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Replication of a plasmid bearing a human *Alu*-family repeat in monkey COS-7 cells

(replication origin/*Dpn* I resistance/cell transformation/tumor antigen)

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ABSTRACT **Monkey COS-7 cells were transformed with BLUR8 DNA, a pBR322 plasmid containing a human *Alu*-family sequence at the *Bam*HI site. Within 24 hr of transformation 2–5% of the BLUR8 molecules recovered resisted cleavage by *Dpn* I, indicating they had replicated. Electron microscopy revealed appropriately sized circular molecules with replication bubbles whose centers were mapped to the *Alu* insert. A 16-base-pair deletion within the *Alu* sequence prevented replication. The results indicate that certain *Alu* sequences can serve as origins of replication in COS-7 cells.**

The *Alu* sequences comprise a family of base sequence-related DNA segments, ≈270 base pairs (bp) long, that are present at $>3 \times 10^5$ different positions in primate DNAs (1–3). A region of the *Alu* "consensus" sequence is similar to the sequence at the origin of DNA replication of simian virus 40 (SV40), polyoma, and BK viral DNAs (ref. 4 and Fig. 1). This region contains the pentanucleotide GAGGC, which has been shown to make contact with SV40 tumor (T) antigen (6, 7). Because of this sequence similarity it has been suggested that *Alu* family members may serve as origins of DNA replication (4). Cells may show specificity with regard to the use of different origins during development or at different times during the cell cycle. It might therefore be advantageous to examine initiation on transfected DNA sequences in cells primed to initiate at a class of origins. Monkey COS-7 cells synthesize T antigen and support replication of DNA bearing the SV40 origin (8). We report here that a cloned *Alu* family member initiates DNA replication in monkey COS-7 cells.

MATERIALS AND METHODS

Transfection of COS-7 Cells and Extraction of Low Molecular Weight DNA. COS-7 cells were grown to 10–20% confluence as monolayers as described by Gluzman (8) and transformed by addition of 2.0 μg of plasmid DNA and 20 μg of mouse liver carrier DNA in a calcium phosphate precipitate (9) to $\approx 2 \times 10^6$ cells in 5.0 ml of medium at time 0. Immediately afterward, chloroquine diphosphate was added to a final concentration of 0.1 mM (10). After 4 hr, the cells were rinsed three times with fresh medium and then grown for various times. Cells transformed in this manner resumed doubling with a generation time of ≈28 hr. Low molecular weight DNA was extracted from the cell monolayers by the procedure of Hirt (11) as modified by Yang *et al.* (12). DNA in each Hirt supernatant was purified and aliquots were analyzed by restriction enzyme cleavage, gel electrophoresis, and electron microscopy.

Electron Microscopy of Replicating Plasmid Molecules. DNA was diluted to a concentration of 1.0 μg/ml in 50% (vol/vol) formamide/0.1 M Tris·HCl/10 mM EDTA, pH 8.5. Then, cytochrome *c* was added to a concentration of 50 μg/ml, and samples were spread onto a hypophase of 20% (vol/vol) formamide/10 mM Tris·HCl/1.0 mM EDTA, pH 8.5. The spread DNA was picked up on collodion grids, stained with uranyl acetate, and rotary-shadowed with Pt/Pd as described (13). Length measurements were made on enlarged micrographs using a Numonics digital integrator.

CONSENSUS	CCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGC
BLUR8	CCTGGAATCCCAGCTACTTAGGAGGCTGAGACAGAAGAATCCC
BLUR8Δ1	CCTGGAATCCCAGxxxxxxxxxxxxxxxxGACAGAAGAATCCC
SV40 ori	ATAGCTCAGAGGCCGAGGCGGCCTC

FIG. 1. Comparison of sequences from human *Alu* repeats with the sequence at the origin of replication of SV40. The *Alu* "consensus" base sequence and the sequence of BLUR8 are taken from ref. 3. The base sequence of the region of the SV40 origin of DNA replication is from Reddy *et al.* (5). BLUR8Δ1 was obtained by *in vitro* deletion of 16 bp of the *Alu* family member of BLUR8. The base sequence of BLUR8Δ1 was confirmed by conventional DNA sequence determination methodology and the region of interest is given. The positions of the 16 deleted nucleotides are indicated by the letter "x," and the region of base sequence similarity between the *Alu* sequences and the SV40 *ori* region is underlined.

Deletion of 16 Nucleotides from the BLUR8 *Alu* Sequence. The region of base sequence between the *Alu* I restriction site and the most distant *Dde* I site of the *Alu* family member in the BLUR8 plasmid was deleted by treatment with the two restriction enzymes followed by repair of the *Dde* I end with phage T4 DNA polymerase and religation of the resultant DNA fragments. Base sequence analysis showed that three more nucleotides than expected were removed from the *Dde* I side of the deletion (Fig. 1).

RESULTS

***Dpn* I Resistance of BLUR8 Plasmid DNA After Transformation of COS-7 Cells.** COS-7 cells were transformed with BLUR8 DNA, a pBR322 plasmid containing a 265-bp human *Alu* sequence. This plasmid had been amplified in *Escherichia coli* strain HB101, a *dam*$^+$ strain that methylates the adenine base in the sequence GATC, which can be cleaved by *Dpn* I. Replication in the monkey cells would result in nonmethylated or hemimethylated double-stranded DNA that would resist *Dpn* I cleavage (14, 15). Initially, the DNA was purified from Hirt supernatants isolated from the COS-7 cells at various times after transformation, treated with *Dpn* I or left untreated, and then analyzed by agarose gel electrophoresis. At 4 hr after transformation, all of the BLUR8 DNA could be cleaved by the enzyme but, by 24 hr, a significant portion of the plasmid DNA had become *Dpn* I

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Abbreviations: bp, base pair(s); SV40, simian virus 40; T antigen, tumor antigen; kb, kilobase(s).

resistant (data not shown). To examine this further, COS-7 cells were transformed with the BLUR8 plasmid and equal quantities of cells were harvested 0, 4, 24, and 48 hr later. Hirt supernatant DNA was digested with *Pvu* II, which cleaves once in the BLUR8 plasmid, and assayed for resistance to cleavage by *Dpn* I as described in Fig. 2*A*. At 0 hr, no *Dpn* I-resistant linear BLUR8 DNA could be observed. At 4 hr, a small amount was observed, which was increased at 24 hr and then remained approximately the same to 48 hr. During this time, there was a continued decrease in the overall amount of BLUR8 DNA per cell (Fig. 2*B*). Therefore, for BLUR8 molecules that entered the COS-7 cells, the overall rate of loss exceeded the rate of replication but, from 24 hr after transformation, the level of *Dpn* I-resistant DNA remained relatively constant. Based on densitometric scanning of radioautographic films, we estimated that 2–5% of detectable BLUR8 DNA was *Dpn* I-resistant 24 hr after transformation. To confirm that the *Dpn* I-resistant BLUR8 DNA shown in Fig. 2*A* did not result from partial digestion by the enzyme, COS-7 cells were transformed with BLUR8 DNA and, at 48 hr, DNA was isolated, cleaved with *Pvu* II, and subsequently treated with increasing amounts of *Dpn* I (Fig. 2*C*). Even at the highest concentration of *Dpn* I, some of the linearized BLUR8 DNA resisted digestion. (The lower intensity of the band in the 10-unit gel lane resulted from variability in sample recovery during the double restriction enzyme digestion procedure; see Fig. 2*C*). It could be that *Dpn* I-resistant BLUR8 DNA was generated by repair synthesis but, since there are 22 *Dpn* I sites in the plasmid, it is unlikely that only the full-length molecules shown in Fig. 2*A* would be observed. Furthermore, when COS-7 cells were transformed with pBR322 DNA devoid of any insert, no full-length *Dpn* I-resistant DNA could be detected at any time after transformation (see below). Therefore, if repair synthesis is responsible for the generation of the *Dpn* I-resistant BLUR8 molecules, the repair reaction would have to favor repair at all the *Dpn* I sites in pBR322 molecules that contain an *Alu* insert (BLUR8) over those that do not (pBR322 DNA without any insert). This prospect seems unlikely. *Dpn* I-resistant BLUR8 DNA is therefore most likely the product of semiconservative replication.

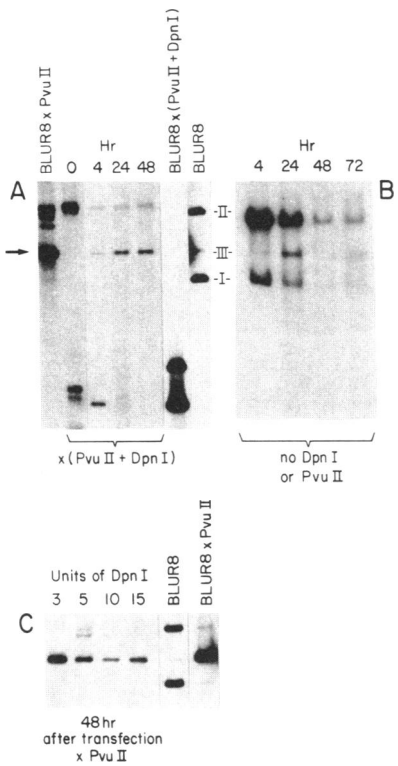

FIG. 2. *Dpn* I resistance of BLUR8 DNA after transformation of monkey COS-7 cells. (*A*) COS-7 cells were transformed with circular BLUR8 plasmid DNA and, at the indicated times, low molecular weight DNA was isolated by the method of Hirt (11), treated with *Pvu* II/*Dpn* I (30 units/μg of DNA), and assayed for BLUR8 sequences as follows. The DNA was electrophoresed in a 1.4% agarose gel in 20 mM NaOAc/10 mM EDTA/40 mM Tris·HCl, pH 8.2, partially depurinated, denatured, and blotted onto a GeneScreen*Plus* membrane (New England Nuclear) essentially as described by Wahl *et al.* (16). Hybridization to the filter-bound DNA was with nick-translated pBR322 DNA ($\approx 3 \times 10^4$ cpm/ml, specific activity $1–5 \times 10^7$ cpm/μg). After hybridization for 18 hr at 68°C, filters were washed first with 2× standard saline citrate (2× SSC; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) for 10 min at 20°C, then with 2× SSC/1.0% $NaDodSO_4$ for 30 min at 68°C and finally with multiple changes of 0.1× SSC at 20°C for 4 hr. The filters were blotted dry and exposed to Fuji x-ray film for 7 days. Untransformed BLUR8 DNA was either left untreated (lane BLUR8) or was treated with *Pvu* II (lane BLUR8 × Pvu II) or with *Pvu* II/*Dpn* I [lane BLUR8 × (Pvu II + Dpn I)] to serve as markers in the agarose gel. The position of the *Pvu* II-linearized BLUR8 plasmid DNA is indicated by the arrow. The positions to which form I, form II, and form III BLUR8 DNAs migrated in the gel are indicated. (*B*) COS-7 cells were transformed with circular BLUR8 plasmid DNA and, at the indicated times after transformation, low molecular weight DNA was isolated by the method of Hirt (11). It was then electrophoresed, without restriction enzyme digestion, in a 1.4% agarose gel and assayed by hybridization with radiolabeled pBR322 DNA as described in *A* and autoradiography for 24 hr. (*C*) COS-7 cells were transformed with circular BLUR8 plasmid DNA and, 48 hr later, low molecular weight DNA was isolated by the method of Hirt (11) and digested with *Pvu* II. The DNA was concentrated by ethanol precipitation and resuspended, and aliquots were digested with 3, 5, 10, or 15 units of *Dpn* I. The samples were precipitated with ethanol and assayed for the presence of BLUR8 sequences as described in *A*. A sample of *Pvu* II-digested BLUR8 DNA (lane BLUR8 × Pvu II) and a sample of undigested BLUR8 DNA (lane BLUR8) were also electrophoresed.

Electron Microscopic Mapping of BLUR8 Replication Bubbles Generated in COS-7 Cells. COS-7 cells were transformed with BLUR8 plasmid DNA and, at various times after the transformation, DNA was isolated and examined by electron microscopy for the presence of replicating molecules. Circular DNA molecules were seen with average sizes of 4.6, 9.6, and 15.2 kilobases (kb). Only those of the 4.6-kb class (the expected size of monomeric BLUR8 plasmid DNA) were scored. The 9.6-kb molecules are most likely BLUR8 dimers and the 15.2-kb molecules could be trimers but they could also be mitochondrial DNA molecules, which are approximately this size (17). Of identifiable, open circular, 4.6-kb molecules, 3.4% possessed replication bubbles 48 hr after transformation. The remainder of the molecules, even 72 hr after transformation, were predominantly supercoiled or were partially supercoiled. Representative circular molecules with replication bubbles are shown in Fig. 3 *A–C*. The molecule in Fig. 3*C* is a "θ form" in which the replication bubble has nearly completed its course leaving two interconnected circles, one partially supercoiled. No circular molecules of 4.6 kb were seen that could unequivocally be construed to possess more than one replication bubble.

To determine whether initiation of replication occurred within the *Alu* sequence, DNA in Hirt supernatants of transformed cells was cleaved with *Pvu* II and examined by electron microscopy. *Pvu* II cleaves the BLUR8 plasmid once, approximately opposite the *Alu* insert to give full-length, linear molecules within which replication bubbles could be mapped. Only linear molecules of the 4.6-kb class that possessed a single replication bubble (multiple bubbles were not seen) were scored. A typical molecule is shown in Fig. 3*D*. Several molecules were seen with compound loops at one end of the replication bubble as shown in Fig. 3*D*. This could be the result of secondary initiation but is more likely the result of branch migration, as observed previously in replication bubbles (18). The positions of the bubble in

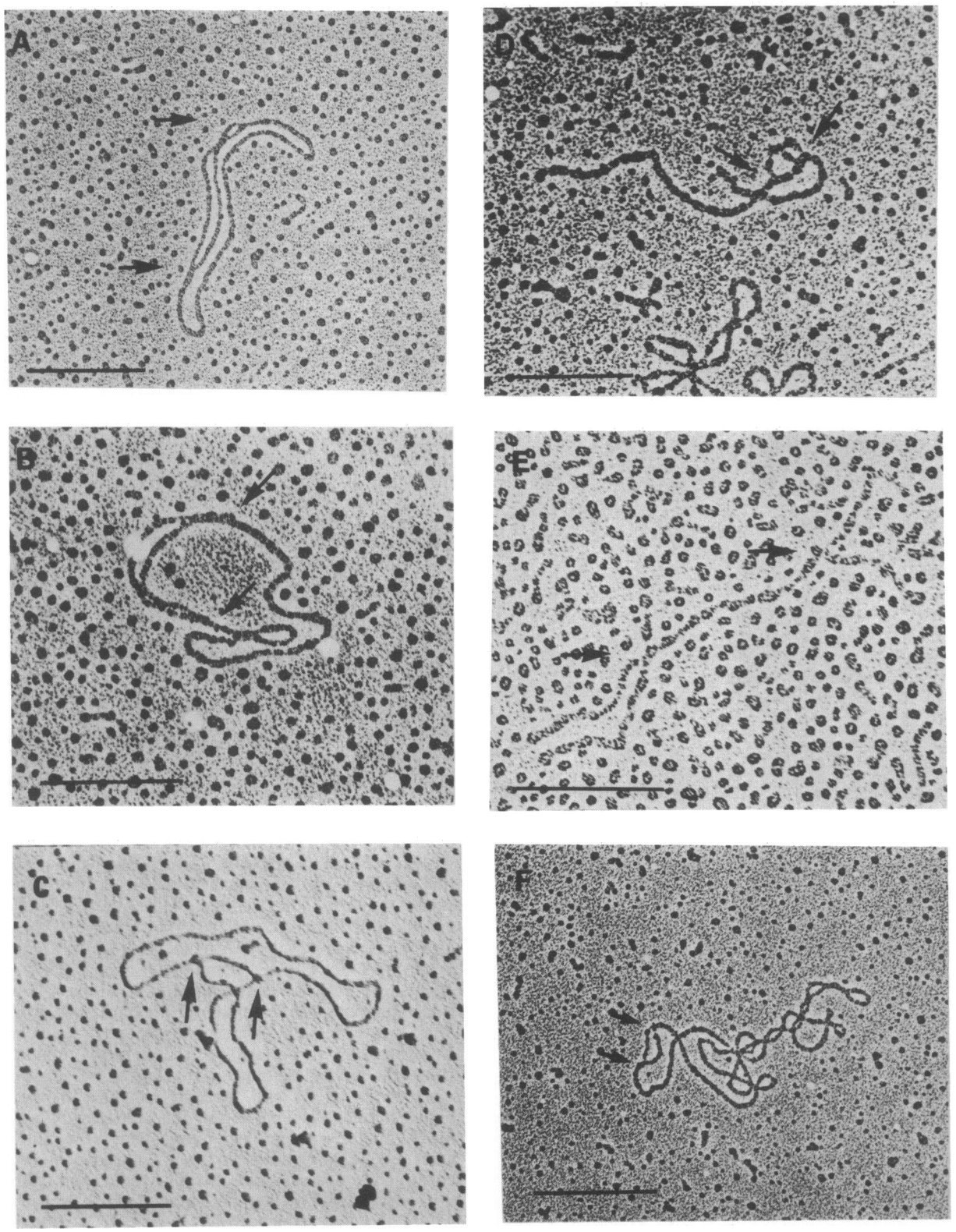

FIG. 3. Electron microscopy of BLUR8 plasmid DNA isolated from transformed monkey COS-7 cells. Cells were transformed and low molecular weight DNA was isolated and spread for electron microscopy with elevated temperature steps eliminated to minimize branch migration. (*A–C*) Replication bubbles in a circular plasmid DNA spread from COS-7 cells 24 hr after transformation. (*D*) A linear DNA molecule of BLUR8 size bearing a replication bubble. DNA was isolated from COS-7 cells 24 hr after transformation and cleaved with *Pvu* II. (*E*) A late θ-form DNA molecule of BLUR8 size. DNA was isolated from COS-7 cells 24 hr after transformation and cleaved with *Bam*HI, yielding an H-form structure. (*F*) A replicating circular plasmid DNA spread from COS-7 cells 48 hr after transformation. The arrows indicate branch points of replication bubbles. Bars = 1.0 kb, based on a value of 0.338 nm per base pair of DNA in B-helical form.

several linear molecules and a plot of the difference in distance between the center of the linear molecules and the center of the replication bubble they each contain are shown on the right- and left-hand sides, respectively, of Fig. 4. As can be seen, the bubbles are distributed about the *Alu* insert, whose position in the linearized molecules is indicated at the tops of the drawings. The clustering of the bubble positions indicates that initiation occurred at a preferred region of

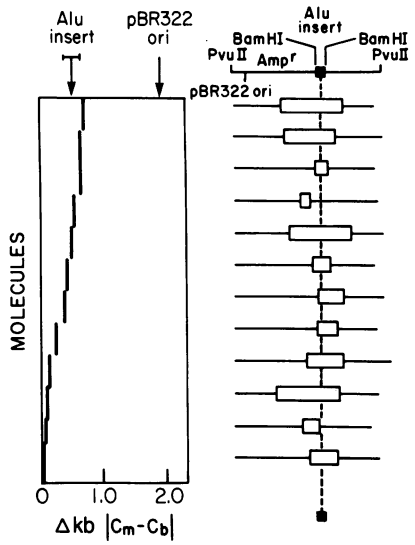

FIG. 4. Electron microscopic mapping of replication bubbles in linearized BLUR8 plasmid DNA. COS-7 cells were transformed with BLUR8 plasmid DNA and, 24 and 48 hr later, low molecular weight DNA was isolated according to the method of Hirt (11, 12) with steps at elevated temperature eliminated to minimize possible branch migration at replication bubbles (18). DNA was cleaved with *Pvu* II and spread for electron microscopy as described in Fig. 3. All linear DNA molecules of 4.6 kb with replication bubbles were recorded. No molecules were seen that unequivocally had more than one replication bubble. (*Left*) Mapping positions of replication bubbles. For each measured molecule the absolute value of the difference between the positions of the center of the observed replication bubble (C_b) and the center of the molecule (C_m) was determined. The positions of the *Alu* insert and the pBR322 origin of replication are indicated by arrows. (*Right*) Depiction of 12 molecules spread from COS-7 cells transfected with BLUR8. Molecules such as that of Fig. 3*D* were measured and positioned with long arms at the left below a map of *Pvu* II-linearized BLUR8 DNA.

sequence either within or near the *Alu* insert. The replication bubbles in the linear molecules ranged in size from ≈400 bp to 2 kb, with an average of 1.2 kb. We therefore would have expected to observe bubbles that had initiated at the position of the pBR322 origin of replication (indicated in Fig. 4) had they existed. None were detected.

These results were confirmed by treatment of DNA in Hirt supernatants with a second restriction enzyme, *Bam*HI. This enzyme excises the *Alu* insert from BLUR8 (Fig. 4). If initiation had occurred in the *Alu* sequence, cleavage of late θ forms would leave "H-form" molecules with one fork near each end and four double-stranded branches. Several such molecules were observed, an example of which is shown in Fig. 3*E*. Assuming bidirectional fork movement was synchronous, or nearly so, the length of the replicated segment at one end should be approximately equal to that at the other end. Ten H-form molecules with an average replicated length of 1.6 kb were examined. The average ratio of lengths of the branches at one end to those at the other end was 0.87 (± 0.14), a value consistent with initiation within the *Alu* insert.

One possible artifact to consider is that plasmid molecules that had initiated DNA replication in *E. coli* could be preserved during the transformation and subsequently detected by electron microscopy. A search of several hundred BLUR8 molecules isolated directly from *E. coli* failed to reveal any that contained a replication bubble. Therefore, the bubbles we observed in the BLUR8 molecules isolated from the COS-7 cells must have originated in those cells.

Studies on the Selectively Mutated *Alu* Sequence. To determine whether initiation of replication was dependent on specific sequences within the *Alu* insert of BLUR8, 16 bp from the BLUR8 *Alu* sequence, including most of the region with similarity to the SV40 origin of replication, were deleted. This mutated plasmid, BLUR8Δ1 (Fig. 1) failed to replicate in COS-7 cells by two criteria. Several hundred appropriately sized DNA molecules isolated from COS-7 cells 4, 24, and 48 hr after transformation with BLUR8Δ1 were examined by electron microscopy. None were detected that had a replication bubble nor were *Dpn* I-resistant form I, form II, or form III molecules detected by agarose gel electrophoresis (Fig. 5*B*). Likewise, when pBR322 DNA lacking an insert was used in the transformation no replication was detected in the COS-7 cells either by electron microscopy or by the gel electrophoretic method used to identify form I molecules that resisted cleavage by *Dpn* I (Fig. 5*B*). These observations are consistent with the hypothesis that the region of the *Alu* sequence showing base sequence similarity to that at the SV40 replication origin is required for initiation of DNA replication of the BLUR8 plasmid in COS-7 cells. To replicate the BLUR8 plasmid might have to interact with SV40 T antigen, and the deletion of BLUR8Δ1 has eliminated the base sequences that participate in that interaction. The BLUR8Δ1 plasmid may have experienced some repair synthesis because with increasing time after transformation, bands appeared in the gel above the largest band produced by *Dpn* I cleavage (Fig. 5*B*). However, no intact molecules were observed following *Dpn* I cleavage.

As a precaution against the possibility that the observed replication of the BLUR8 plasmid resulted from the creation of an artificial sequence resulting from juxtaposition of the *Alu* sequence with adjacent pBR322 sequence, the orientation of the *Alu* sequence in the BLUR8 plasmid was reversed and the electron microscopic observations described above were repeated. Replication bubbles in the plasmid containing the *Alu* sequence in the reverse orientation were observed with approximately the same frequency as in the original BLUR8 plasmid after transformation into COS-7 cells. It therefore seems unlikely that some artificially created sequence is responsible for the observed BLUR8 replication.

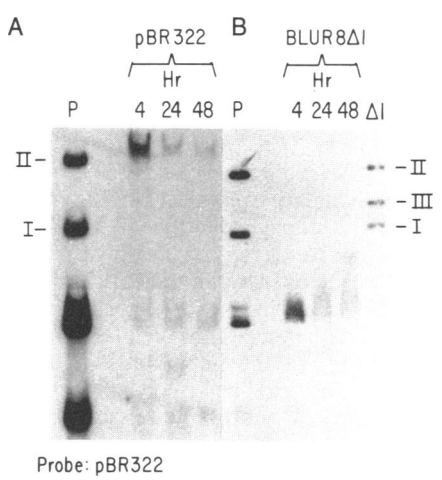

FIG. 5. *Dpn* I sensitivity of BLUR8Δ1 DNA and pBR322 DNA after transformation of monkey COS-7 cells. COS-7 cells were transformed with either pBR322 DNA (*A*) or BLUR8Δ1 DNA (*B*) and, 4, 24, and 48 hr later, low molecular weight DNA was isolated, treated with *Dpn* I, and electrophoresed. The resulting blots were hybridized to nick-translated pBR322 DNA as described in Fig. 2. Lanes P: a partial *Dpn* I restriction digest of pBR322 DNA showing forms I and II (indicated on the left) as well as the 1.4-kb *Dpn* I-generated fragment. Lane Δ1: purified, untransfected, uncleaved BLUR8Δ1 DNA showing forms I, II, and III. The band that migrated above the position of the form II band in the lanes loaded with pBR322 DNA is probably digestion-resistant aggregates that arose during the calcium phosphate precipitation. This band decreases with increasing time during the course of the experiment.

DISCUSSION

The observations described here indicate that an *Alu* sequence can initiate DNA replication in mammalian cells. In COS-7 cells the BLUR8 plasmid initiated replication within or near the *Alu* sequence to generate *Dpn* I-resistant molecules. It has previously been reported (19) that BLUR8 replicates in a cell-free extract optimized to replicate SV40 DNA, but other workers (20) have failed to repeat this observation. The level of replication initiation of BLUR8 observed here was low relative to that of authentic SV40 DNA and of a plasmid bearing an authentic SV40 *ori* sequence. It was also previously reported that, in COS-7 cells, *Dpn* I-resistant copies of the plasmid pSV2neo increased approximately 3-fold in number over 28 hr (21). Recombinant plasmids bearing SV40 ori sequences replicated 1–5% as efficiently as authentic SV40 DNA (22), and BLUR8 (this report) replicated ≈7% as efficiently as pSV2neo. We estimate that transformation of 10^6 COS-7 cells with 1 ng of BLUR8 DNA yielded 60–70 molecules of BLUR8 DNA per cell 48 hr after transformation. Similar estimates of pSV2neo DNA and authentic SV40 DNA are ≈10^3 and 10^6 molecules per cell, respectively (22, 23).

The base sequence of BLUR8, although similar in one region, is not identical to the SV40 *ori* sequence. The pentanucleotide GAGGC, to which T antigen binds (6, 24, 25), is present four times in the SV40 *ori* sequence, and all four copies bind T antigen (25). There are four copies of this pentanucleotide in BLUR8 and nine more with a single nucleotide mismatch, but only two are clustered as in the SV40 *ori* sequence. Since T antigen binding is required for SV40 DNA replication (26, 27), reduced binding should reduce replication. We also note that BLUR8 contains an 11-nucleotide region identical except for a single nucleotide to the "consensus" sequence derived from a number of yeast autonomous replicating sequences (*ARS* sequences; ref. 28), but its involvement in the replication reported here has not been assessed. Other factors, such as the presence of the "poison" sequence (22) in pBR322 DNA or the rate of resolution of replicating molecules might also influence replication efficiency. In this respect we note that the frequency of catenated (unresolved) dimers observed with BLUR8 was greater than previously observed with pSV2neo. One such molecule that initiated a second round of replication is shown in Fig. 3*F*.

It has already been reported that SV40 T antigen binds *in vitro* to the *Alu* sequence present in BLUR8 (ref. 19; see also ref. 24). That the sequence identified in Fig. 1 is involved in the replication reported here (presumably by T-antigen binding) is shown by the observation that BLUR8Δ1, which has a deletion that disrupts this sequence, did not replicate.

On infection, SV40 induces host cell DNA replication, and T antigen is required for the induction (29). Our data suggest that *Alu* sequences may be involved. The observations reported here do not address the issue of whether *Alu* sequences function as origins of DNA replication in cells that do not contain T antigen. If they do, then only a fraction of them must do so, because they are present at a frequency substantially higher than has been estimated for the number of chromosomal replication origins used during a single round of DNA replication (30). Preliminary experiments with BLUR8 gave no indication of replication in CV-1 cells. In this regard it is interesting that particular viral origins of DNA replication function in some mouse embryo cells but not in others, depending on the stage of development (14). It is also of interest that the *Alu* family is a group of related, but not identical, sequences and only some may function in a particular set of circumstances. We note that *Alu* sequences are present in short DNA fragments extruded by branch migration from chromosomal replication bubbles in an abundance somewhat higher than expected from their genomic frequency (31, 32).

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