Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5572–5576, September 1982
Cell Biology

Dissection of a replication origin of *Xenopus* DNA

(recombinant plasmids/origin of replication/initiation of transcription)

JASEMINE CHOY CHAMBERS*, SHINICHI WATANABE†, AND J. HERBERT TAYLOR

Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

Contributed by J. Herbert Taylor, June 14, 1982

ABSTRACT **A previously cloned 503-base pair (bp) *Eco*RI segment of genomic DNA from *Xenopus laevis* selected for enhancement of replication of its vector plasmid was moved to the *Eco*RI site of pBR322. This plasmid designated pJCC31 and five other clones, which were made by cleaving the 503-bp segment in relation to a dispersed repeated sequence and subcloning, were compared with pBR322 for replication by microinjection into *Xenopus* eggs. The replication measured by incorporation of a ^{32}P-labeled nucleotide as well as semiconservative segregation and dilution of N^6-methyladenine at the *Eco*RI sites showed pJCC31 to be about 15 times as efficient as pBR322. The next most efficient subclone, pJCC31-2, contains an insert with a complete 320-bp dispersed repeated sequence bracketed by an 8-bp direct repeat. This observation, along with our previous report that repeated sequences of the *Alu* family in the human genome enhanced replication of the vector plasmid nearly as much as that of the presumptive *Xenopus* origin, leads to the hypothesis that members of a subset of the short dispersed repeated sequences in vertebrates function as origins for chromosomal replication. Preliminary studies also show that the presumptive *Xenopus* origin contains a RNA polymerase promoter that increases the transcription of the plasmid when it is microinjected into *Xenopus* oocytes.**

In previous communications (1, 2) we reported the screening of cloned segments of vertebrate chromosomal DNA for origins by injection of plasmids containing the segments into unfertilized eggs of the frog, *Xenopus laevis*. One plasmid (pSW14) with 503 base pairs (bp) of *Xenopus* DNA was found which replicated 10–20 times more efficiently than the vector. The *Xenopus* sequence was assumed to contain an origin of replication. There are now two confirming reports that support the concept that there is a significant quantitative difference between the functioning of bacterial and homologous origins in *Xenopus* eggs. Hiraga *et al.* (3) screened a number of segments cloned into pBR322 and selected two, pXY65 and pXY62. The first, pXY65, contained 3.2 kilobases (kb) of *Xenopus* DNA and incorporated 2- to 4-fold more [^{3}H]thymidine than did the vector alone or the other plasmid, pXY62 with 4.3 kb of *Xenopus* DNA, which was selected as a negative control. In an electron microscopic study of the "eyes" produced by partial replication in a *Xenopus* egg extract, the origin was shown to be localized in a relatively small region of the *Xenopus* segment of pXY65. Hines and Benbow (4) injected into *Xenopus* eggs a plasmid, pXlr11, containing a segment of *Xenopus* ribosomal DNA and compared it with the vector plasmid ColE1 for efficiency of replication as well as the position of eyes by electron microscopy; pXlr11 was 5-fold more efficient than ColE1, but both formed eyes at specific loci.

All of the above-mentioned studies indicate that specific sequences are involved in the beginning of replication, in contrast to a study by Harland and Laskey (5) which they interpreted to indicate that origins representing specific sequences might not be utilized in chromosomal DNA. Their conclusion was based on the fact the DNAs from bacterial viruses, the plasmid ColE1, and recircularized fragments of simian virus 40 all replicate in the *Xenopus* egg to a limited extent. The replication of bacterial plasmids certainly occurs in *Xenopus* eggs at a low efficiency but is, perhaps, initiated at or near the plasmid origin as the electron microscopic studies indicate (4). Until more is known about the mechanism of priming DNA replication in this system, the implications of such events cannot be understood.

In an attempt to further characterize vertebrate chromosomal origins, we have moved the 503-bp segment in pSW14 containing the presumptive *Xenopus* origin from the original plasmid used for its isolation into the better-characterized pBR322; the new combination is designated pJCC31. The *Xenopus* segment was originally selected solely on the basis of its efficiency in stimulating the replication of its vector plasmid when injected into *Xenopus* eggs. However, sequence determination and further analysis have revealed a 320-bp sequence bracketed by an 8-bp direct repeat. The fragment also contains sequences similar enough to hybridize with an abundant family of repeated sequences distributed throughout the *Xenopus* genome which is perhaps analogous to the human *Alu* family of repeats (6). Four subclones containing various parts of the 320-bp segment and one clone with a piece of the 503-bp segment outside the 320-bp sequence have been compared with the parental plasmid pJCC31 and the vector, pBR322, for the ability to replicate in *Xenopus* eggs. Preliminary studies of the transcription of the segment in *Xenopus* oocytes are also included.

MATERIALS AND METHODS

Biological Materials. *X. laevis* (Carolina Biological Supply) were maintained at 19–21°C in the laboratory. *Escherichia coli* SF8 (r_k^-, m_k^-, lop-11, rec B$^-$, rec C$^-$) and pACYC189 were obtained from S. N. Cohen (Plasmid Reference Center, Stanford University) and pBR322 was from H. W. Boyer (University of California, San Francisco). The *Xenopus* fragment being analyzed as a possible origin of replication is a 503-bp segment produced by *Eco*RI digestion of the genomic DNA isolated from liver of *X. laevis*. It was one of a number of *Xenopus Eco*RI fragments ligated into the *Eco*RI site of pACYC189 which interrupts the chloramphenicol-resistance gene. The plasmids produced were tested for efficiency of replication by microinjection into unfertilized eggs of *Xenopus*. The one designated pSW14 (1) is a head-to-head dimer of pACYC189 produced when two linearized plasmids joined head-to-head with the 503-bp *Xenopus* fragment inserted into one *Eco*RI junction. Both copies of the chloramphenicol-resistance gene are inactive and when the plasmid is digested with *Eco*RI the products are the linear

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: bp, base pair(s), kb, kilobase(s).

* Present address: Dept. of Microbiology and Immunology, Duke Univ. Medical Center, Durham, NC 27710.
† Present address: McArdle Laboratory for Cancer Research, Univ. of Wisconsin, Madison, WI 53706.

pACYC189 and the 503-bp *Xenopus* fragment. To have uniform vectors for comparison in the *Xenopus* eggs, the 503-bp fragment was moved to pBR322, the sequence of which is known (7). Subclones were prepared with various parts of the sequence inserted into pBR322. Methods for construction and selection of these clones have been described (1, 2).

Isolation of Plasmids, Purification, and Microinjection into *Xenopus* Eggs. Plasmids were isolated from cleared lysates prepared as described (1) and the supercoiled forms were separated by centrifugation to equilibrium in CsCl/ethidium bromide. The concentration was determined by optical density after extraction of the dye and extensive dialysis. However, separation of the ultraviolet absorbing materials by gel filtration (Bio-Gel A-15m; Bio-Rad) showed that only about 10% of the material was plasmid DNA; the remainder was small fragments of nucleic acids, presumably ribosomal DNA resistant to RNase. An aliquot of each plasmid was separated by gel filtration and the concentration was adjusted according to the ratio of the two components.

Microinjection and recovery of plasmids from eggs along with methods for separation of the DNA by gel electrophoresis after appropriate restriction enzyme digestion have been described briefly (1). Unfertilized eggs that had been recently ovulated were injected with 50 nl of solution containing 1.5 ng of plasmid DNA. Tests showed that this amount of a plasmid with the *Xenopus* origin saturates the DNA replication system. The increase in incorporation is linear with amount of plasmid injected up to 1.5 ng per egg, but the incorporation does not increase with higher amounts. Our previous reports (1, 2) did not correct for the extra ultraviolet absorbing material and were therefore probably incorrect by a factor of 10.

Microinjection of Plasmids into *Xenopus* Oocytes, Extraction of the RNA, and Analysis of the Transcription. Clusters of oocytes were obtained by surgery on frogs anesthetized by immersion in ice water. The oocytes were separated and oriented with the animal pole up on a nylon grid (0.8-mm mesh) in small plastic Petri dishes where they remained for 12–24 hr. The oocytes were centrifuged at 1,000 × *g* for 10 min at 20°C to bring the nucleus to the top just before injection. Each oocyte then was microinjected with 30 nl of buffer containing plasmid DNA (25 μg/ml) into the nucleus. After 24–36 hr of incubation at 19–21°C, the oocytes were lysed and the RNA was extracted by using the procedure of Probst *et al.* (8).

RNA Transfer and Hybridization. The RNA was separated on an agarose gel, transferred to nitrocellulose membrane, and hybridized to a ^{32}P-labeled probe, essentially as described by Thomas (9). The blot was hybridized for 36 hr at 42°C and then was washed and exposed to x-ray film at −70°C for 4–8 days with an intensifying screen. The probe was either the end-labeled *Xenopus* fragment or *Taq* I fragments of the vector pBR322.

RESULTS

Subcloning Segments of the Presumptive Origin from *Xenopus*. The 503-bp *Xenopus* fragment was moved from pSW14 into the *Eco*RI site of pBR322 by ligating the fragment to an *Eco*RI digest of pBR322 treated with bacterial alkaline phosphatase. *E. coli* cells were transformed and selected for resistance to both ampicillin and tetracycline. Resistant clones were examined for a plasmid that was about 500 bp larger than pBR322. The 31st clone examined proved to be one that was shown to contain the fragment. It was designated pJCC31.

Based on the assumption that the cloned segment may represent a significant region for initiating replication in *Xenopus* chromosomes, we prepared several subclones containing parts of the segment (Fig. 1*A*). Fragments a, b, and c produced by digesting the segment with *Sau*3A had previously been cloned and preliminary studies were reported (2) which indicated that none of the subclones was as efficient as the original pSW14. After subcloning these three fragments, we noticed that the 503-bp segment contained a 320-bp region bracketed by a short direct repeat of 8 bp. In Fig. 1*B* the repeat is shown in capital letters. Because none of the three subclones of *Sau*3A pieces contained the whole 320-bp piece, we decided to prepare two other clones, one with fragments a plus b and another with fragments b plus c. The first of these would contain the whole 320-bp segment which we supposed to be a dispersed repeat analogous to the *Alu* family of repeats in the human genome (11).

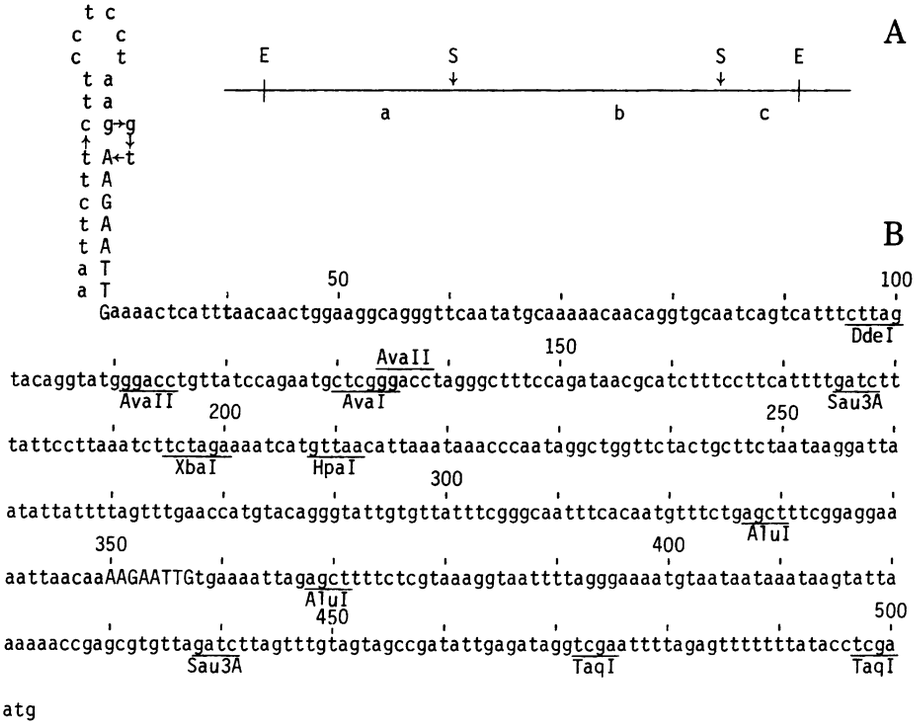

FIG. 1. Restriction map and sequence of the *Xenopus* segment that is being tested for its function as an origin for replication. (*A*) The segment is included in the region between two *Eco*RI (E) sites. It contains two *Sau*3A sites used for subcloning. (*B*) The sequence given was determined by Watanabe (10) and is arranged here to show an imperfect palindrome, the 320-bp segment bracketed by an 8-bp direct repeat (capital letters) and a number of restriction sites.

We had noted that the 503-bp *Xenopus* fragment hybridized with many *Eco*RI fragments of the total *Xenopus* genome when these were separated on an agarose gel and transferred to a nitrocellulose membrane by blotting. A comparison by spot hybridization between pJCC31, pSW14, and random *Eco*RI segments of *Xenopus* genomic DNA also indicated that several hundred thousand copies of a sequence similar to the probe were present (data not shown).

From a partial *Sau*3A digest of the *Xenopus* segment, two fragments were isolated from an 8% polyacrylamide gel. One contained 329 bp (fragments b plus c) and the other 437 bp (fragments a plus b). These were ligated into the large fragment of pBR322 produced by digestion with *Eco*RI and *Bam*HI. These ligated plasmids were used to transform *E. coli* SF8, and the clones resistant to ampicillin and sensitive to tetracycline were screened for plasmids that could be linearized by *Xba* I. Both *Xenopus* fragments contain this restriction site, whereas pBR322 lacks the site. After this screening additional restriction digests were separated on gels to identify each *Xenopus* fragment. Seven plasmids were now available for comparison of replication efficiency in *Xenopus* eggs (Table 1). Inspection of the sequence suggested that if transcribed by polymerase III, as many of the *Alu* and *Alu*-equivalent sequences are, the orientation of the transcript should be identical to the strand shown in Fig. 1*B* (compare with transcripts from mammalian sequences given in ref. 12). Clusters of adenines are more prominent at the 3′ end near and including the second copy of the short repeat than in the complementary chain.

Assay of the Subclones for Efficiency in Replication. We decided to use two indicators of replication in the assay. One was incorporation of [α-^{32}P]dATP into plasmid DNA. The other was an indicator used previously (1)—i.e., semiconservative segregation of N^6-methyladenine at the *Eco*RI sites. The simplest assay is the relative amount of incorporated ^{32}P shown in column 1 of Table 2. The plasmids are arranged in the order of highest to lowest efficiency. Relative radioactivity is given in decimal fractions with the highest incorporation per eight eggs shown for pJCC31 (2,657 cpm) set equal to 1.0. The injection of eggs was carried out in two separate experiments about 3 wk apart. Aliquots of the same DNA solutions were used and eggs obtained from the same frog were injected each time. The relative efficiency of replication was similar in both experiments (data for the second experiment are shown in Table 2). The data in Table 2 were obtained by lysing 30–50 eggs and by using fractions of the isolated DNA for determinations of radioactivity after separation of labeled plasmids on agarose gels. Unlabeled plasmid of the same type was added to each aliquot from a batch of eggs before digestion with *Pst* I to linearize and release the plasmids that would otherwise be trapped at the top of the gel (see Fig. 2). The added plasmid was sufficient to stain in the gel, to determine if digestion was complete, and to locate the labeled bands for cutting out and counting the radioactivity. After counting the radioactivity in slices of all the agar in some lanes to show that essentially all radioactivity was in the band of linear plasmids, only these bands were removed and the radioactivity counted for comparisons of the different plasmids shown in Table 2. Duplicate samples equivalent to labeled DNA in two eggs each were also assayed directly from the phenol-extracted solution of DNA. Those data (not shown) were very similar to the data in column 1 of Table 2.

Table 1. Comparison of vector and derived plasmids

Plasmid	Fragment(s) inserted	Length of insert, bp
pBR322	—	0
pJCC31	a plus b plus c ("Xori")	503
pJCC31-2	a plus b	437
pJCC31-1	b plus c	329
pSW142	a	174
pSW143	b	263
pSW141	c	66

To describe the results of segregation of the methylated adenine is a bit more complicated. The assumption was made that if the *Eco*RI sites are methylated this base would not be eliminated except by semiconservative dilution during replication. Vertebrates are not known to have either N^6-methyladenine in their DNA or the enzyme for its maintenance. It is not lost readily if at all by repair in eggs (1); therefore, its distribution may be useful to reveal rounds of replication. If both chains are methylated with *Eco*RI methylase, two rounds of replication are required to make one-half of the DNA chains free of methyladenine and susceptible to cutting by *Eco*RI endonuclease.

Each of the plasmids was methylated *in vitro* with *Eco*RI methylase. To test for completeness of methylation, a sample of the DNA was digested with *Eco*RI endonuclease and run on a gel in a lane next to the digested, unmethylated control. Because the DNA to be methylated was free of linear plasmids, any significant cleavage could be detected. The methylation reaction was continued until all plasmids were resistant to *Eco*RI endonuclease.

To estimate the rounds of replication we assumed that the DNA is symmetrically methylated on both chains. If that is correct, the percentage of plasmid (z) that has replicated two times at any interval during incubation is calculated by the following equation:

$$z = \frac{y/2}{x} \cdot 100,$$

in which x = radioactivity in the uncleaved plasmids and y = radioactivity in the fragments produced by cleavage. To obtain the data for calculating z, a sample of each replicating plasmid was run before and after digestion with either *Pst* I to linearize the circles or *Pst* I and *Eco*RI to reveal the plasmids that had lost the methyladenine at the *Eco*RI sites. Fig. 2 illustrates an example of one plasmid, pJCC31-2. Lane A shows the undigested plasmid that has in this case been converted to open circles by ^{32}P decay; lane B shows the *Pst* I digest with circles converted to linear form; lane C shows the ratio of DNA protected from *Eco*RI (upper band of whole linear plasmid) and below the two fragments produced by those plasmids that are digested by *Eco*RI. The bands were cut out and the radioactivity was counted to give the values for x and y. Radioactivity (counts) in the upper band (lane C) is the value for x and the sum of the

Table 2. Comparison of the efficiency of replication of pBR322 and derivatives with *Xenopus* inserts

Plasmid	Radioactivity incorporated: Total, $x + y$	Radioactivity incorporated: y/x	Plasmids replicated two times, %
pJCC31	1.00*	0.47/0.53	44
pJCC31-2	0.71	0.34/0.37	46
pJCC31-1	0.43	0.16/0.27	30
pSW142	0.42	0.19/0.23	41
pSW141	0.22	0.09/0.13	35
pSW143	0.19	0.06/0.13	23
pBR322	0.06	0.02/0.04	25

See text for discussion of x and y.
* The amount of [α-^{32}P]dATP incorporated was 2,657 cpm per eight eggs.

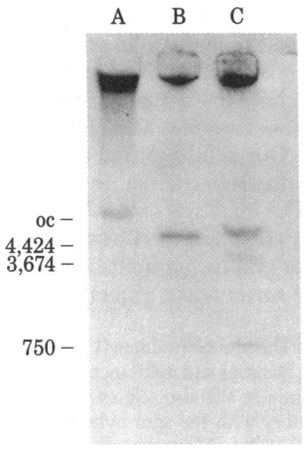

FIG. 2. Autoradiogram illustrating how the data in column 2 of Table 2 were obtained. An aliquot of DNA isolated from the equivalent of eight eggs microinjected with pJCC31-2 was placed in the well for each lane before or after digestion by restriction endonucleases; numbers on the left indicate bp. Lanes: A, without digestion but decay of ^{32}P has relaxed all plasmids (oc, open circles); B, digested with *Pst* I to linearize the plasmid; C, digested with *Pst* I and *Eco*RI (in the absence of methylation two fragments are produced with 3,674 and 750 bp).

counts in the two lower bands is the value for y. Counts in other parts of the lane were usually quite low and were not considered in the calculations. Most of the large material (trapped at the top of lane A) was converted to linear form by digestion with *Pst* I and some of it represents multimers of the plasmid.

The percentage of molecules that have been through two or more rounds of replication is only ≈50% of the molecules that replicated once for the efficiently replicated plasmids and only 25% for the plasmids with the lowest efficiency of replication. For pBR322 with an efficiency for replication of only 6% of pJCC31, the indication is that once a molecule has replicated, the chance that it will undergo a second round of replication is relatively high compared to that for a previously unreplicated molecule.

Comparison of Replication Efficiency by Injection of Two Plasmids in the Same Eggs. To better compare plasmids with different potentials for replication, two plasmids were injected into the same eggs and the incorporation of [α-^{32}P]ATP was compared in the same cellular environment. For this test only pJCC31-2 and pJCC31-1 were compared with pBR322. The plasmids could be separated on a gel because pBR322 has one site for each endonuclease, *Pst* I and *Bam*HI, but pJCC31-2 and pJCC31-1 lack the *Bam*HI site. The cut plasmids were separated on a gel and the ratio of counts in each pair of plasmids, pJCC31-1 with pBR322 and pJCC31-2 with pBR322, was determined. The incorporation showed pJCC31-2 to be 7-fold more efficient than pBR322 and pJCC31-1 to be 4-fold more efficient. Although these data indicate a slightly higher efficiency for pBR322 in the combination than when injected alone, there is probably no synergistic effect between two plasmids replicating in the same egg. However, more quantitative data are necessary before the question can be finally answered.

Initiation of Transcription by the *Xenopus* Fragment. Because some repeated sequences of the *Alu* family and equivalent sequences in other mammals are transcribed *in vitro* and *in vivo* by RNA polymerase III (12), we injected pJCC31 and pJCC31-2 into *Xenopus* oocytes and incubated for 36 hr at 20°C to see if a transcript could be detected. The controls were pBR322, a plasmid (pLu103) with a sea urchin 5S gene, BLUR 8 (2), and oocytes without injected plasmids. The RNA from 30–50 oocytes was isolated and separated on an agarose gel. The RNA was labeled in some experiments by coinjection with [α-^{32}P]GTP and autoradiograms were prepared. Although pLu103 induced the formation of large amounts of 5S RNA, no bands of small RNA could be detected when pJCC31, pJCC31-2, or BLUR 8 was injected (data not shown). If present, larger RNA transcripts would have been obscured by synthesis of large RNAs in the control oocytes. We therefore tried again and transferred the RNA to a nitrocellulose filter by the RNA transfer

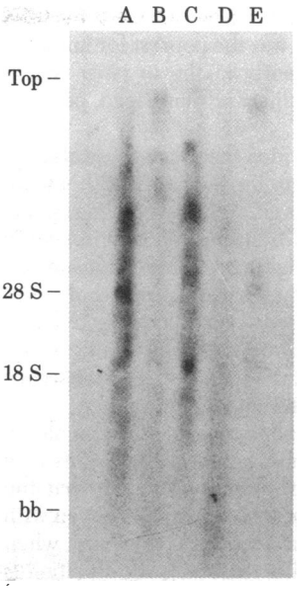

FIG. 3. Autoradiogram of a RNA blot transferred from an agarose gel to nitrocellulose and hybridized with ^{32}P end-labeled *Taq* I restriction fragments of pBR322. RNA was isolated after microinjection of oocytes with the following plasmids; pJCC31-2 after 36 hr of incubation (lane A); pJCC31 without incubation (lane B); pJCC31 after 36 hr of incubation (lane C); pBR322 after 36 hr of incubation (lane D). Lane E is a control of oocytes without microinjected DNA. On the left size markers are indicated: 28S RNA, 18S RNA, and the bromophenol blue dye (bb).

blotting procedure. We then end-labeled the *Xenopus* fragment with ^{32}P and used it as a probe for hybridization. The oocytes injected with pJCC31 showed a marked increase in hybridizable material of many sizes compared to the pBR322 control, but there was only one rather fuzzy band slightly larger than 28S RNA or about the length of the whole plasmid. The other region of high concentration of transcripts was smaller than the 18S RNA, or about 1,000 nucleotides (data not shown).

Because the transcripts were larger than the *Xenopus* fragment, we decided to use *Taq* I fragments of pBR322 as a ^{32}P-labeled probe. Fig. 3 shows these results. Transcripts of the plasmids were produced by pJCC31 and pJCC31-2 (lanes A and C). No distinct bands can be seen, but the termination of transcripts does not appear to be completely random. The amount of hybridizable material is definitely greater than in the controls—pJCC31 injected but lysed without incubation (lane B), pBR322 (lane D), and uninjected oocytes that were incubated for 36 hr (lane E).

DISCUSSION

The evidence presented here and elsewhere (1–4) indicates that DNA replication in chromosomal DNA is initiated at specific regions. The nature of these regions is not yet clear, but the sites include all or part of one of the dispersed repeated sequences typical of the DNA of higher animals (2, 3). These sequences have been called the *Alu* family of repeated sequences in the human genome (11) because they include one *Alu* I endonuclease site about 130 nucleotides from one end of the 300-bp sequence typical of this family. Some of these sequences have been cloned into pBR322 after ligating *Bam*HI linkers to them (13). A number of these plasmids (BLUR clones) were microinjected in *Xenopus* eggs (2) and compared to a plasmid with the *Xenopus* origin analyzed in this report for replication efficiency. Most of the BLUR plasmids replicated as well as the plasmid with a *Xenopus* origin, but one—BLUR 19, which has the *Alu* I site—was only about one-third as efficient as the best ones. Many of the dispersed repeats are characterized by having promoters that lead to their transcription *in vitro* by RNA polymerase III (12). Similar sequences are also present in other mammalian genomes, but the *Alu* I site is missing and the repeats vary in length and sequence. However, considerable sequence homology was seen when a number of mammalian repeats were compared (14) and these were classified into type

I (not transcribed) and type II (transcribed *in vitro* by RNA polymerase III). BLUR 19, which was the poorest for initiating replication, is also transcribed poorly in the *in vitro* system (Shella Fuhrman, University of California, San Diego, personal communication).

Such observations support the idea that most replication in chromosomes is primed at origins by segments of RNA approaching the size of these repeats (15). However, the connection between DNA initiation and transcription requires more careful study. Our preliminary tests reported here indicate that both pJCC31 and pJCC31-2, which include the whole *Alu*-equivalent repeat, are transcribed when microinjected into *Xenopus* oocytes. However, there does not appear to be a very efficient termination signal because many of the transcripts were very long in comparison to the repeat size.

The question of whether the *Xenopus* egg is as discriminating as other cells might be in initiating replication at origins is of concern in designing further experiments. The fact that the origin of the bacterial plasmid appears to function (4) even with a low efficiency has caused us some concern. However, when one considers the experiments with yeast cells the situation is similar. Though it is true that the plasmids with only a bacterial origin fail to function in the yeast by the criterion of increased transforming efficiency, it is difficult to make good comparisons. Yeast cells have been used for screening the replication of DNAs from a variety of sources by comparing the transformation efficiencies of plasmids without a yeast origin but with foreign DNA segments (16). Sequences from *Neurospora crassa, Dictyostelium discoideum, Caenorhabditis elegans, Drosophila melanogaster,* and *Zea mays* that increased the efficiency of transformation and therefore replication of a plasmid in yeast cells were found. In contrast, no sequences from *E. coli* were effective, including the known chromosomal origin of replication. The origin of simian virus 40 was also ineffective in yeast (16). Whether the *Xenopus* egg is a good test system for origins of replication can only be determined by further tests. One might expect it to be less discriminating than other cells because many more origins appear to be used in early embryos than at later stages in development (17, 18), but because the yeast cell can use a variety of sequences from distantly related DNAs, there may be considerable flexibility in usable sequences. We compared the sequence in the *Xenopus* origin with the Chinese hamster *Alu*-equivalent sequences reported by Haynes and Jelinek (12) without finding any sequence similarities except the overall one in which a sequence bracketed by direct repeats ends with a cluster of As.

The significance of a promoter sequence for transcription is also difficult to assess. The *Xenopus* segment appears to have such a sequence and one would suppose that it is in the region bracketed by the direct repeats. However, the fact that removal of any part of the 503-bp segment appears to have some effect on efficiency of replication is puzzling. It is possible that the cluster of Ts in the distal fragment c is a terminator for RNA polymerase, but this cannot explain the increase in efficiency that fragment c adds when cloned into pBR322 far from the plasmid origin. Because we do not have the sequence in either direction from this 503-bp segment in *Xenopus*, a careful study of other clones will be necessary. Our failure to find a strong band among the short transcripts may indicate that the terminator was deleted in the cloning. The small size of this *Xenopus* segment has the advantage of restricting the structures that we must compare in considering other origins but limits its value for determining the importance of surrounding sequences.

We thank Prescott L. Deininger and Theodore Friedman (University of California, San Diego) for providing facilities and assistance for S.W. to learn DNA sequence analysis techniques. We also acknowledge the assistance of Dan Riggs of this laboratory with the spot hybridization between pSW14 and *Xenopus* genomic DNA. This research was supported in part by a contract with the U.S. Department of Energy (EV05854) and a grant from the National Institute on Aging (1-RO1-AG-01807-03).

1. Watanabe, S. & Taylor, J. H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5292–5296.
2. Taylor, J. H. & Watanabe, S. (1981) in *The Initiation of DNA Replication*, ICN–UCLA Symposia on Molecular and Cellular Biology, ed. Ray, D. S. (Academic, New York), Vol. 22, pp. 597–606.
3. Hiraga, S., Sudo, T., Yoshida, M., Kubota, H. & Ueyama, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3697–3701.
4. Hines, P. J. & Benbow, R. M. (1982) *Cell*, in press.
5. Harland, R. M. & Laskey, R. A. (1980) *Cell* **21**, 761–771.
6. Houck, C. M., Rinehart, F. P. & Schmid, C. W. (1979) *J. Mol. Biol.* **132**, 289–306.
7. Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
8. Probst, E., Kressman, A. & Birnstiel, M. L. (1979) *J. Mol. Biol.* **135**, 709–732.
9. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
10. Watanabe, S. (1980) *Dissertation* (Florida State Univ., Tallahassee).
11. Jelinek, W. R., Toomey, T. P., Leinwand, L., Duncan, C. H., Biro, P. A., Choudary, P. V., Weisman, S. M., Rubin, C. M., Houck, C. M., Deininger, P. M. & Schmid, C. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1398–1402.
12. Haynes, S. R. & Jelinek, W. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6130–6134.
13. Rubin, C. M., Houck, C. M., Deininger, P. L., Friedman, T. & Schmid, C. W. (1980) *Nature (London)* **284**, 372–374.
14. Haynes, S. R., Toomey, T. P., Leinwand, L. & Jelinek, W. R. (1981) *J. Mol. Cell. Biol.* **1**, 573–583.
15. Taylor, J. H. (1982) in *Molecular Events in the Replication of Viral and Cellular Genomes*, ed. Becker, Y. (Martinus Nijhoff, The Hague), in press.
16. Stinchcomb, D. T., Thomas, M., Kelly, J., Selker, E. & Davis, R. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4559–4563.
17. Blumenthal, A. B., Kriegstein, H. J. & Hogness, D. S. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 205–223.
18. Callan, H. G. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 195–201.