

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 *Bam*HI 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 *Bam*HI 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, polyoma 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 supE hsdR*), a derivative of *Escherichia coli* K-12, was used as the host (18).

Reagents. The enzymes used and their sources were: *Bam*HI, *Hind*III, *Eco*RI, *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''-P¹,P⁴-tetrphosphate (Ap₄A) was purchased from P-L Biochemicals and [α -³²P]dCTP (2,000-3,000 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels), [*methy*-³H]dTTP (30 Ci/mmol), and [⁶⁻³H]thymidine (27 Ci/mmol) were obtained from Amersham International.

Cloning Procedures and Biohazard. DNA extracted from *X. laevis* sperm was digested with *Bam*HI and ligated to *Bam*HI-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 μ g/ml) for 15 hr. The cells were harvested, lysed in a buffer solution with lysozyme, EDTA, and NaDodSO₄. 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''-P¹,P⁴-tetrphosphate; 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 23°C for 4 hr in modified Barth's saline and then de-jellied 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 80°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 diphenylloxazole, 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 pre-treated 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 × 10⁷ cpm; 10⁷ cpm/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 de-jellied 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 CEC1).

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 dTTP, 250 μM CTP, GTP, 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 fractions, 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, rotatory-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 *Bam*HI, 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 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 *Bam*HI segment had no sites for *Xho* I, *Sst* I, *Eco*RI, *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 DNA.

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 chromosomal DNA. The DNA probe hybridized with *Xenopus* DNA fragments of various lengths produced by complete digestion with *Bam*HI 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 280 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

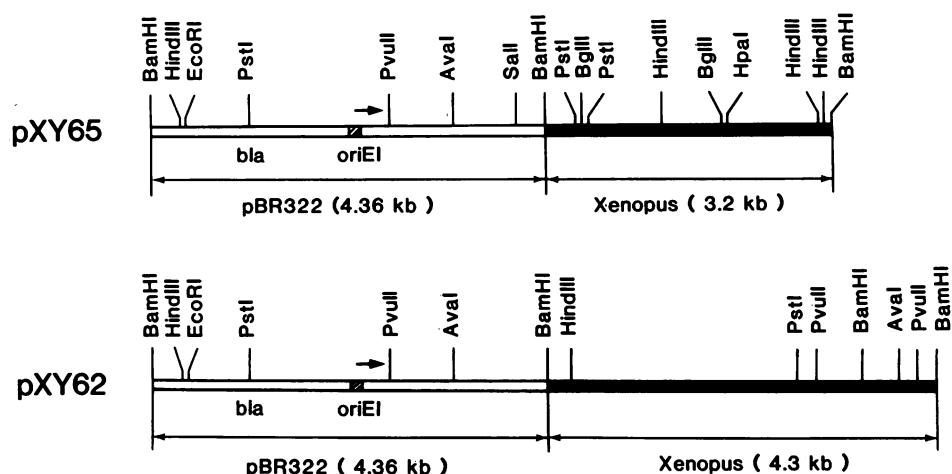


FIG. 1. Cleavage map of pXY65 and pXY62. *bla*, Gene for β -lactamase; *oriEI*, *in vivo* replication origin of the pBR322 vector; arrow, direction of replication *in vivo* from *oriEI* (26).

(10–100 $\mu\text{g/ml}$), 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 α -amanitin (0.2 $\mu\text{g/ml}$) was low; there was only a 30% reduction compared to the control. Ap_4A (0.1 $\mu\text{g/ml}$) (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 - ^{32}P]dTTP. The DNA was extracted with phenol and centrifuged in an alkaline sucrose gradient. The ^{32}P -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 ^{32}P -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 vitro* 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-

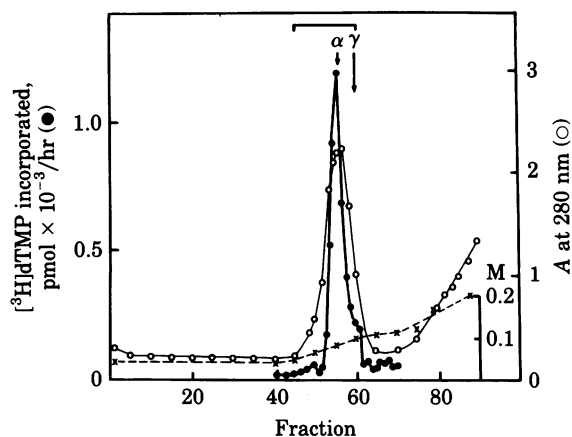


FIG. 2. DEAE-Cellulose column chromatography of supernatant prepared from unfertilized eggs. Fractions 46–61 were collected as indicated, concentrated 20-fold by dialysis, and used for DNA synthesis *in vitro*. ●, Activities of DNA polymerase using activated calf thymus DNA as the template; ○, A_{280} ; x, concentration of Tris buffer.

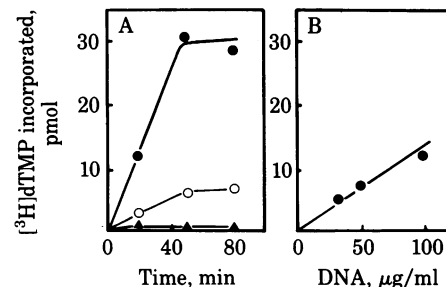


FIG. 3. Incorporation of [^3H]dTTP into acid-insoluble fraction in an enzyme system. (A) Time course of incorporation without DNA (Δ), with pXY65 DNA (\bullet), and with pBR322 DNA (\circ) as template. (B) Effect of varying amounts of pXY65 DNA on the amount of incorporation during 20-min incubation.

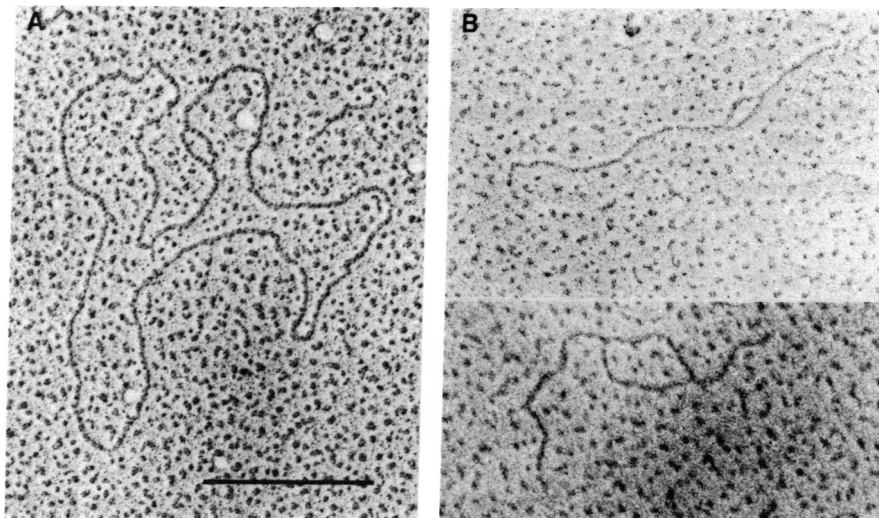


FIG. 4. Electron micrograph of eye structures in the pXY65 DNA formed *in vitro*. Supercoiled pXY65 DNA was incubated for 40 min in the enzyme system prepared from *X. laevis* eggs. (A) Eye structure in a dimeric molecule. (B) Eye structures in the 3.2-kb fragments observed after *Bam*HI digestion of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

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 *Bam*HI, extracted again with phenol, and observed in the electron microscope (Fig. 4B). *Bam*HI 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 non-

randomly 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 *Hind*III after incubation for DNA replication, the 1.7-kb *Hind*III 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 *Bam*HI 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 *Bam*HI vector fragment. Most of eye

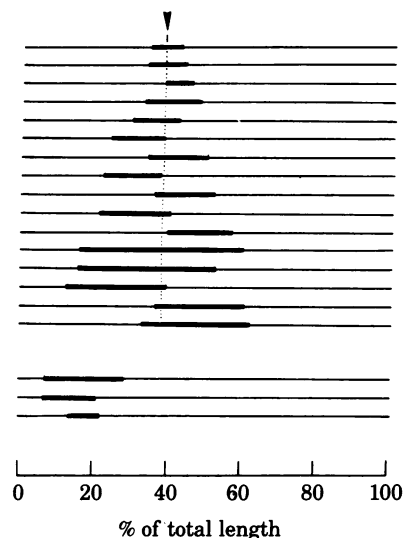


FIG. 5. Diagram of the location of the eye structure of the *Bam*HI 3.2-kb *Xenopus* DNA fragment of pXY65. pXY65 DNA was incubated for 40 min in the enzyme mixture, recovered by phenol extraction, and digested with *Bam*HI. Electron micrographs of the 3.2-kb *Bam*HI fragments containing a replication 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).

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

DNA	Reaction time, min	Number of molecules*			
		SC	OC or RC	RI	Total
pXY65	0	1,915	95	5 (0.2)	2,015
pXY65 [†]	0	—	1,444	3 (0.2)	1,447
pBR322	0	968	41	1 (0.1)	1,010
pXY62	0	956	48	1 (0.1)	1,005
pXY65	20	205	232	11 (2.5)	488
pBR322	20	784	848	0 (<0.1)	1,632
pXY62	20	478	135	0 (<0.1)	613
pXY65	40	241	579	19 (2.3)	839
pBR322	40	186	790	3 (0.3)	979
pXY62	40	371	1,205	5 (0.2)	1,581

* 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 μ g of ethidium bromide per ml to make relaxed circular forms.

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 *Bam*HI *Xenopus* segment that appears to be essential for efficient initiation of replication *in vitro*. 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 vitro* 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 *Bam*HI 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 vivo* replication origin of pBR322. A similar result has been found when a chimeric plasmid containing the pMB9 DNA and yeast 2- μ m plasmid DNA was incubated with a crude extract of yeast; eye structures were formed in either one of the two *Eco*RI segments of the 2- μ m 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 *Bam*HI 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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