of the insect oocytes. In the ovariole tips of adult beetles most of the amplified rDNA occurs in late pachytene or early diplotene oocytes, which show no autoradiographically detectable incorporation of \(^{3}H\)thymidine (Fig. 1). We believe, therefore, that the circles we have examined are all forms present after replication has occurred.

The ribosomal RNA of Dytiscus marginalis consists of 28S and 18S components in the usual 2:1 ratio, as determined by sucrose gradient centrifugation (unpublished observa-

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**Fig. 4.** Frequency distribution of molecular weights of 51 circular rDNA molecules from the beetle Colymbetes fuscus. Molecular weights have been multiplied by \(10^{-6}\).

**Fig. 5.** Frequency distribution of molecular weights of 46 circular rDNA molecules from the beetle Dytiscus marginalis.

**Fig. 6.** Plot of the mean molecular weights for each size class of circular rDNA molecules. For Colymbetes the rDNA unit is \(10.04 \times 10^6\), for Dytiscus \(19.17 \times 10^6\).
Partial denatured circular rDNA molecules of *Dytiscus* represented by white (denatured) and black (native) segments. Shown are molecules containing 1, 2, or 3 rDNA units, aligned by eye to bring apparently homologous regions into registry. Most of the 2-unit and 3-unit molecules contain 2 or 3 major denatured regions, respectively.

Denaturation map prepared from the molecules shown in Fig. 7. For the 34 rDNA units contained in these 19 molecules, the number of molecules denatured in a given region was plotted as a function of distance along the unit.

Although accurate molecular weight determinations have not been made, it is probable that the combined molecular weight of the two major rRNA molecules is about 2.2 to 2.3 × 10^6. The coding region of the rDNA would thus be about 4.5 × 10^6 daltons. Accordingly, the coding region would amount to about 45% of the total rDNA length in *Colymbetes*, but only 23% in *Dytiscus*. In the partially denatured *Dytiscus* molecules, segments of about this length appear to denature before the rest of the molecule. It seems probable that the more easily denatured regions correspond to the sequences for rRNA. If this is the case, then the beetle rDNA is similar in overall organization to the rDNA of *Xenopus* (15), consisting of a very high (G+C) spacer separating somewhat lower (G+C) regions coding for the rRNA. In *Colymbetes* the ratio of spacer to coding region is about 1:1, whereas in *Dytiscus* the spacer is about three times longer than the coding region. Recently the amplified rDNA of the cricket, *Acheta*, has been shown by Miller's technique to have a repeat length of about 22 × 10^6 daltons (16). In this case approximately half of the molecule is a nontranscribed spacer, but the transcribed region appears to have at least twice the length of the combined 18S and 28S rRNA molecules. Since the function of spacers (transcribed and nontranscribed) is unclear, variations in their length are of unknown significance. Very long spacer regions have been described in the 5S rDNA of *Xenopus laevis* and *Xenopus mulleri* (17), where approximately 6/7 and 17/18 of the molecule is a spacer of unknown function between the repeated coding sequences.

Our earlier study of *Colymbetes* and *Dytiscus* had suggested that the spacer regions were longer than those reported here, especially in *Dytiscus* (7). These earlier data were misleading, in part because they were based on heterologous hybridization with *Xenopus* rRNA, and also because in *Dytiscus* we failed to distinguish the rDNA from the very large satellite DNA of nearly the same buoyant density.

We do not know whether all of the amplified rDNA occurs as circles in vivo. Since 13.5–19% of the molecules that we have isolated by a relatively crude procedure are intact circles, it is quite possible that all the amplified rDNA was circular before extraction. Certainly molecules larger than 80 × 10^6 daltons, the largest intact circle we have seen, would be easily broken by shear during isolation and spreading for electron microscopy. In the case of *Dytiscus*, approximately half of the molecules in the heavy peak are satellite DNA unrelated to the rDNA, so that the percentage of rDNA present as circles is actually closer to 35–40%.

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