In vitro replication of recombinant plasmids carrying chromosomal segments of Xenopus laevis

(eukaryotic DNA/replication origins/DNA initiation/cloning/electron microscopy)

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ABSTRACT Recombinant plasmids carrying a segment of Xenopus laevis chromosomal DNA were constructed with plasmid pBR322 as the vector. A recombinant plasmid pXY65 carrying a 3.2-kilobase BamHI segment of the chromosome of X. laevis has been found to contain a repetitive sequence dispersed throughout the X. laevis chromosomes. This plasmid initiated replication in vitro when the supercoiled circular molecules were incubated in a replication system. The other recombinant plasmids tested and the pBR322 vector were not replicated. Electron microscopic analysis of the replicative intermediates showed that the replication was initiated at a specific site in the 3.2-kilobase BamHI segment of pXY65 and that the replication usually proceeded bidirectionally. Analysis of the reaction products by centrifugation in alkaline sucrose gradients indicated that short pieces were synthesized in the in vitro replication system. DNA synthesis was inhibited in vitro by the addition of aphidicolin and by omission of dNTPs. These results indicate that the X. laevis segment cloned in pXY65 contains a site capable of initiating replication in vitro.

The replication of eukaryotic DNA occurs by initiation at many chromosomal sites which are usually clustered in specific regions characteristic of cell type and the stage of the cell cycle (1-9). Recently, several investigators have tried to isolate replication origins by using in vitro recombination techniques (10-13). Many eukaryotic "autonomously replicating sequences" of yeast as well as other higher organisms have been described. These sequences from higher organisms may act as initiators of replication in yeast cells (9, 10). However, there is no evidence that these cloned eukaryotic DNA segments can also act as replicators in cells of the original higher organism.

Watanabe and Taylor (13) have described the isolation of a recombinant plasmid which had a 500-base-pair segment of Xenopus laevis DNA and was replicated semiconservatively after microinjection into unfertilized Xenopus eggs. Harland and Laskey (14) also reported that circular double-stranded DNA molecules of simian virus 40, poloma virus, bacteriophages, and plasmids were replicated in unfertilized eggs of X. laevis. However, they concluded that a specialized DNA sequence was not essential for initiation of DNA replication in the Xenopus egg.

To study DNA replication in vitro, Benbow et al. (15) prepared an extract from unfertilized Xenopus eggs and fractionated it by DEAE-cellulose chromatography. The active fraction contained several activities including DNA polymerases α, β, and γ, RNA polymerases, DNA ligase, a DNA-binding protein, and RNase H (16). Using this system, Benbow et al. were successful in obtaining replication of a recombinant plasmid that consisted of the vector pMB9 and a segment that coded for rRNA in X. laevis.

In this paper, we describe a recombinant plasmid carrying a chromosomal segment of X. laevis which initiates replication in vitro in an extract prepared from unfertilized eggs of X. laevis according to the procedure of Benbow et al. (15). The replication was initiated at a specific site on the Xenopus segment and usually proceeded bidirectionally.

MATERIALS AND METHODS

Bacterial Strain and Vector Plasmid. Plasmid pBR322 (17) was obtained from H. W. Boyer (University of California, San Francisco, CA). Strain KH802 (met gal sup E hsdR), a derivative of Escherichia coli K-12, was used as the host (18).

Reagents. The enzymes used and their sources were: BamHI, HindIII, EcoRI, Pst I, Sal I, and T4 DNA ligase (Takara Shuzo, Kyoto, Japan); Pvu II, Ava II, and Xho I (New England Biolabs); Kpn I, Xho I, Xba I Kpn I, and Sst I (Bethesda Research Laboratories); E. coli DNA polymerase I grade I, Boehringer Mannheim), and Bgl II and Hpa I (H. Ohmori). Aphidicolin was supplied by M. Onodera, and α-amanitin was from A. Ishihama. Diadenosine 5',5''-P',P'-tetraphosphate (Ap4A) was purchased from P-L Biochemicals and [α-32P]dCTP (2.000-3.000 Ci/mm mol; 1 Ci = 3.7 × 1010 becquerels), [methyl-3H]dTTP (30 Ci/mm mol), and [6-3H]thymidine (27 Ci/mm mol) were obtained from Amersham International.

Cloning Procedures and Biohazard. DNA extracted from X. laevis sperm was digested with BamHI and ligated to BamHI-digested pBR322 DNA with T4 ligase. The ligated DNA mixture was used to transform KH802 cells, and ampicillin-resistant tetracycline-sensitive cells were selected (19). All procedures for cloning were carried out under P2 conditions.

Preparation of Plasmid DNA. Bacterial cells were grown in L broth and were incubated in the presence of chloramphenicol (150 μg/ml) for 15 hr. The cells were harvested, lysed in a buffer solution with lysozyme, EDTA, and Na2-EDTA. Sodium chloride was added to 1 M according to the procedure of Guerry et al. (20). The supernatant fluid from salt-precipitated lysates was diluted with the same volume of distilled water and treated with RNase A. DNA was extracted by shaking with phenol and precipitated with ethanol. The plasmid DNA was purified by banding in a CsCl/ethidium bromide gradient by centrifugation in an SW 50.1 rotor at 37,000 rpm at 10°C for 40 hr; it then was dialyzed against TES buffer (50 mM Tris·HCl, pH 8.5 mM EDTA/50 mM NaCl).

Microinjection of Plasmid DNA into X. laevis Eggs. Unfertilized eggs were obtained by microinjection (21) of 500 units

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Abbreviations: Ap4A, diadenosine 5',5''-P',P'-tetraphosphate; kb, kilobase(s).
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of gonadotropin (Tehkoku Zohki Pharmaceutical) into adult female frogs. Plasmid DNA in 15 mM Tris HCl, pH 7.5/88 mM NaCl/[3H]thymidine (10 mCi/ml, 27 Ci/mmol) was injected (50 ± 10 nl per egg) into 30 or 50 unfertilized eggs that had been irradiated for 60 sec with ultraviolet light (25,000 erg/cm²) immediately before the injection. The injected eggs were incubated at 25°C for 4 hr in modified Barth’s saline and then dejellied with 2% (wt/vol) sodium thioglycolate. The eggs were washed twice in saline and suspended in 0.5 ml of ice-cold TES buffer to which 0.05 ml of 10% NaDodSO₄ was added. The eggs were homogenized in a Vortex mixer. The homogenate was incubated at 37°C for 2 hr with Pronase (2 mg/ml, heated at 70°C for 20 min) and RNase A (0.1 mg/ml, heated at 90°C for 20 min), and the DNA was extracted by shaking twice with 1 ml of chloroform/isoamyl alcohol, 24:1 (vol/vol). The aqueous phase was removed and shaken with an equal volume of phenol saturated with TES buffer. The DNA was precipitated with ethanol and dissolved in 50 μl of TES buffer.

Agarose Gel Electrophoresis and Fluorography. Plasmid DNAs or DNA fragments were separated by electrophoresis on agarose gels with a buffer containing 10.8 g of Tris, 0.93 g of EDTA, and 5.5 g of boric acid per liter. DNA in the gel was visualized with 254-nm light after staining with ethidium bromide (1 μg/ml). For fluorography of [3H]-labeled DNA, gels were equilibrated sequentially with ethanol, ethanol containing 10% of diphenylxazole, and water, dried, and exposed to x-ray film (Fuji).

DNA-DNA Hybridization. DNA was transferred from agarose gels to BA85 membrane filters (Schleicher & Schuell) according to the method of Wahl et al. (22). The filters were pretreated with Denhardt’s medium. The filters were incubated at 65°C for 18 hr with sonicated denatured E. coli DNA (50 μg/ml) and plasmid DNA (7 × 10⁶ cpn; 10⁶ cpn/μg of DNA); the latter had been labeled with [α-32P]dCTP (Amersham) by nick-translation (23). The DNA-bearing filters were washed and exposed to x-ray film at −70°C.

Preparation of Enzyme Fraction for DNA Synthesis. The enzyme fraction for in vitro DNA synthesis was prepared from 25 g of dejellied unfertilized eggs of X. laevis according to Benbow et al. (15). Active fractions (fractions III, IV, V, and VI; see ref. 15) eluted from the DEAE-cellulose column were collected without subfractionation and concentrated 20-fold at 0°C with an Amicon on-line column elute concentrator (model CECI).

Conditions for in Vitro DNA Replication. The reaction mixture (total volume, 50 μl) contained 50 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 25 mM NaCl, 25 mM KCl, 2 mM 2-mercaptoethanol, bovine serum albumin at 200 μg/ml, 50 μM dATP, dCTP, dGTP, and dTPP, 250 μM CTP, CTP, and UTP, 2.25 mM ATP, 36 μl of the enzyme fraction, and 100 μg of plasmid DNA per ml. After the mixture was incubated for 20 or 40 min at 26°C, the reaction was terminated by the addition of phenol, and the extracted DNA was immediately observed in the electron microscope (15). [3H]dTTP was used for the measurement of deoxyribonucleotides incorporated into acid-insoluble fraction and [α-32P]dCTP was used for the analysis of the reaction products by alkaline sucrose gradients.

Electron Microscopy. DNA was extracted from the reaction mixture as described by Benbow et al. (15), and 5-μl aliquots of the extracted DNA were immediately spread on collodion/carbon-coated copper grids by a microaqueous procedure (24, 25). Grids were stained with uranyl acetate, rotary-shadowed with Pt/Pd, and photographed in a JEM100B (JEOL, Japan) electron microscope.

Alkaline Sucrose Gradient Centrifugation. [32P]-Labeled DNA products synthesized in vitro were analyzed by centrifugation in a 5–20% alkaline sucrose gradient (10 ml) in a Spinco L2-65B and SW 41 rotor for 14 hr at 21,000 rpm at 4°C. Fractions were collected from the bottom, and the radioactivity in the acid-insoluble material of each fraction was measured.

RESULTS

Cloning of X. laevis Chromosomal DNA. Recombinant plasmids that carried a DNA fragment of the X. laevis chromosome were isolated by in vitro recombination techniques using pBR322 as the vector. To screen plasmids that could replicate autonomously in unfertilized eggs, plasmid DNAs were extracted from 12 groups of 5 clones each (a total of 60 clones) and injected into unfertilized eggs of X. laevis with [3H]thymidine. After 4-hr incubation at 23°C, DNA was extracted from these eggs and analyzed by electrophoresis in agarose gels. Results of fluorography of these gels showed that the incorporation of [3H]thymidine into plasmid DNA was low in the vector pBR322 and in most of the recombinant plasmids. However, in three recombinant plasmids, the incorporation was 2- to 4-fold higher than in the vector plasmid. One of the three plasmids, pXY65 was picked for further tests for the ability to replicate in vitro. The vector pBR322 and another recombinant plasmid pXY62 which showed a low incorporation in vivo were used as controls.

 Cleavage maps of pXY65 and pXY62 were determined by using restriction endonucleases (Fig. 1). When the plasmid DNA was digested with BamHI, 4.36- and 3.2-kilobase (kb) fragments were obtained. This indicates that pXY65 consists of a 3.2-kb DNA segment of the X. laevis chromosomal DNA and the vector plasmid pBR322 segment (4.36 kb). The contour length of pXY65 molecules measured in the electron microscope was consistent with these values. The 3.2-kb BamHI segment had no sites for Xho I, Sst I, EcoRI, Sal I, Ava I, Xba I, or Kpn I. The plasmid pXY62, on the other hand, consists of a 4.3-kb Xenopus segment and the pBR322 segment.

Plasmid pXY65 Contains Repetitive Sequence. To test whether these plasmids contained any repetitive sequences which are widely dispersed in Xenopus chromosomes, plasmid DNA was labeled heavily with [32P]dCTP by nick-translation, denatured, and hybridized with denatured X. laevis erythrocyte DNA transferred onto nitrocellulose filters by the Southern blot technique. [32P]-Labeled pXY65 DNA hybridized efficiently with the X. laevis chromosomal DNA but not with a control human chronological DNA. The DNA probe hybridized with Xenopus DNA fragments of various lengths produced by complete digestion with BamHI or Bgl II. Because no clear discrete bands could be observed in autoradiography, we suggest that pXY65 carries a X. laevis DNA segment that is repeated many times in the Xenopus DNA, whereas pXY62 appears to contain no repetitive sequence but represents a unique sequence of X. laevis chromosome.

In Vitro Replication of Recombinant Plasmid DNA. Plasmid pXY65 was tested for its ability to replicate in vitro. The enzyme fraction used in the test was prepared as described by Benbow et al. (15) and was derived from the eluted fractions containing the first absorbance peak at 290 nm (Fig. 2). These fractions were collected, concentrated, and used for the test. When supercoiled circular molecules of the plasmid pXY65 were added to the system, DNA-dependent incorporation of [3H]dTTP into acid-insoluble materials was observed (Fig. 3A). The amount of incorporation with pBR322 was lower than that with pXY65. When pXY65 was present, the rate of incorporation proceeded linearly up to 50 min and then decreased. Under the reaction conditions the amount of the template plasmid DNA present was not in excess (Fig. 3B).

This DNA-dependent incorporation with pXY65 as the template was inhibited 80–90% by the addition of aphidicolin.
(10–100 μg/ml), which is a specific inhibitor of DNA polymerase α [EC 2.7.7.7] (27, 28). As expected, the incorporation also was nearly completely inhibited when three dNTPs were absent. The inhibitory effect of a-amanitin (0.2 μg/ml) was low; there was only a 30% reduction compared to the control. ApA (0.1 μg/ml) (29) did not stimulate the incorporation of [3H]dTTP in the in vitro system with pXY65; in fact, it inhibited the incorporation about 30%.

The pXY65 DNA was incubated for 20 min in the enzyme system containing [α-32P]dTTP. The DNA was extracted with phenol and centrifuged in an alkaline sucrose gradient. The 32P-labeled product sedimented slowly whereas the small amount of radioactivity in the bulk or template DNA rapidly sedimented to the bottom of the tube. These preliminary results suggest that 32P-labeled short pieces were not covalently bound to the template and that incorporation was not due to the filling of a gap or to nick-translation.

**Formation of Eye Structures by in Vitro Replication.** In order to visualize the structure of the replicated DNA produced in the in vitro replication system, electron micrographs were taken. Plasmid DNA molecules containing an eye structure (θ form) were observed with a relatively high frequency (about 3%) in the products derived from pXY65. The size of the eye formed in a 20-min incubation was 200–400 bp. In a 40-min incubation, it was 200–1,500 bp. There were also dimers of pXY65, and eye structures were observed both in the monomers and dimers of pXY65 (Fig. 4A). Each replicating molecule contained only one eye structure. By contrast, the frequency of formation of eye structures was very low in pBR322 and pXY62 (Table 1).

To determine whether or not the formation of the eye in vivo was specific for pXY65, dimers of pXY65 and monomers of pBR322 were incubated together for 20 min in the enzyme system and the products were examined by electron microscopy. Dimers (15 kb) of pXY65 could be distinguished from the monomers (4.4 kb) of pBR322 by the difference in their molecular lengths. The frequency of molecules having an eye form was 2.5% in pXY65 but <0.1% in pBR322. The formation of the eye structures was also low in dimers of pBR322 as well as in monomers when a pBR322 DNA sample that contained 90% dimeric molecules was used as template. To see the eye structures more clearly, the pXY65 DNA was spread on grids in the presence of a low concentration of ethidium bromide, which results in the relaxation of most of the supercoiled molecules (30). We were able to confirm that the frequency of preexisting eyes is low (Table 1).

The plasmid pXY65 cannot replicate in polA1 mutant cells of E. coli which also do not support the replication of pBR322. Moreover, all the deletion plasmids—which were prepared from pXY65 DNA by Sal I digestion followed by digestion with nuclease BAL-31, ligation, and transformation of polA+ cells—have the origin of pBR322 but most of them had lost the Xenopus segment. These results indicate that the origin in the Xenopus segment is incapable of initiating replication in bac-
terial cells. Therefore, the eyes that were observed at a low frequency in the pXY65 DNA preparations must have been produced by initiation from the origin of the vector in polA* cells (KH802).

The amount of synthesized DNA which was estimated based on the average length and frequency of the eye structure of pXY65 in 20-min incubation was about 1/3 the amount of DNA measured by incorporation of [3H]dTTP into the acid-insoluble fraction during 20-min incubation (data not shown). The lower amount of synthesized DNA calculated from the frequency of eye structure may be due to the extrusion of newly synthesized DNA segments and the snapback of parental strands.

**Origin and Direction of Replication.** To determine whether pXY65 initiates the replication at a specific site, pXY65 DNA was incubated in the enzyme system and then extracted with phenol, digested with BamHI, extracted again with phenol, and observed in the electron microscope (Fig. 4B). BamHI digestion of pXY65 generated two fragments: the larger fragment (4.36 kb) is the vector DNA and the smaller fragment (3.2 kb) is the Xenopus DNA (see Fig. 1). Replication intermediates were observed among both fragments, although the frequency in the vector fragment was lower. Eye structures were located nonrandomly in the Xenopus fragment (Fig. 5). This result suggests that the replication in most of the molecules started at a site located 40% (1.3 kb) from one end of the Xenopus fragment and that replication proceeded bidirectionally in about half of the molecules; in the other half, replication proceeded asymmetrically either in the clockwise or counterclockwise direction. In addition, when the pXY65 DNA was digested with HindIII after incubation for DNA replication, the 1.7-kb HindIII fragments with an eye structure were also observed. These results suggest that the initiation site of replication is located about 1.3 kb from the right BamHI site of the 3.2-kb Xenopus segment (Fig. 1). As shown in Fig. 5, in three molecules the position of the eyes were significantly different. This suggests that another weak origin might exist in the Xenopus segment.

On the other hand, eye structures were also located nonrandomly in the 4.36-kb BamHI vector fragment. Most of eye

**Table 1.** Electron microscope analysis of plasmid DNAs incubated in enzyme system prepared from *X. laevis* eggs

<table>
<thead>
<tr>
<th>DNA</th>
<th>Reaction time, min</th>
<th>Number of molecules*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
<td>OC or RC</td>
<td>RI</td>
</tr>
<tr>
<td>pXY65</td>
<td>0</td>
<td>1,915</td>
<td>95</td>
</tr>
<tr>
<td>pXY65</td>
<td>0</td>
<td>—</td>
<td>1,444</td>
</tr>
<tr>
<td>pBR322</td>
<td>0</td>
<td>968</td>
<td>41</td>
</tr>
<tr>
<td>pXY62</td>
<td>0</td>
<td>955</td>
<td>48</td>
</tr>
<tr>
<td>pXY65</td>
<td>20</td>
<td>205</td>
<td>232</td>
</tr>
<tr>
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<td>848</td>
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<td>790</td>
</tr>
<tr>
<td>pXY62</td>
<td>40</td>
<td>371</td>
<td>1,205</td>
</tr>
</tbody>
</table>

*All molecules exhibiting more than three superhelical turns were classified as supercoiled. SC, supercoiled circle; OC, open circle; RC, relaxed circle; RI, replicating intermediate. Values in parentheses represent percentage of total molecules observed.

*pXY65 DNA was spread on grids in the presence of 1 μg of ethidium bromide per ml to make relaxed circular forms.

**Fig. 4.** Electron micrograph of eye structures in the pXY65 DNA formed in vitro. Supercoiled pXY65 DNA was incubated for 40 min in the enzyme system prepared from *X. laevis* eggs. (A) Eye structure in a dimeric molecule. (B) Eye structures in the 3.2-kb fragments observed after BamHI digestion of pXY65 DNA which was incubated in the enzyme system. (Bar represents 0.5 μm.)

**Fig. 5.** Diagram of the location of the eye structure of the BamHI 3.2-kb Xenopus DNA fragment of pXY65. pXY65 DNA was incubated for 40 min in the enzyme mixture, recovered by phenol extraction, and digested with BamHI. Electron micrographs of the 3.2-kb BamHI fragments containing a replication loop were taken, and the location and size were measured. The figure was constructed by lining up one end and plotting the position of the eyes against the percentage replication. Thick lines represent the replicated regions. The arrow indicates the most probable point of the origin which is located where most eye structures overlap (see the text).
structures appeared to be produced by initiation of replication at a site about 1.7 kb from one end of the fragment—i.e., the location of the site was different from that of the pBR322 origin that acts in E. coli cells (Table 1). It may be assumed, on the basis of their locations, that a minor fraction of the eye structures were already initiated in E. coli cells.

**DISCUSSION**

We have obtained a plasmid, pXY65, carrying a 3.2-kb BamHI Xenopus segment that appears to be essential for efficient initiation of replication in *vivo*. Replication does not seem to be initiated randomly. Most of the eye structures in the 3.2-kb Xenopus segment appear to cluster around one region (Fig. 5). If one assumes that the replication proceeds unidirectionally or that the replication always proceeds symmetrically, our data might suggest that the replication could be initiated nearly randomly at one of many sites of the segment. Alternatively, if the replication proceeds either symmetrically or asymmetrically under the in *vivo* conditions, the origin might be fixed at a site of the segment. We favor the second alternative: the origin seems to be located at a site 1.3 kb from the right BamHI site of the Xenopus segment of pXY65.

When pBR322 alone or the mixture of pBR322 and pXY65 was used as the template in the enzyme system, the frequency of eye formation in pBR322 was low. On the other hand, when the pXY65 DNA was incubated with the *in vitro* mixture, eye structures were formed not only in the 3.2-kb Xenopus segment but also in the vector segment in which the replication appears to start at a site about 0.6 kb from the *in vitro* replication origin of pBR322. A similar result has been found when a chimeric plasmid containing the pMB9 DNA and yeast 2-μm plasmid DNA was incubated with a crude extract of yeast; eye structures were formed in either one of the two EcoRI segments of the 2-μm DNA or the pMB9 vector segment (31).

Lusky and Botchan (32) described the inhibition of simian virus 40 replication in simian cells by specific pBR322 DNA sequences. We do not yet know whether a pXY65 derivative lacking the specific sequences can replicate more efficiently in *Xenopus* eggs. Recently, Zakian (33) described a recombinant plasmid carrying the origin of the *X. laevis* mitochondrial DNA which can replicate in yeast cells. Our cleavage map of the 3.2-kb BamHI DNA segment in pXY65 shows a pattern different from that of the mitochondrial DNA (34) or ribosomal DNA (35, 36) and, therefore, the segment probably is not derived from the mitochondrial DNA or ribosomal DNA of *X. laevis*. Moreover, the result of the DNA-DNA hybridization suggests that pXY65 contains a repetitive sequence(s) from the *X. laevis* chromosomes. Our recent data suggest that a repetitive sequence is located near or at the origin in the Xenopus segment of pXY65 (unpublished data), but we do not know whether the repetitive sequence is essential for the initiation of replication.

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