Replication forks are underrepresented in chromosomal DNA of *Xenopus laevis* embryos

(replication intermediate/single-stranded DNA/strand separation/chromosomal DNA replication/embryogenesis)

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ABSTRACT Chromosomal DNA was isolated from rapidly dividing cells of *Xenopus laevis* embryos at blastulation, at gastrulation, and at the beginning of hatching. Few, if any, replication forks were seen by electron microscopy in DNA isolated at any stage of embryogenesis. Instead, unbranched DNA, which appeared to be single-stranded, was abundant at all stages. Chromosomal DNA that was single-stranded was quantitated by electron microscopy and by monitoring the release of acid-soluble radioactivity during digestion of labeled chromosomal DNA with nuclease specific for single-stranded DNA. The amount of single-stranded DNA was inversely correlated with the length of S phase during embryogenesis. We postulate that chromosomal DNA replication in *X. laevis* embryos takes place by a mechanism in which strand separation is uncoupled from DNA synthesis.

Conventional semidiscontinuous, bidirectional mechanisms of chromosomal DNA replication (refs. 1-5; figure 5-38 of ref. 6) explicitly predict that the highest frequency of replication forks will be found in chromosomal DNA isolated from the most rapidly dividing cells. Such replication forks have been observed and extensively characterized in prokaryotes (7), lower eukaryotes (8, 9), and Dipiterans (2, 10-13). However, replication forks have been reported only infrequently in DNA isolated from higher eukaryotes (e.g., enterocoelomates) (14-17). In addition, replication forks are much less abundant than extensive regions of stably denatured or single-stranded DNA (ss DNA) in molecules isolated from rapidly dividing cells of higher eukaryotes (reviewed in 18 and 19). It has been suggested that the frequently observed ss DNA is an artifact generated by the procedures used to isolate the DNA (18, 20, 21). Alternatively, we have proposed that extensive single-stranded regions of parental DNA are key intermediates in normal chromosomal DNA replication in rapidly dividing cells (19). We have further speculated that chromosomal DNA replication in enterocoelomate embryos may not involve replication forks at all (19).

To test these hypotheses, chromosomal DNA was isolated from cells of *Xenopus laevis* embryos with genetically different lengths of S phase. The isolated DNA was analyzed by quantitative electron microscopy and by digestion with nucleases specific for single-stranded DNA. No replication forks were observed in chromosomal DNA isolated from embryonic cells with the shortest S phase. In addition, abundant single-stranded regions were found specifically in DNA isolated from cells with the shortest S phase (i.e., during the time of most rapid chromosomal DNA replication during *X. laevis* embryogenesis).

MATERIALS AND METHODS

Frogs. Adult *X. laevis* were acquired from Xenopus I (Ann Arbor, MI). Eggs were obtained and fertilized in *vitro* as described (22). Embryos were allowed to develop at 22°C in dechlorinated water until they reached the blastula (stage 7), gastrula (stage 11), or hatched embryo (stage 34/35) stages of Nieuwkoop and Faber (23).

DNA Isolation. Dejellied embryos were gently homogenized in a loose-fitting glass/Teflon hand homogenizer in 0.5% citric acid (24), spermine was added to 1.5 mM (25), and the homogenate was centrifuged at 1000 × g. The pellet, which contained the nuclei, was sequentially washed with 100 mM KCl/1.5 mM spermine; 50 mM Tris-HCl, pH 8.0/1.5 mM spermine; and 10 mM Tris-HCl, pH 8.0/1.5 mM spermine. The nuclei were lysed at 60°C in 10 mM 3-cyclohexylamino-1,1-propanesulfonate (P-L Biochemicals)/200 mM EDTA, pH 10.4/1% Sarkosyl (NL97, Geigy) (10, 26). The resulting DNA suspension was dialyzed against 10 mM Tris-HCl, pH 8.0/1 mM EDTA/10 mM NaCl at 4°C for up to 12 hr. Dialysis was necessary to remove the Sarkosyl, which otherwise made it difficult to distinguish between ss DNA and double-stranded DNA (ds DNA). No replication forks were seen in undialyzed DNA samples. This isolation method was selected because it resulted in the longest median length chromosomal DNA molecules. Similar results (no replication forks and abundant ss DNA) were also obtained with chromosomal DNA extracted by more conventional procedures (refs. 27, 28; unpublished observations). Nuclear cages were isolated by the method of ref. 29. DNA from these preparations gave similar results. Chromosomal DNA isolated from stage 10 embryos treated with psoralen at 10 μg/ml and heavily crosslinked [more than 1 crosslink per 0.1 kilobase (kb)] by exposure to 365-nm light also showed no replication forks or "eyes" and abundant ss DNA.

Labeling of Chromosomal DNA. To label chromosomal DNA, embryos were dejellied after the first cleavage, microinjected with 25-75 nl of [α-32P]dTTP [3000 Ci/mmol, 10 μCi/ml (1 Ci = 37 GBq); ICN] between the 2-cell and the 8-cell stages, and allowed to develop into blastulae. Chromosomal DNA was then isolated as described above.

Electron Microscopy. DNA was diluted 1:20 or 1:40 into 10 mM Tris-HCl, pH 8.0/1 mM EDTA/1.3 mM NaOAc, pH 4.6/10 mM NaCl/0.05 mM ZnSO4/5%/vol/vol) glycerol/3 M CsCl (to dissociate it from the DNA proteins that remain during our isolation procedure) and was spread in the presence of formamide as described (30). Electron micrographs were taken on a Zeiss EM 109, usually at 7000×. At least 105 kb of DNA was photographed at each stage. More than 105 kb of DNA was examined for blastulae, but not all

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Abbreviations: kb, kilobase(s) or kilobase pair(s); ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.

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were photographed because replication forks were never observed. Contour length measurements were made on a Hewlett-Packard 9874A digitizer and converted to kilobases by comparison with the length of pBR322 (4.3 kb) spread under the same conditions. All DNA molecules in each field were photographed with the exception of a few small fragments (<1 kb) and rare 17.4-kb circular DNA molecules (presumably mitochondrial DNA). The median length of chromosomal DNA molecules in each preparation was ≈100 kb, except for DNA isolated from blastulae, which was variably shorter (<55 kb) depending on the preparation.

Nuclease Digestion. 32P-labeled DNA isolated from blastulae was digested with S1 nuclease (Bethesda Research Laboratories), mung bean nuclease, and Neurospora crassa single-strand nuclease (P-L Biochemicals) as described in the legend to Table 2. 3H-labeled, alkali-denatured (80% sensitive to S1 nuclease) Escherichia coli DNA (New England Nuclear, 5.4 mCi/mg) and 14C-labeled, native (<0.1% sensitive to S1 nuclease) E. coli DNA (New England Nuclear, 0.011 mCi/mg) were included in each reaction mixture as positive and negative controls for nuclease specificity. Acid-insoluble radioactivity was measured as described (31).

**RESULTS**

Underrepresentation of Replication Forks. We had expected to observe frequent replication forks or replication eyes in chromosomal DNA isolated from cleaving embryos (see the calculations in table III of ref. 32). Few, if any, were observed. In particular, no replication forks were seen in >10^6 kb of chromosomal DNA isolated from blastulae (Table 1). We had expected to find at least one fork every 15–20 kb to account for the rapid rate of DNA replication taking place at this stage.

In DNA isolated at later stages of embryogenesis, a few branched structures that might be interpreted as replication forks were observed (Table 1). These structures were, however, unconvincing (i.e., extremely small and lacking the appropriate single-stranded regions in trans). Moreover, these structures were still too few in number, by at least an order of magnitude, to permit replication of the complete genome during the limited time available. We thus conclude that replication forks are underrepresented in chromosomal DNA isolated from *X. laevis* embryos.

**Extensive Regions of ss DNA.** Chromosomal DNA isolated from embryos at all three stages studied exhibited an unexpectedly large percentage of DNA that appeared to be single-stranded (Fig. 1 and Table 1). Chromosomal DNA isolated by the same procedure from nondividing cells did not show any detectable ss DNA, other than a few small terminal single-stranded regions presumably generated by shear forces (Table 1). Molecules as long as 75 kb that appeared to

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Table 1. Electron microscopic analysis of frequency of replication forks and single-stranded regions in chromosomal DNA isolated from *X. laevis* embryos

<table>
<thead>
<tr>
<th>X. laevis</th>
<th>Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage of development</strong></td>
<td><strong>Blastula</strong></td>
</tr>
<tr>
<td>Duration of S phase, min</td>
<td>10–20</td>
</tr>
<tr>
<td>Replication forks per 10^4 kb</td>
<td>0*</td>
</tr>
<tr>
<td>Observed</td>
<td></td>
</tr>
<tr>
<td>Minimum required to replicate genome in S phase</td>
<td>1000</td>
</tr>
<tr>
<td>ss DNA (% DNA)</td>
<td>40</td>
</tr>
</tbody>
</table>

*More than 10^6 kb of DNA from blastulae was examined without detecting a single replication fork prior to the midblastula transition.

†Corrected for fusion of adjacent replication eyes (see ref. 2).

Based on 10^6 kb of DNA photographed and digitized.
Table 2. Analysis of [3H]dTMP-labeled blastula chromosomal DNA by digestion with single-strand-specific nuclease

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction of radioactivity released, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 nuclease</td>
<td>30</td>
</tr>
<tr>
<td>Mung bean nuclease</td>
<td>37</td>
</tr>
<tr>
<td>N. crassa endonuclease</td>
<td>69</td>
</tr>
</tbody>
</table>

[3H]dTMP-labeled blastula DNA (1000 counts per assay, assays performed in duplicate) was digested with S1 nuclease, mung bean nuclease, or N. crassa single-strand nuclease. S1 nuclease digestions were carried out in 30 mM NaOAc, pH 4.6/5.0 mM NaCl/1 mM ZnSO4/5% (vol/vol) glycerol containing 7.2 ng of [3H]-labeled E. coli ss DNA, 0.36 µg of [14C]-labeled E. coli ds DNA, 35 µg of [32P]-labeled X. laevis blastula chromosomal DNA, and 100 units of S1 nuclease. The reaction mixture was incubated at 37°C for 1 hr with aliquots removed at 0 min, 5 min, 10 min, 20 min, 30 min, and 1 hr or for 19 hr with aliquots removed at 0 min, 5.5 hr, 9 hr, and 19 hr. N. crassa nuclease digestions were carried out in 100 mM Tris-HCl, pH 8.2/10 mM MgCl2/5% (vol/vol) glycerol containing 7.2 ng of [3H]-labeled E. coli ss DNA, 0.36 µg of [14C]-labeled E. coli ds DNA, 35 µg of [32P]-labeled X. laevis blastula chromosomal DNA, and 150 units of N. crassa nuclease. The reaction mixture was incubated at 37°C for 19 hr with aliquots removed at 0 min, 5.5 hr, 9 hr, and 19 hr. Mung bean nuclease digestions were carried out in 30 mM NaOAc, pH 5.0/50 mM NaCl/1 mM ZnCl2/5% (vol/vol) glycerol containing 7.2 ng of [3H]-labeled E. coli ss DNA, 0.36 µg of [14C]-labeled E. coli ds DNA, 35 µg of [32P]-labeled X. laevis blastula chromosomal DNA, and 420 units of mung bean nuclease. The reaction mixture was incubated at 37°C for 19 hr with aliquots removed at 0 min, 5.5 hr, 9 hr, and 19 hr. In all cases, duplicate samples of each aliquot were spotted on Whatman no. 3 filter disks. Trichloroacetic acid precipitations were carried out as described in ref. 31.

*Normalized to exactly 80% digestion of the control [3H]-labeled alkali-denatured DNA.
*Collins (31) has similarly observed that N. crassa endonuclease digests two-fold more DNA isolated from HeLa cells in S phase than does S1 nuclease. The reason for this is not understood.

labeled chromosomal DNA was isolated from X. laevis blastulae, and aliquots were digested with one of three different single-strand-specific nuclease. Digestion conditions were standardized so that 80% of the [3H]-labeled single-stranded control DNA and <0.1% of the [14C]-labeled native DNA were digested in the same reaction mixture. Digestion with S1 nuclease converted 30% of the input precipitable label into acid-soluble radioactivity (Table 2). N. crassa nuclease liberated 69% of the input precipitable radioactivity, and mung bean nuclease liberated 37% of the input precipitable radioactivity (Table 2). These values were in general agreement with the amount of ss DNA quantitated by electron microscopy. In contrast, RNase A did not release acid-soluble radioactivity from the labeled chromosomal DNA isolated from blastulae.

Control Experiments Using Mixtures of Nuclei Isolated from Drosophila and X. Laevis Embryos. Nuclei were isolated from Drosophila preblastoderm embryos using the procedure in ref. 10. Chromosomal DNA was extracted (33) either before or after mixing with nuclei isolated from X. laevis stage 8/9 embryos (33); the DNA was examined by electron microscopy (33). The frequency of replication forks was ~200/10⁴ kb in the DNA isolated from Drosophila nuclei alone, 200/10⁴ kb in the DNA isolated from the Drosophila and X. laevis nuclei mixture, and 0/10⁴ kb in the DNA isolated from the X. laevis nuclei alone. [3H]-labeled (34) chromosomal DNA from Drosophila preblastoderm embryos and [32P]-labeled chromosomal DNA from X. laevis stage 7 blastulae were isolated and digested in the same test tube with nuclease S1. Reaction conditions were as in the legend to Table 2, but the [3H]-labeled denatured and [14C]-labeled native DNA standards were omitted. Less than 0.1% of the [3H]-labeled but 24.6% of the [32P]-labeled DNAs were released by digestion with nuclease S1.

DISCUSSION

The data presented here suggest there is a dramatic underrepresentation of replication forks in chromosomal DNA at each stage of X. laevis embryogenesis studied. This underrepresentation is not explained by inability to isolate replication forks. The expected frequency of replication forks was observed in chromosomal DNA we isolated from Drosophila melanogaster embryos (Table 1) and polyoma viral DNA (35). Moreover, polyoma replicative intermediates microinjected into cleaving X. laevis embryos were recovered, showing that the contents of these embryos were not incompatible with replication forks.

It is likely that some replication forks might not have been observed because of selective destruction during DNA isolation. We believe, however, this is unlikely to be the entire explanation in view of the long median length of the isolated chromosomal DNA molecules (~100 kb), the extremely long molecules (~200 kb) frequently seen, and the entirely single-stranded molecules >70 kb long isolated during the later stages of embryogenesis.

In contrast to the surprising absence of replication forks, the amount of ss DNA observed was directly correlated with rapid rates of chromosomal DNA replication (Table 1). In X. laevis blastulae, the S phase ranges from 10 to 20 min in length. The highest percentage of ss DNA was observed at this stage. In hatched embryos, however, the S phase is >3 hr long (i.e., ~12 times longer than S phase in blastulae). Only 3% of the DNA isolated at this stage was single-stranded (i.e., about 1/12th of the amount observed in chromosomal DNA isolated from blastulae). If the extensive regions of ss DNA were simply artifacts produced during isolation, we would expect the percentage to remain relatively constant throughout embryogenesis. This was clearly not the case since the amount of ss DNA varied dramatically with the stage of embryogenesis. In addition, DNA isolated from Drosophila embryos by these procedures was not made single-stranded, suggesting that the isolation procedure per se did not generate extensive regions of ss DNA.

ss DNA has been reported in association with newly replicated DNA in a variety of eukaryotic organisms (reviewed in refs. 18 and 19). In particular, there have been frequent reports that extensive regions of ss DNA have been isolated from rapidly dividing embryonic cells in a number of systems, including sea urchin (36–39) and other enterocladic larvae (reviewed in ref. 18) and in DNA isolated at S phase from Ehrlich ascites (40) and from mammalian cells grown in tissue culture (41–43).

Many investigators have dismissed these single-stranded regions as artifacts of the DNA isolation technique used or as structures unrelated to DNA replication (18–21). These single-stranded regions, however, were found preferentially in chromosomal DNA isolated from eukaryotic cells that have entered S phase (44–48). Furthermore, the ss DNA has been shown to be largely of parental origin (31, 45, 46) and often as long as 70 kb (42). This minimizes the possibility that the ss DNA was generated entirely by shear forces.

In X. laevis embryos, it is also extremely unlikely that the extensive single-stranded regions were generated by nuclease activity (49–52). ss DNA either incubated in extracts of eggs or microinjected into fertilized and unfertilized eggs is remarkably stable under a wide variety of conditions. DNA polymerase-primase activity on single-stranded templates can be detected even in crude extracts of X. laevis eggs (49–51).

It is difficult to establish conclusively whether the chromosomal DNA within the embryo is genuinely single-stranded or simply destabilized. If synthesis occurs discontinuously on both strands, one might expect the short nascent, "Okazaki"-type fragments to be somewhat destabilized (but
see the psoralen control experiments in Materials and Methods). Preliminary experiments with bromoacetaldehyde (53), however, suggest that a substantial fraction of DNA in X. laevis embryos reacts as if it were single-stranded in vivo (P. Hines and E. Hoehn-Saric, personal communication). In any event, the loss of small destabilized fragments is not sufficient to explain the underrepresentation of replication forks or the presence of the extensive single-stranded regions.

The observations presented above are difficult to reconcile with current models of eukaryotic DNA replication that require replication forks. These models explicitly predict that replicating DNA should contain double-stranded replication eyes and, furthermore, that DNA isolated from embryos in early stages of development should exhibit more of these structures than DNA from later stages. In addition, these models do not readily explain the extensive amounts of single-stranded and stably denatured DNA we and others have observed.

**Strand-Separation Hypothesis.** To interpret our observations, we suggest an alternative hypothesis for chromosomal DNA replication in X. laevis embryos. It is based on the underrepresentation of replication forks, on the extensive single-stranded regions specific to S phase, and on the following additional observations. (i) Leading-strand synthesis has not been detected in either chromosomal DNA from X. laevis embryos (unpublished observation) or DNA templates microinjected into X. laevis eggs (unpublished observation). This is in agreement with experiments in other higher eukaryotic organisms that support a mechanism of discontinuous synthesis of both strands of chromosomal DNA (40, 54-58). (ii) X. laevis DNA polymerase α₁ has been shown to have an associated DNA primase (51, 59, 60) that preferentially synthesizes on primed ss DNA and catalyzes self-primed synthesis at many sites on single-stranded templates. (iii) The DNA polymerase–primase complex is extremely abundant in fertilized eggs (61) (2 × 10⁶ molecules per egg). This corresponds to ~1 × 10⁵ DNA polymerase α₁ molecules per replicative domain, which is in gross excess of the amount required to replicate the entire genome in the available time. (iv) There is no rigorous requirement for replication origins to replicate DNA microinjected into X. laevis eggs (49, 62, 63).

The strand-separation hypothesis is diagrammed in Fig. 2. We postulate that replication begins with rapid strand destabilization and strand separation throughout an entire chromosomal DNA domain. This step is uncoupled from strand synthesis. Although the possibility of strand synthesis being coupled from strand unwinding has been proposed previously (18), it was in the context of processive replication fork movement. Strand separation could begin at specific sites such as the putative anchorage sites (64, 65). Topological constraints introduced by this process would be relieved by DNA topoisomerases I and II, which allow single-strand single strand and double-strand double-strand passage, respectively (reviewed in refs. 66 and 67). The resulting single strands, stabilized by a strand-stabilization factor that protects the DNA from nucleolytic attack (refs. 68, 69; unpublished observation), then serve as substrates for the DNA polymerase–primase complex (51, 59, 70, 71). Totally discontinuous synthesis would occur along the entire length of both parental strands (i.e., no leading-strand synthesis).

This mechanism would facilitate extremely rapid rates of DNA replication in early cleavage when the duration of S phase is quite short and DNA polymerase–primase is in gross excess. At the blastula stage, there are still ~10 molecules of polymerase–primase for each chromosomal domain (61). At blastulation, the duration of S phase begins to increase, presumably because the levels of DNA polymerase–DNA primase become rate limiting. Self-primed DNA synthesis should nevertheless still occur at independent sites within each domain. This would result in formation of the alternating short regions of ss and ds DNA observed. By gastrulation, however, there is less than one polymerase α₁ molecule per chromosomal domain. The duration of S phase necessarily increases again. The strand-separation hypothesis predicts that the DNA within an entire domain will still separate in preparation for DNA synthesis. As the level of DNA polymerase α₁ per domain decreases, however, more long regions of ss DNA should be observed, even though the percentage of the genome that is single-stranded decreases.

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