Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5292–5296, September 1980
Cell Biology

Cloning of an origin of DNA replication of *Xenopus laevis*

(recombinant plasmids/origins of replication/*Eco*RI methylase)

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Contributed by J. Herbert Taylor, June 3, 1980

ABSTRACT DNA fragments of *Xenopus laevis*, the African frog, were cloned in the *Eco*RI site of the *Escherichia coli* plasmid pACYC189 and tested for ability to initiate and complete replication of the recombinant plasmid when injected into unfertilized eggs of *X. laevis*. After measurement of the [^{3}H]-thymidine incorporation per egg for a number of recombinant plasmids, pSW14 and pSW9, which respectively contain a small segment (550 base pairs) and several kilobases of frog DNA, were selected for more extensive analysis. In spite of the small size of the segment in pSW14, it incorporates in 2 hr at least 3 times as much labeled thymidine as either pSW9 or the vector alone. The DNA synthesis in pSW14 was shown to be replication rather than repair synthesis, based on a buoyant density shift of the product when iododeoxyuridine was used for labeling. To determine the number of replications of pSW14, a novel method was employed. Because pSW14 is a head-to-head dimer of the vector with the *Xenopus* fragment inserted at an *Eco*RI site, the plasmid has three methylatable sites—two bracketing the *Xenopus* fragment and one opposite the fragment. By co-transformation of *E. coli* with pSW14 and pBR322 containing the *Eco*RI methylase gene, supercoiled pSW14 was methylated and injected into eggs with [^{3}H]thymidine. Disappearance of modified *Eco*RI sites by semiconservative replication was followed by measuring the sensitivity to *Eco*RI endonuclease over time. The results showed that about 50% of the labeled, supercoiled DNA recovered from eggs after 4 hr was sensitive to EcoRI digestion, which indicates that most of the DNA that incorporated [^{3}H]thymidine had replicated twice during the 4 hr in the unfertilized eggs of *X. laevis*. We conclude that pSW14 has a functional origin in the *Xenopus* DNA segment.

Initiation of DNA synthesis is the controlling step in chromosome replication. Unlike the situation in the genome of viruses and bacteria, which usually have only one initiation site, chromosomes of higher cells have many sites (origins) per chromosome. Clusters of sites operate simultaneously in a chromosome. Yet, once a site has functioned in an S phase (DNA replication phase of the cell cycle), it must be altered in some way so that it is unavailable to the proteins involved in initiation. Furthermore, the regions of a chromosome are controlled in replication so that some replicate early in S phase whereas others replicate late or at some intermediate stage. The late-replicating DNA contains much of the DNA that is nonfunctional in transcription, such as certain repetitive sequences and, in mammals, the nonfunctional portion of the X chromosome in females. The nonfunctional X chromosome contains the same genes as its homolog but is suppressed in genic expression by some modification, which occurs in the early embryo and is inherited by all descendants of each cell in which the modification occurs. To understand the significance of these observations or to learn much more about the control of replication, it is essential to isolate and clone small segments of DNA containing functional origins.

The nucleotide sequences of segments including origins has been determined in bacteria (1–3). However, to date the origins of replication in nuclear DNA of higher eukaryotes have not been isolated and identified, and their sequences have not been determined. In Chinese hamster cells, functional origins are found at intervals of about 74 μm [222,000 base pairs (bp)] in fibroblasts in culture (4) in which the S phase is 6–7 hr. In some organisms, cells of the early embryo complete the S phase in a few minutes, and many more functional origins have been detected in *Drosophila*, for example (5). The time interval between cleavages in sea urchin embryos and some amphibian embryos is so short that origins would have to be close together and operating simultaneously to allow completion of replication in the time observed. Callan (6), by the use of DNA fiber autoradiography, has estimated that the interval in *Triturus vulgaris* (a salamander) might be as small as 10 μm (30,000 bp). However, a study by DNA fiber autoradiography of Chinese hamster (CHO) cells in culture indicated that potentially functional origins might be as close as 4 μm (12,000 bp) in the DNA that replicates early in S phase (7).

With these studies in mind we initiated studies on the cloning and characterization of the origins in *Xenopus laevis* (the African clawed frog), which has been widely used to study the expression (transcription and translation) of foreign genes in oocytes (8–12). It also has been shown that unfertilized *X. laevis* eggs have the ability to support DNA replication from injected exogenous DNA (13–15).

A number of derivatives of pACYC189 containing segments of *Xenopus* DNA have been injected into unfertilized eggs and, as predicted from the frequency of origins, several appear to initiate replication of the whole plasmid, which would not otherwise replicate to a significant extent in the frog egg. One in particular, pSW14, contains only about 550 bp of frog DNA but replicates at least two times after injection. One other derivative, pSW9, contains a much larger segment but does not replicate better than the vector without a recombinant segment. In this communication we present evidence of the semiconservative replication of pSW14 after injection and isolation several hours later in a supercoiled state.

MATERIALS AND METHODS

Biological Materials. *Xenopus laevis* were obtained from Mogul-Ed (Oshkosh, WI) and were maintained at 19–21°C in this laboratory. Eggs were obtained by injecting *X. laevis* females with 350 units of chorionic gonandotropin (choriogonadotropin; Parke, Davis); the animal started laying eggs approximately 11–12 hr later.

Escherichia coli SF8 ($r_k^-m_k^-$ lop-11, rec B$^-$, rec C$^-$) and plasmid pACYC189 were provided by S. N. Cohen through the Plasmid Reference Center (Stanford University). Plasmid pBR322 and *E. coli* RY13 were obtained from H. W. Boyer (University of California, San Francisco) and J. H. Stuy (Florida State University), respectively. Both plasmids were transferred

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Abbreviations: bp, base pairs; EtdBr, ethidium bromide.

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into SF8 by transformation before the isolation of plasmids for cloning experiments. *E. coli* SF8 was grown in L broth (Difco). To prepare plasmids, cleared lysates were made by the method of Guerry *et al.* (1973) and extracted over phenol twice. After ethanol precipitation, nucleic acids were treated with RNase A (50 μg/ml) for 2 hr at 37°C. Phenol extraction and ethanol precipitation were repeated and nucleic acids were dissolved in CsCl/ethidium bromide (EtdBr) centrifugation buffer (30 mM Tris/5 mM EDTA/50 mM NaCl, pH 7.8) and dialyzed against the same buffer. CsCl/EtdBr centrifugation was carried out by the method of Clewell (16). DNA was then dialyzed against the appropriate buffers.

X. laevis DNA was purified from young *X. laevis* liver by mincing and lysis in 1% sodium dodecyl sulfate/0.1 M EDTA/0.1 M $NaHCO_3$, pH 9.5. After several phenol extractions, ethanol precipitation, and RNase digestion, nucleic acids were extracted again over phenol and finally precipitated with ethanol. High molecular weight DNA was spooled out on a glass rod and dissolved in TEN buffer (10 mM Tris/1 mM EDTA/10 mM NaCl, pH 7.8).

Construction of Recombinant Plasmids. *X. laevis* and pACYC189 DNA were digested with *Eco*RI (Miles) and precipitated in ethanol. *X. laevis* DNA (2.1 μg) and pACYC189 DNA (1.4 μg) were dissolved in ligase mixture (17). The ligation was carried out at 0°C for 48 hr, and the DNA was used for transformation without further purification.

To clone the *Eco*RI methylase gene separately from the endonuclease gene, DNA from a cleared lysate of *E. coli* RY13 was mixed with pBR322, treated with *Hin*dIII (Bethesda Research Laboratories, Rockville, MD) and ligated with T4 ligase (6.25 units/ml). After 10-hr incubation at 0°C, the reaction mixture was diluted 10-fold in T4 ligase buffer with an additional 2.5 units of T4 ligase per ml and was left at 0°C for an additional 48 hr. This reaction mixture was used for transformation without further purification.

Transformation of $CaCl_2$-treated *E. coli* SF8 was optimized by modifying Cohen's method (18) to give approximately 250–300-fold higher efficiency for the transformation. An overnight *E. coli* SF8 culture was diluted 200-fold into 30 ml of L broth, and cells were harvested at an OD of 0.5–0.6 at 590 nm. Cells were washed once in cold 0.15 M NaCl/5 mM Hepes, pH 7.0, and were resuspended in 3 ml of cold 20 mM $CaCl_2$/4 mM $MgCl_2$. A cold pipette was used to mix 0.2 ml of cell suspension with 0.1 ml of DNA (with the $CaCl_2$ and $MgCl_2$ concentrations maintained at 20 mM and 4 mM, respectively), and the mixture was left in an ice water bath for 50–60 min. A 42°C heat pulse was applied for 30 sec and was terminated by plunging the mixture into an ice water bath. After cells were diluted 10-fold with L broth, they were grown for at least 1 hr before selection pressure was applied. Transformation and growth of transformed cells were carried out in a P2 containment facility in compliance with National Institutes of Health guidelines for recombinant DNA research.

For the cloning of *X. laevis* DNA, the D-cycloserine enrichment procedure for recombinant transformants was used (19). For the cloning of *Eco*RI methylase gene, DNA from the initial culture with transformants was lysed and digested with *Eco*RI, and *E. coli* was transformed again with it. Several colonies from the second transformation were grown to test the sensitivity of their DNA to *Eco*RI digestion.

Microinjection and Recovery of Plasmids from Eggs. Eggs obtained as described above were placed on wet filter paper and irradiated with UV light (20). Each egg received 50 nl of a solution containing DNA (300 μg/ml) and [^{3}H]thymidine (4 mCi/ml; 1 Ci = 3.7×10^{10} becquerels) in injection buffer (10 mM Hepes/0.5 mM EDTA/88 mM NaCl, pH 7.4) and was incubated in sterile incubation buffer (0.11 M NaCl/2 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.5, with 10 mg of benzyl penicillin and 10 mg of streptomycin sulfate per liter) at 19–21°C (10). All eggs were stored at −20°C until further analysis.

Eggs were thawed and lysed in 0.1 M Tris/0.05 M NaCl/0.01 M EDTA/0.5% sodium dodecyl sulfate with 50 μg of proteinase K (EM Laboratories, Elmsford, NY) or 500 μg of Pronase B (Calbiochem) per ml (15). The lysing solution was always predigested for 2 hr at 37°C. The volume of the lysing solution was kept at 0.3 ml per egg. For lysis, each egg in a small volume of lysing solution was mixed with a Vortex in a 1.5-ml Eppendorf tube and incubated at 37°C for 3 hr. In other cases, the eggs were lysed by using one gentle stroke in a Dounce homogenizer and were incubated at 37°C for 3 hr.

Agarose Gel Electrophoresis. Agarose gel was made in E buffer (40 mM Tris/5 mM Na acetate/1 mM EDTA, pH 7.7) and run in the same buffer. The radioactivity in agarose gel slices was determined in a 1:2 toluene/Triton X 100 mixture with 4 g of Omnifluor per liter after the gel was melted in a small volume of water.

RESULTS

Variations in Template Activity of Plasmids Injected into Unfertilized Eggs. *X. laevis* DNA was spliced into the *E. coli* plasmid ACYC189 at the *Eco*RI site. These recombinant plasmids were used to transform *E. coli*. A number of clones were selected which had plasmids about 2 times the size of the vector (pACYC189). Cultures were grown, the DNA was isolated, and the supercoiled (form I) rings were purified by banding in a CsCl solution containing EtdBr. The plasmids were injected into recently ovulated, unfertilized eggs of *X. laevis* and incubated at 20°C for 2 hr. Eggs were lysed individually in a small amount of buffer and extracted once with phenol (found necessary to reduce the high background radioactivity). The radioactivity of the acid-insoluble fraction, which was precipitated on a glass filter, was determined for each egg by liquid scintillation counting. After these preliminary tests, three recombinant plasmids were selected for more careful comparisons by using the same techniques as described above. As seen in Fig. 1, there was a wide range of radioactivity among the eggs, but if one ignores the points near the ordinate, which presumably represent injection failures, the differences among the plasmids are clearly visible. One recombinant plasmid, pSW9, the vector pACYC189, and random *Eco*RT segments of *Xenopus* DNA (shown at the lowest level in Fig. 1) stimulated about the same amount of incorporation, which was clearly less than the recombinant plasmids pSW14 and pSW18. Eggs injected with [^{3}H]thymidine without template DNA incorporated very little of the radioactivity (shown in the upper level of Fig. 1 and designated "saline").

The plasmid pSW14 was compared with the vector mentioned above for the ability to induce incorporation of [^{3}H]-thymidine. Eggs were lysed at intervals of 30 min up to and including 2 hr, the DNA was extracted from pooled batches of eggs, and the radioactivity was assayed. These measurements showed that incorporation in the eggs with vector alone had nearly leveled off after about 30 min, while eggs with the recombinant plasmid were still incorporating at an increasing rate (data not shown). An experiment was then designed to follow incorporation into pSW14 over a period of 5 hr (Fig. 2). The curve produced suggests an exponential increase and gave us the idea that the recombinant plasmid pSW14 might be replicating more than once.

The Supercoiled State of the Product of DNA Synthesis. After injection of about 200 frog eggs with the recombinant plasmid pSW14, the lysate was pooled and the DNA was extracted and further purified by separation on the basis of

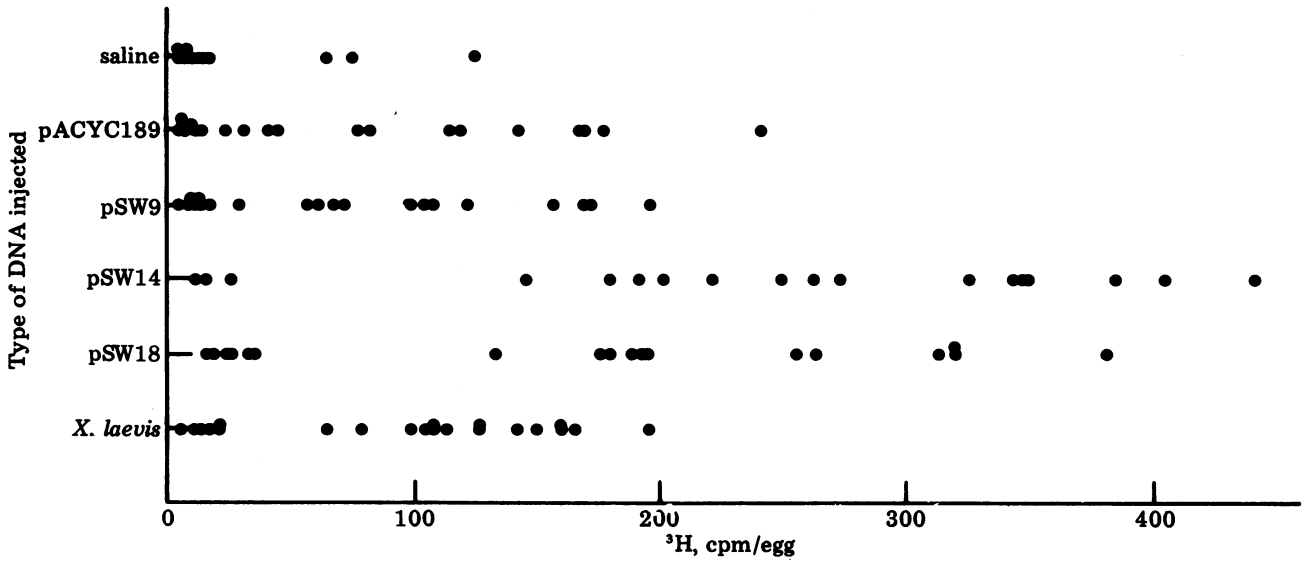

FIG. 1. Microinjection of various substrates. Unfertilized eggs were injected with about 50 nl of solution containing [³H]thymidine (4 mCi/ml) and saline (injection buffer) with or without various DNAs (300 μg/ml). After a 2-hr incubation, each egg was lysed separately.

buoyant density in a CsCl/EtdBr solution spun in the preparative centrifuge. The radioactivity was localized by counting an aliquant of each fraction removed from the bottom of the centrifuge tubes. Two bands were located (Fig. 3); the higher-density band is supercoiled (form I) DNA and the lower-density one contains both relaxed circles (form II) and linear segments (form III). A further analysis (Fig. 4) of the bands containing the pSW14 form II or III DNA by separation in a 1% agarose gel by electrophoresis showed that all detectable radioactivity was incorporated in form II rather than in linear form III DNA. This observation excludes any involvement of endogenous DNA in the [³H]thymidine incorporation, because it would be either linear, chromosomal DNA or circular, mitochondrial DNA of a larger size than the plasmid. Most of the band of higher buoyant density moved in the gel as expected for fully supercoiled DNA; a small amount was apparently relaxed by the manipulations after removal from the gradient.

Because DNA was injected in the supercoiled state and would have to be relaxed during replication, some agent would have to restore the supercoiled state. Perhaps histones complex with the DNA before replication or a gyrase activity is present in the egg; the former is most probable from what is known of the synthetic capacity of frog eggs.

Is the DNA Synthesis in the Egg Repair Replication or Semiconservative Replication? Since eggs contain large endogenous pools of nucleotides, labeling the DNA with a density label sufficient to see a density shift after replication is a bit of a problem. Bromodeoxyuridine was not effective, but iododeoxyuridine injected with the [³H]thymidine proved sufficient to get a detectable buoyant density shift. In Fig. 5 is shown the distribution of the labeled DNA isolated from eggs injected with a mixture of [³H]thymidine and iododeoxyuridine. The density shift of all of the incorporated radioactivity in supercoiled, relaxed, and linear DNAs indicates that repair

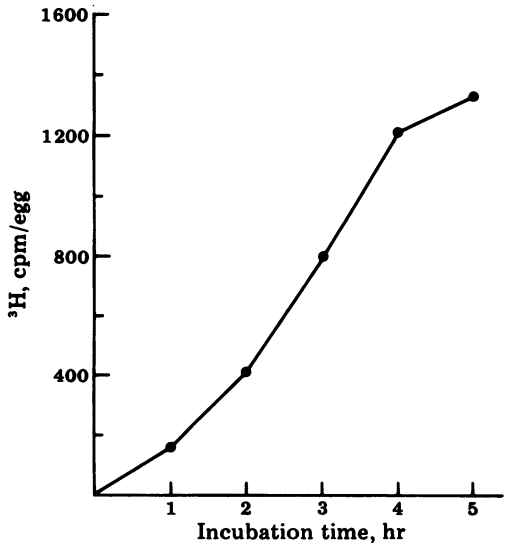

FIG. 2. Time course of DNA synthesis in unfertilized eggs injected with pSW14. Unfertilized eggs were injected with [³H]thymidine (4 mCi/ml) and form I pSW14 (300 μg/ml). After various incubation periods, eggs were lysed separately. Each point is the average of 20–25 eggs.

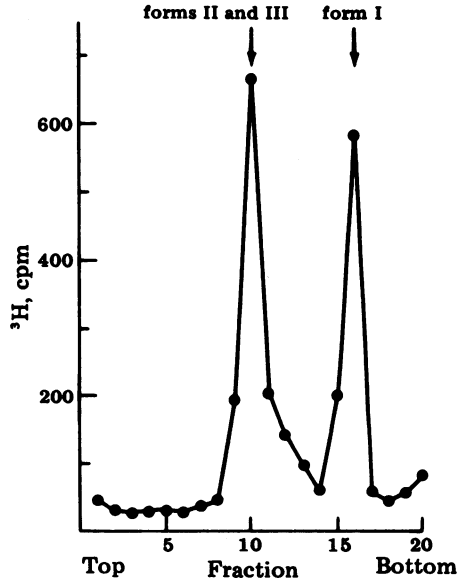

FIG. 3. CsCl/EtdBr centrifugation of pSW14 incubated in unfertilized eggs. Form I pSW14 (150 μg/ml) and [³H]thymidine (4 mCi/ml) were injected into unfertilized eggs. After a 4-hr incubation, the DNA was subjected to CsCl/EtdBr centrifugation. The bottom of the centrifuge tube was punctured and 15 drops were collected for a determination of radioactivity and further experimentation (see Fig. 4).

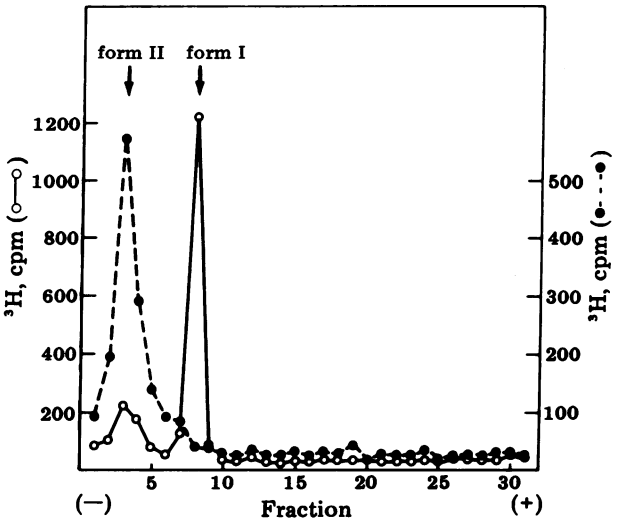

FIG. 4. 1% agarose gel electrophoresis of pSW14 DNA injected into unfertilized eggs. The DNA was obtained from the CsCl/EtdBr centrifugation (Fig. 3). 1% agarose gel electrophoresis was run at 3mA/tube for 5 hr with a marker pSW14. After electrophoresis, the gel was stained in EtdBr (0.5 μg/ml) and the locations of the markers were recorded. The gel was sliced into 5 mm thicknesses and the radioactivity was determined. ○, DNA from the form I peak in Fig. 3; ●, DNA from the forms II and III peak in Fig. 3.

replication probably does not account for the incorporation. The observation (see below) that pBR322 containing the *Eco*RI·M (methylase) gene, injected along with pSW14, failed to incorporate a measurable amount of [^{3}H]thymidine into the supercoiled form makes the case for semiconservative replication even more likely. However, the next experiment with methylated DNA was necessary to complete the evidence and measure the number of rounds of replication.

Determination of the Number of Replications of pSW14 in Unfertilized Eggs. The plasmid SW14 has a molecular size of about 6.8×10^6 daltons as measured by electrophoresis. The vector pACYC189 has a molecular weight of 3.4×10^6, and in the early experiments we had assumed that the recombinant plasmid contained a segment of frog DNA of about the same size as the vector. However, careful analysis of a complete digest showed only two bands, one identical to the vector and a small fragment of frog DNA of about 550 bp. We propose that pSW14 consists of a head-to-head dimer of the vector with the fragment of *Xenopus* DNA inserted at one of the *Eco*RI sites; therefore, it has three *Eco*RI sites—two bracketing the *Xenopus* fragment and one opposite the fragment. After this fact was learned, we prepared a dimer of the vector and used it as a control rather than the monomer. It had no significantly increased template activity in the *Xenopus* eggs.

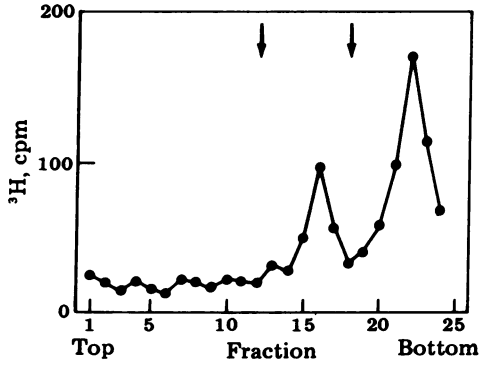

FIG. 5. CsCl/EtdBr centrifugation of normal density and iododeoxyuridine-density-labeled pSW14. Unfertilized eggs were injected with [^{3}H]thymidine (3 mCi/ml), form I pSW14 (150 μg/ml), and 50% saturated iododeoxyuridine. After a 4-hr incubation, CsCl/EtdBr centrifugation was performed. Arrows at fractions 18 and 12 indicate the form I peak and the forms II and III peak, respectively, of unsubstituted pSW14.

pACYC189, pSW14, and pSW9 (which does not function as a template for replication in the *Xenopus* egg) were hybridized with *Xenopus* DNA bound to cellulose nitrate filters to confirm that pSW14 and pSW9 contained *Xenopus* DNA; pACYC189 would serve as a background, nonspecific binding control. Although both pSW14 and pSW9 bound significantly more than pACYC189, pSW14 was bound about 3 times as much as pSW9.

A novel approach was taken to measure the number of replications of the recombinant plasmids. Because the recombinant plasmid SW14 is a dimer of the original vector cut with *Eco*RI and has an *Eco*RI fragment of frog DNA in one of its *Eco*RI sites, it should have a total of three *Eco*RI sites, two bracketing the frog DNA fragment and one opposite the fragment. The *Eco*RI M (methylase) gene was cloned into *E. coli* in the plasmid BR322 without the linked endonuclease locus functional. Plasmid SW14 was used with the pBR322 (*Eco*RI M) to cotransform a modification negative mutant of *E. coli*. Most of the plasmids produced by the transformed cells were methylated as indicated by their resistance to *Eco*RI endonuclease. The methylated plasmids were purified in CsCl/EtdBr after *Eco*RI digestion. After being shaken with butanol to remove the dye, the methylated plasmids were injected into frog eggs. The DNA from a pooled batch of eggs was isolated, then purified in a CsCl/EtdBr gradient; the dye was removed by shaking over butanol. The supercoiled DNA (form I) was digested with *Eco*RI and electrophoresed on a 1% agarose gel. Forms I and II pSW14, from III pACYC189, and form I pBR322 (*Eco*RI M) were added as markers. Their positions in the gel are shown in Fig. 6 along with the radioactivity in slices of the gel.

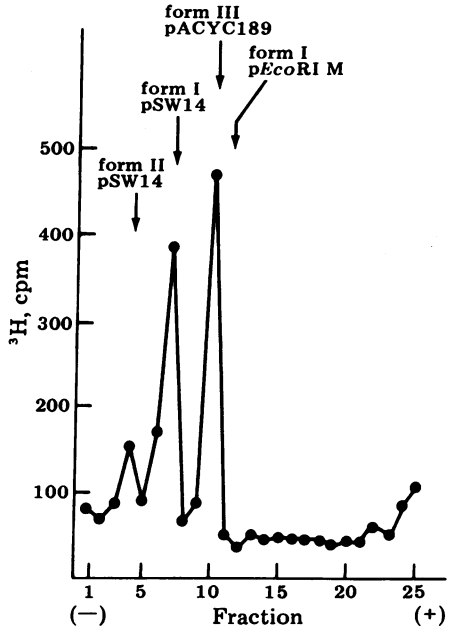

FIG. 6. *Eco*RI digestion of methylated pSW14 after incubation in unfertilized eggs. Form I pBR322 (*Eco*RI M), methylated form I pSW14 (150 μg/ml), and [^{3}H]thymidine (7 mCi/ml) were injected into unfertilized eggs. The form I DNA was purified by using CsCl/EtdBr centrifugation after a 4-hr incubation. DNA was digested with *Eco*RI and electrophoresed in 1% agarose gel (3mA/tube, 5 hr). The forms I and II pSW14, form III pACYC189, and form I pBR322(*Eco*RIM) were added as markers. The positions of markers were identified by staining the gel in EtdBr (0.5 μg/ml) after electrophoresis. The gel was sliced into 6-mm thicknesses and the radioactivity was determined.

At the position of pBR322 (*Eco*RI M) there was no radioactivity, which indicates that the *E. coli* plasmids do not replicate in the frog egg. Nearly equal amounts of radioactivity were at the positions of linear (form III) pACYC189 and supercoiled (form I) pSW14. This indicates that about one half of the supercoiled DNA had lost its methyl groups, which happens if it replicated semiconservatively through two rounds under conditions that would not maintain methylation. A small amount of radioactivity was associated with the form II pSW14, which would be produced by nicking of the supercoiled DNA in handling after removal from CsCl or by contamination with the form II band in fractionation.

The conclusion drawn from these data is that pSW14 replicates at least two times in 4 hr and, therefore, contains an active origin that functions well in the unfertilized *Xenopus* eggs. The plasmid vector without frog DNA and the recombinant plasmid pSW9, which has a large fragment of frog DNA, probably do not replicate at all in spite of the fact that some incorporation of [^{3}H]thymidine was measured in eggs receiving only these plasmids. Some synthesis cannot be ruled out, but the failure of the supercoiled pBR322 (*Eco*RI M) to incorporate detectable radioactivity means that only the nicked circles could have been labeled; careful experiments with more eggs would be necessary to determine the extent of such synthesis, if any.

DISCUSSION

Both pSW14 and pSW18 contain a functional origin for DNA replication that operates effectively in *X. laevis* eggs. The size of the frog DNA fragment in pSW14 is only 550 bp whereas that in pSW18 is several kilobases; yet, both replicate equally well. Another recombinant plasmid, pSW9, contains several kilobases of frog DNA but does not stimulate incorporation of [^{3}H]thymidine better than the vector pACYC189 does. Only pSW14 has been studied enough to know that replication is complete and proceeds through at least two rounds before the unfertilized eggs fail to support a high level of synthesis. Because the plasmids injected were all supercoiled and the product of pSW14 was recovered in the supercoiled state, the DNA synthesis measured has no gap filling, nick translation, or strand displacement with synthesis. It appears to be a normal initiation with complete replication of the circular plasmid, separation of the products of replication, and restoration of the supercoiled state.

The initiation of DNA replication is probably controlled by a nucleotide sequence present in the *Xenopus* fragments of pSW14 and pSW18 but missing in pSW9. Furthermore, since supercoiled pBR322 with incorporated [^{3}H]thymidine was not recovered from the frog eggs in which it was injected along with pSW14, it is unlikely that complete replication of any plasmid without a eukaryotic origin can be induced.

Unfertilized *X. laevis* eggs contain enormous amounts of mitochondrial DNA (3.8 ng; 21). The stored mitochondrial DNA, however, does not replicate until tadpole stages (21). Neither chromosomal DNA nor mitochondrial DNA were activated during the course of these experiments because (*i*) significantly lower DNA synthesis occurred when saline alone was injected (Fig. 1), (*ii*) all radioactivity from the form I peak of a CsCl/EtdBr centrifugation moved with form I or II pSW14 in agarose gel electrophoresis (Fig. 5), (*iii*) all radioactivity from the forms II and III peak of CsCl/EtdBr centrifugation moved with the form II pSW14 in agarose gel electrophoresis (Fig. 5), and (*iv*) no peaks appeared in the gradient profile when saline was injected (data not shown). These observations strongly indicate that there was no involvement of endogenous DNA in the injected eggs.

Although the density shift technique used initially by Meselson and Stahl (22) to demonstrate semiconservative replication has since then been the primary method used for this purpose and for determination of the number of rounds of replication, an alternate procedure introduced here may prove useful in some instances. Since methylated adenine is rare or absent in the DNA of most higher eukaryotes, we assumed that the *Eco*RI methylation pattern would not be maintained in the frog egg. Its dilution by semiconservative replication could then serve as a test to distinguish between that process and repair replication if enough sites can be labeled. The product is easily tested by digestion with the restriction endonuclease and separation of the products by gel electrophoresis. It worked remarkably well in the frog eggs as reported here. Incorporation of density and radioactive labels in plasmids before injection and the monitoring of their distributions in a buoyant density gradient after recovery might be preferred but would be more expensive in time and materials. During the course of these experiments, two papers appeared on an autonomous replication unit in eukaryotes. Stinchcomb *et al.* (23) found a plasmid that transforms yeast cells at a high frequency. Huttner *et al.* (24) found a plasmid that replicates autonomously in mouse cells.

We gratefully acknowledge helpful suggestions of microinjection from Drs. J. C. Gerhart and J. E. Mertz. This research was supported by a grant from the U.S. Department of Energy (EY-76-S-05-2690) and by a grant from the National Institutes of Health (1-R01 AGO1807-01).

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