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The Amplification of Ribosomal RNA Genes Involves a Rolling Circle Intermediate

(DNA replication/*Xenopus laevis*/gene amplification/repetitive DNA/electron microscopy of DNA)

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ABSTRACT During the development of *Xenopus* oocytes there is a special DNA synthesis that leads to a thousandfold amplification of the genes that code for ribosomal RNA. We have used the electron microscope to study this process. Our primary observation is the presence of ribosomal DNA in rolling-circle intermediates at the time of amplification. We believe that these intermediates are involved in the amplification process, and as such offer the first example of the involvement of a rolling circle in the replication of eukaryotic DNA.

Gene Amplification. During oogenesis in many organisms a specific replication of the genes that code for ribosomal RNA occurs (for a review, see ref. 1). The purpose of this well-controlled and selective DNA synthesis is apparently to provide additional template for the massive ribosomal RNA synthesis that occurs during the development of the oocyte. The product RNA is packaged into ribosomes which are stored until they are used for the extensive protein synthesis of embryogenesis.

Amplification of ribosomal DNA has been best documented in the studies of Brown and Dawid (2) and Gall (3) with *Xenopus laevis*. In this species of South African toad, there is about a thousandfold amplification of the ribosomal RNA genes. The product DNA appears in multiple nucleoli which, in contrast to the two nucleoli of somatic cells, are found in the nucleoplasm unattached to the chromosomes (1, 4–7). The amplified DNA, as it serves as a template for ribosomal RNA transcription, has been observed with the electron microscope by Miller and Beatty (8).

Fine Structure of Amplified Genes. The structure of the *Xenopus laevis* genes that code for 18S and 28S ribosomal RNA (rDNA) has been extensively studied in the laboratories of Brown, Birnstiel, and Miller. These studies show that ribosomal DNA is a repetitive DNA in which each repeating sequence has a molecular mass of about 8 million daltons, or about 13,000 base pairs (9). Each repeat of ribosomal DNA is composed of two regions. One of these regions contains the nucleotide sequences coding for 18S and 28S rRNA (10, 11) and is transcribed into a 40S piece of RNA that serves as a common precursor (12). The remainder of the rRNA gene consists of a spacer region that is not transcribed and whose function is unknown (8, 10, 11, 13). A diploid *Xenopus* somatic cell contains about 900 copies of the rRNA gene (10, 11). Some, perhaps all, of these rRNA genes are arranged in a tandem array, head to tail, along the DNA (9, 14, 15). The DNA product of the selective amplification of ribosomal RNA genes is very similar in organization to somatic ribosomal DNA (9, 13).

The mechanism by which ribosomal RNA genes are selectively amplified is an unsolved problem. We have used the electron microscope to search for intermediates in the amplification process. Our results indicate that amplification of ribosomal RNA genes involves a rolling-circle intermediate.

METHODS

Isolation of ribosomal DNA during amplification

Xenopus laevis tadpoles were obtained from the Amphibian Facility of the University of Michigan. The tadpoles had been exposed to estradiol, a hormone that causes almost all of the 50% of the animals that are genetically male to develop into fertile females (16). About 1 week after metamorphosis, when ribosomal DNA was being amplified (3, 17), the young frogs were injected daily for 3 days with 10 μl of tritiated thymidine (50 Ci/mmol, 5 μg/ml; New England Nuclear Corp.). The label was injected into the intraperitoneal cavity through the tip of a 27-gauge needle connected to calibrated intramedic tubing. On the fourth day, ovaries from about 50 frogs were removed into 20 ml of lysing solution [10 mM Tris·HCl–20 mM EDTA (pH 8.5); 2% sodium *N*-dodecyl-sarcosinate (Sigma); 0.1% diethyl oxydiformate (Eastman); and self-digested Pronase, 1 mg/ml] and incubated for at least 3 hr at 37°. In the preparation of the ovarian lysates, care was taken not to shear the DNA.

To analyze the ribosomal DNA, it was necessary for us to separate it from the chromosomal DNA which was also present in the lysate. We took advantage of the fact that amplified ribosomal DNA, because of its relatively high GC content (67%), bands in CsCl at a higher density than bulk chromosomal DNA (40%) (18). Thus, unfractionated ovarian lysates could be centrifuged in a CsCl density gradient, and ribosomal DNA could be recovered as material in a dense, satellite peak (Fig. 1). The satellite fractions were pooled and dialyzed into a buffer suitable for electron microscopy.

RESULTS

Circles. Although most of the molecules in the rDNA satellite fraction are linear, 2–5% of the DNA forms are circular (Fig. 2*A*). These circles range in size from 8 million to 140 million daltons. Often the circles are supercoiled (19), in which case they contain two interlocked and covalently-continuous polynucleotide chains.

Contour measurements of the circles were made. Most circles with a molecular mass less than 40 million daltons readily fall into discrete size classes—monomers, dimers, trimers, and tetramers (Fig. 3). The smallest circles have a length that corresponds to a molecular mass of 8 million daltons—that is, to the size of the ribosomal RNA gene (9). Those circles which have circumferences larger than five-times

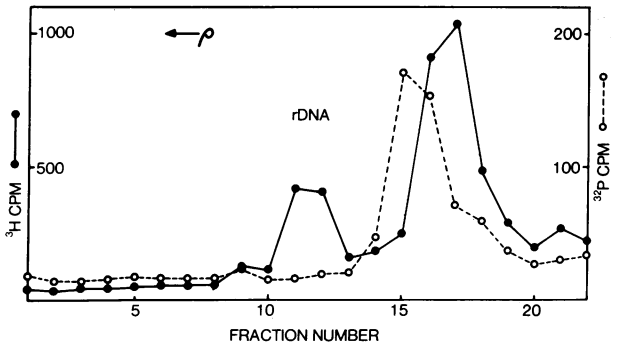

FIG. 1. Isolation of amplified ribosomal DNA. Ribosomal DNA (ρ = 1.729) was separated from chromosomal (ρ = 1.699) and mitochondrial (ρ = 1.701) DNA by CsCl centrifugation. 20 ml of ovarian lysate was mixed with 24.6 g of CsCl and centrifuged at 31,000 rpm for 60 hr at 15° in a Beckman angle 40 rotor. 10% of each fraction was counted for tritium (*solid line*), which represents synthesis of DNA in ovarian cells during the 3 days before harvest. The denser peak contains ribosomal DNA; the lighter peak contains chromosomal and mitochondrial DNA. *Dashed line* shows the banding position of ^{32}P-labeled lambda DNA, which had been added as a marker. Ribosomal DNA fractions (11 and 12) were dialyzed into a buffer for electron microscopy [0.1 M Tris·HCl–0.01 M EDTA (pH 8.5)–5% formamide].

monomer length do not readily group into discrete size classes. This result is, in fact, expected because there is a characteristic standard deviation of ±5% in the length measurement of the DNA (20); thus, for circles larger than five-times monomer size, the length variation causes an overlap of neighboring multimeric size classes.

The largest circles have circumferences that would correspond to at least 15 ribosomal RNA genes. Peacock (6) and Miller (7) have observed even larger ribosomal DNA circles. It is possible that the large linear molecules we see are derived by breakage of such circles.

Rolling Circles. About one circle in six is a rolling circle (Fig. 2*B*). Just as the circles correspond to ribosomal RNA gene monomers, dimers, trimers... so too, the rings of the rolling circles follow the monomer, dimer, trimer pattern (Fig. 3).

Up to now we have photographed, traced, and measured about 60 rolling circles. In about 85% (51 of 60) of the rolling circles, the tails are longer than the rings to which they are attached. Thus, these molecules cannot be broken Cairns forms (21) because if a Cairns form were broken at one fork, the resulting rolling circle would have a tail less than the size of the circle.

Other Forms. In addition to linear molecules, circles, and rolling circles, two other DNA forms have been observed.

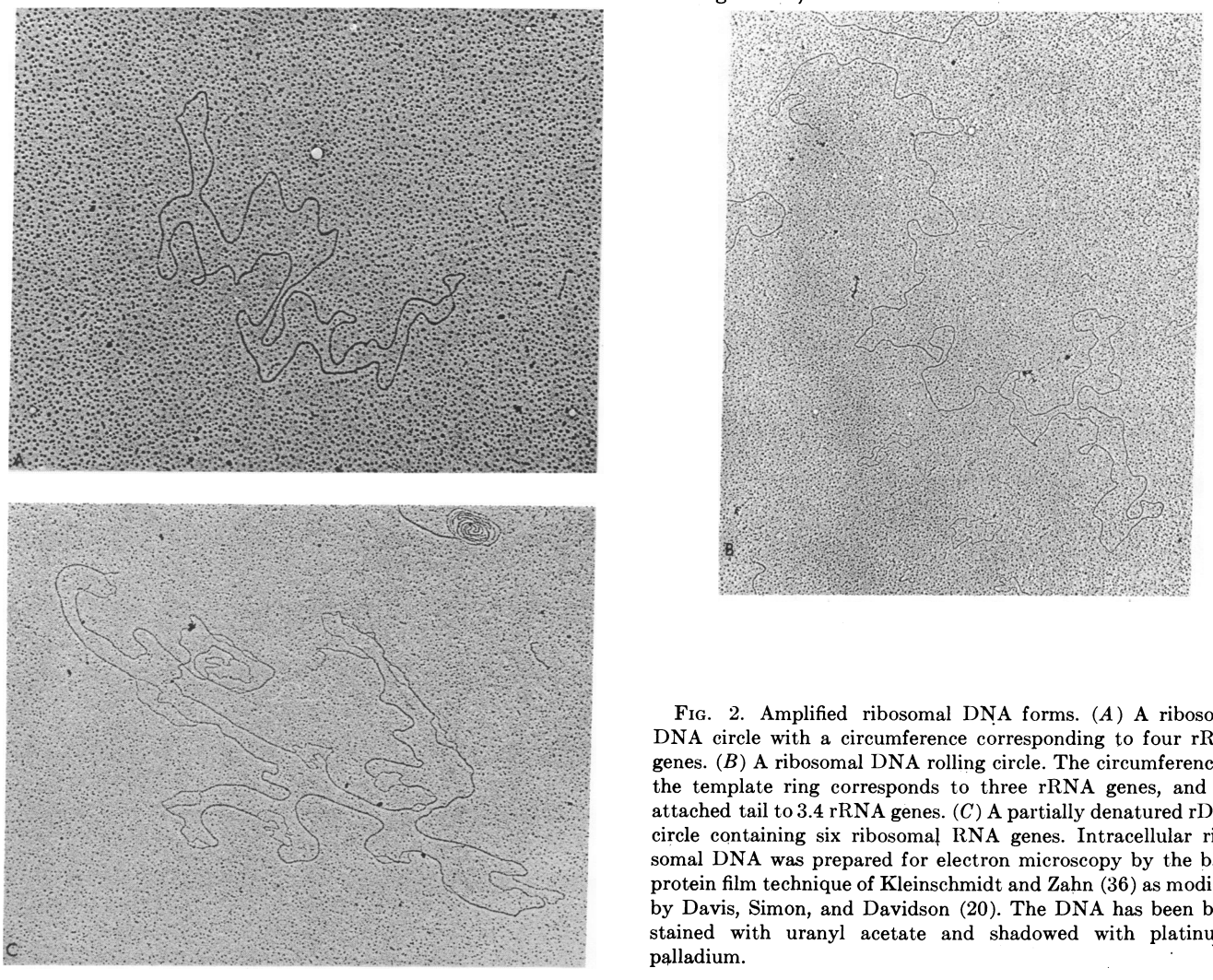

FIG. 2. Amplified ribosomal DNA forms. (*A*) A ribosomal DNA circle with a circumference corresponding to four rRNA genes. (*B*) A ribosomal DNA rolling circle. The circumference of the template ring corresponds to three rRNA genes, and the attached tail to 3.4 rRNA genes. (*C*) A partially denatured rDNA circle containing six ribosomal RNA genes. Intracellular ribosomal DNA was prepared for electron microscopy by the basic protein film technique of Kleinschmidt and Zahn (36) as modified by Davis, Simon, and Davidson (20). The DNA has been both stained with uranyl acetate and shadowed with platinum–palladium.

One is a Y-shaped structure with three unequal arms, representing about 25% of the forked forms. These structures could be broken rolling circles. In any event, their presence is in no way inconsistent with the use of a rolling-circle intermediate for the amplification of ribosomal DNA; this is because the products of rolling-circle synthesis (long tails and free concatemers) necessarily contain intact origins identical to those on the original circles. Thus, these molecules can serve as a substrate for the initiation of DNA synthesis (22).

A more serious question is raised by our finding of two Cairns forms among the circles and rolling circles. While only two in number, the presence of these molecules may indicate that a Cairns pattern of replication (21, 23) precedes a rolling-circle pattern (22, 24) for ribosomal DNA amplification, as has been suggested for the two-stage replication of bacteriophage lambda DNA (25). Alternatively, the two Cairns forms might be the product of a recombination event between the tail and the ring of a rolling circle.

Partial Denaturation. Because circles and rolling circles are a small fraction of the total DNA in the satellite band, it is important to be sure that these molecules are, in fact, ribosomal DNA. Evidence on this point has been provided first by the finding that the circles and rolling circles band at the high buoyant density characteristic of ribosomal DNA, and second because these molecules occur in size classes that correspond to multimeric repeats of the length of the ribosomal RNA gene. However, identification of the circles and rolling circles as ribosomal DNA is subject to an additional and independent test—DNA partial denaturation (26).

Under certain conditions, such as elevated temperatures (26), high pH (27), or high formamide concentrations (28), the DNA double helix becomes stressed, and those regions that are relatively rich in AT base pairs denature (27) (see Fig. 2*C*). Wensink and Brown have already shown that one half of the ribosomal RNA gene (which codes for 18S and 28S rRNA) is richer in AT base pairs than the other half, and will preferentially exhibit denaturation bubbles under partially denaturing conditions (9). We find that, in essence, all of the linear molecules from the material of the satellite peak possess this denaturation pattern (Fig. 4*A*). In addition, all of the circles and rolling circles (Fig. 4*B*) that have been examined show this denaturation pattern. Thus we can conclude that the linear molecules, circles, and rolling circles all represent ribosomal DNA (Fig. 4*C*, *D*, and *E*).

DISCUSSION

The data of this paper show that, during ribosomal gene amplification in *Xenopus laevis*, circles and rolling circles are present in ovarian lysates. These molecules have been accepted as ribosomal DNA on the basis of three criteria: (*1*) they band at a satellite density in CsCl; (*2*) the circumference of their circular components corresponds to multimeric repeats of the length of the ribosomal RNA gene; and (*3*) upon partial denaturation, they display a melting pattern characteristic of ribosomal DNA.

In recent experiments Bird, Rochaix, and Bakken (29) have confirmed our finding of ribosomal DNA rolling circles. In their experiments they have used labeling of ovaries and isolation of rDNA, followed by autoradiography coupled with electron microscopy. This procedure has allowed a more rapid identification of the small percentage of rolling circles (which, because they are replicating, are radioactive) by first selecting for molecules that have undergone a tritium decay. This procedure also provides evidence that the rolling circles are replicating forms.

We believe that the rolling circles are intermediates in the gene amplification process, as has previously been suggested (22). In terms of the rolling-circle intermediate, the amplification of ribosomal DNA would proceed as follows: beginning with a duplex DNA ring, a mechanism is used that leads to the continuous elongation of the 3′-OH terminus of an open strand using a circular template. This synthesis is pictured as leading to the displacement of a single-stranded tail, which is then converted to the duplex state by the *de novo* formation of fragments that are eventually sealed together by ligase.

As the growing point moves endlessly around the circle, the tail becomes a DNA concatemer composed of multiple copies of the ribosomal RNA gene (Fig. 5). In this state the tail contains many tandem homologous DNA sequences. These can be used in generalized recombination to excise a product DNA circle, or in site-specific recombination to excise a circle in much the same way as lambda prophage is believed to be excised from the chromosome of *E. coli* (30). Product DNA circles could be used directly for ribosomal RNA transcription (8), or they could be used to create more rolling circles and thus account for the increasing rate of ribosomal DNA synthesis during amplification (17). There is no supporting evidence, but the product circles would also have the potential of integrating into the *Xenopus* chromosomal DNA.

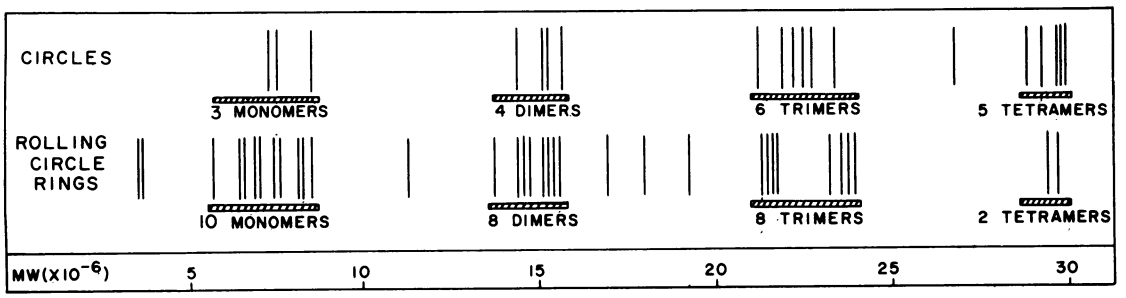

FIG. 3. Size classes of ribosomal DNA circles and rolling circles. Each *vertical line* represents either the circumference of a circle (*top line*) or the circumference of a rolling-circle ring (*bottom line*). The horizontal axis gives the corresponding molecular weights. To determine the circumferences of the circles, we photographed the molecules, magnified 10× during projection on a Nikon shadowgraph, then traced and measured them with a K & E map measurer (620300).

We have no reason to doubt our interpretation of the data, but it must be remembered that only a low percentage of satellite DNA appears in the rolling-circle configuration. The possibility that the forked forms we see might be the product of some nonreplicative process, therefore, cannot be rigorously excluded.

In addition to the rolling-circle model, other models have been suggested to account for amplification of ribosomal DNA.

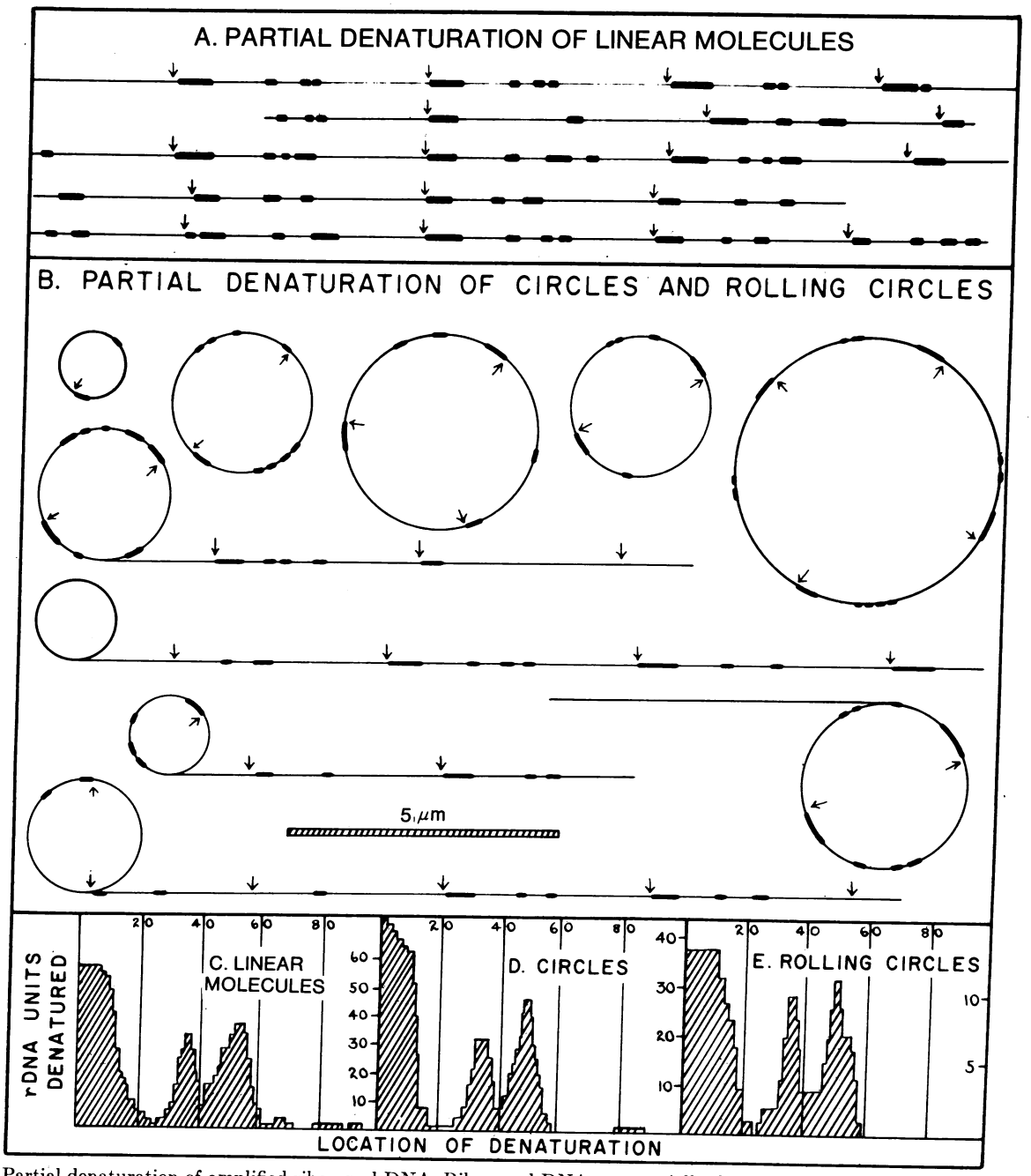

FIG. 4. Partial denaturation of amplified ribosomal DNA. Ribosomal DNA was partially denatured by the combined use of high concentrations of formamide (28) and heat (26). All operations were done in a 38–39° incubator with equilibrated materials. The spreading solution consisted of 10 μl of ribosomal DNA in 0.1 M Tris–0.01 M EDTA (pH 8.5)–5% formamide, 1 μl of cytochrome *c* [0.5 mg/ml in 1 M Tris–0.1 M EDTA (pH 8.5)], and 40 μl of formamide (Matheson, Coleman, and Bell). This mixture was incubated at 38–39° for 5 min, and then spread onto a hypophase [0.3 mM Tris–0.03 mM EDTA, (pH 8.5)]. Parlodion-coated grids were used to pick up the protein–DNA films, which were then stained with uranyl acetate and shadowed with platinum–palladium.

The partially denatured linear molecules, circles, and rolling circles of Fig. 4*A* and *B* have been schematically drawn to a uniform scale. *Black boxes* represent areas of the DNA duplex that are denatured (see Fig. 2*C*). *Arrows* in *A* and *B* mark the estimated borders of adjacent repeating sequences of ribosomal DNA. To construct the histograms in *C*, *D*, and *E*, we normalized the ribosomal DNA units to an arbitrary length of 100 units, and summed the partially denatured areas. The histograms were constructed from 11 linear molecules (59 repeated units) 11 circles (45 repeated units), and 5 rolling circles (18 repeated units).

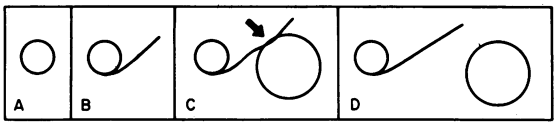

FIG. 5. The diagram shows how a rolling-circle intermediate is expected to produce, by a recombination event in its tail, multimer-length amplified ribosomal DNA circles. This recombination event could also occur if the tail has been detached from the rolling circle and exists as an intracellular concatemer. Single line represents a DNA double helix.

Tocchini-Valentini and Crippa (31) have proposed the involvement of RNA-dependent DNA polymerase, an enzyme capable of participating in the synthesis of DNA duplexes from RNA transcripts (32, 33). Our experiments, as well as the recent experiments of Bird, Rogers, and Birnstiel (34), offer no support for this hypothesis. A second model has been proposed by Buongiorno-Nardelli, Amaldi, and Lava-Sanchez (35); they suggest that the amplification process begins with monocistronic circles, with each successive round of synthesis resulting in circles of doubled circumference (1, 2, 4, 8, 16...) (see ref. 7). Our finding that the circumferences of the circles and rolling circles fit into an arithmetic (1, 2, 3, 4, 5...) rather than a geometric progression is contrary to the prediction of their model. Thus, the present data about synthesis of DNA during ribosomal gene amplification appear to be best explained in terms of a rolling-circle intermediate.

Although the rolling-circle model can account for the DNA synthesis that occurs during ribosomal gene amplification, a number of related questions remain unanswered; foremost among these is the origin of the first rDNA ring.

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