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Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences

(replication origin/*Dpn* I resistance/semiconservative replication)

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ABSTRACT **Twelve clones of origin-enriched sequences (ORS) isolated from early replicating monkey (CV-1) DNA were examined for transient episomal replication in transfected CV-1, COS-7, and HeLa cells. Plasmid DNA was isolated at time intervals after transfection and screened by the *Dpn* I resistance assay or by the bromodeoxyuridine substitution assay to differentiate between input and replicated DNA. We have identified four monkey ORS (ORS*3*, -*8*, -*9*, and -*12*) that can support plasmid replication in mammalian cells. This replication is carried out in a controlled and semiconservative manner characteristic of mammalian replicons. ORS replication was most efficient in HeLa cells. Electron microscopy showed ORS*8* and ORS*12* plasmids of the correct size with replication bubbles. Using a unique restriction site in ORS*12*, we have mapped the replication bubble within the monkey DNA sequence.**

The genomes of most prokaryotes and of some viruses of eukaryotic cells initiate DNA replication at a unique fixed site (1). The larger chromosomes of eukaryotes initiate replication at multiple sites, and, as in prokaryotes, control over the timing and frequency of initiation is expected to be exerted at these sites. Little is known about mammalian origins of replication, but considerable progress has been made in yeast with the isolation of autonomously replicating sequences (ARS) based on their ability to support plasmid replication in *Saccharomyces cerevisiae* (2–4). DNA fragments with ARS activity have also been isolated from human and mouse genomes (5–8), but there is no evidence that they are chromosomal initiation sites.

Increasing biochemical (2, 3, 9, 10, 11) and electron microscopic (12, 13) evidence suggests that mammalian DNA synthesis is initiated at specific sites. In the amplified region (amplicon) of methotrexate-resistant Chinese hamster ovary cells, initiation of DNA synthesis in each amplicon is restricted to a small subset of restriction fragments (9), and the origin of replication associated with this region is contained in a 4.3-kilobase (kb) fragment (10). In murine erythroleukemia cells, the order of replication of immunoglobulin heavy-chain constant-region genes is the same as their physical order along the chromosomes (11).

Using a different approach to identify origins of replication (14), we isolated and cloned, in pBR322, monkey (CV-1) DNA enriched 10^3- to 10^4-fold for nascent sequences that are activated early in the S phase (15). Each of these origin-rich sequences (ORS) should contain an origin of DNA replication at or near its center (16). We have previously reported in detail on the properties of 12 ORS (17).

In this paper we present evidence that at least four of the ORS, ORS*3*, -*8*, -*9*, and -*12*, act as origins of replication in a mammalian system, as determined by their ability to enable transfected pBR322/ORS recombinant plasmids to replicate in monkey (CV-1 and COS-7) and human (HeLa) cells. Plasmid replication in transfected cells was determined by two independent methods: (*i*) resistance to digestion by *Dpn* I (18, 19) and (*ii*) density labeling by bromodeoxyuridine (BrdUrd) incorporation.

MATERIALS AND METHODS

Plasmids. pBR322 and recombinant plasmids pBR/ORS*1* to pBR/ORS*12* (17) were propagated in *Escherichia coli* HB101, and plasmid DNAs were prepared as previously described (14).

Cell Culture. HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). COS-7 (20) and CV-1 African green monkey kidney cell lines were grown in MEM α with 5% FCS.

***Dpn* I Resistance Assay.** Logarithmic phase CV-1, COS-7, or HeLa cells (seeded at 2.5×10^5 cells per 25-cm^2 flask one day prior to transfection) were simultaneously transfected with 3 μg of pBR322 and 3 μg of one of the plasmids pBR/ORS*1* to pBR/ORS*12* (15) by the calcium phosphate coprecipitation method (21). After 4 hr, they were subjected to a glycerol shock (22) and then were given fresh medium containing 5% FCS. At 72 hr posttransfection, low molecular weight plasmid DNA was isolated by the method of Hirt (23), concentrated on Elutip-d columns (Schleicher & Schuell), and precipitated in 70% ethanol. DNA pellets were digested first with *Bam*HI or *Cla* I to linearize the plasmids, then digested with *Dpn* I to cleave unreplicated (fully methylated) input plasmids (19), and electrophoresed on 1.5% agarose gels. Southern blot hybridizations (24) were carried out as previously described (17). Membranes were hybridized with ^{32}P-labeled pBR322 DNA at 65°C, washed, and autoradiographed for 3–6 days.

Semiconservative Replication Assay. HeLa cells were seeded and transfected with 5 μg each of pBR322, pBR/ORS*3*, pBR/ORS*8*, pBR/ORS*9*, or pBR/ORS*12*, as described above. After the glycerol shock, cells were grown for 24 hr in DMEM (5% FCS) containing BrdUrd at 12.5 μg/ml. Hirt extracts were prepared and centrifuged in CsCl gradients (initial refractive index of 1.4150) in a Beckman VTI 80 rotor for 16–18 hr at 65,000 rpm. Fractions of 250 μl were collected from the bottom of the gradient, and the refractive index of every second fraction was read. A 25-μl aliquot from each fraction was diluted 1:1 with distilled H_2O and electrophoresed on a 1% agarose gel that was Southern blotted and probed with pBR322 as described above.

EM. Plasmid DNA isolated from HeLa cells 24 hr after transfection with 5 μg of either pBR/ORS*8* or pBR/ORS*12* was spread for EM in a hyperphase of cytochrome C (120

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Abbreviations: ORS, origin-enriched sequences; LL DNA, light–light DNA; HL DNA, heavy–light DNA; HH DNA, heavy–heavy DNA.

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μg/ml)/40% formamide/50% mM Tris·HCl/10 mM EDTA, pH 8 onto a hypophase of 5% formamide/10 mM Tris·HCl/1 mM EDTA, pH 8. The DNA was picked up on parlodion-coated copper grids (Ladd Research Industries, Burlington, VT), stained with uranyl acetate, and rotary-shadowed with platinum/palladium (80:20) (J.B. EM, Montreal). Electron micrographs were taken with a Zeiss CA10 (courtesy of the Department of Pathology, University of Vermont College of Medicine, Burlington, VT) or a Phillips 410 (Pharmacology Department, McGill University, Montreal, PQ) electron microscope.

RESULTS

***Dpn* I Resistance Assay of pBR/ORS Plasmid DNA.** Twelve mapped and sequenced pBR/ORS plasmids (15, 17) were cotransfected with pBR322 into CV-1, COS-7, and HeLa cells. At 72 hr posttransfection the cells were lysed, and the plasmid DNA from Hirt (23) supernatants was linearized with *Bam*HI or *Cla* I and then digested with *Dpn* I, which cleaves fully methylated DNA at the sequence mGATC. The input plasmids, which were grown in DNA adenine methylase-positive bacteria and contain 22 *Dpn* I cleavage sites, were cleaved into many small size fragments. Plasmids that replicated in the mammalian cells, however, are resistant to *Dpn* I cleavage. Linearized and *Dpn* I-digested DNA was electrophoresed, blotted, and probed with pBR322. The intensity of the *Dpn* I-resistant band from each of the ORS plasmids was compared to that of pBR322. Table 1 shows the results of ORS plasmid transfections in CV-1, COS-7, and HeLa cells. Although there was great variability between experiments, ORS*8*, *-9*, and *-12* plasmids replicated consistently to give *Dpn* I-resistant bands above background intensity (i.e., 2- to 6-fold as intense as pBR alone) in 7 out of 8, 5 out of 7, and 8 out of 8 experiments, respectively, regardless of cell line.

Overall, HeLa cells supported ORS plasmid replication the best; they produced the highest copy number of progeny DNA for all three ORS plasmids as compared to CV-1 and COS-7 (see also Fig. 1). ORS*3* plasmid replicated with a lower efficiency (i.e., produced a much lower copy number of progeny DNA) in both COS-7 and HeLa cells and did not replicate at all in CV-1 cells (Table 1).

An example of the *Dpn* I-resistant bands obtained with ORS*8*, *-9*, and *-12* plasmid DNAs in each of the three cell lines is shown in Fig. 1. These results clearly show that the most efficient replication for the three ORS plasmids takes place in HeLa cells (Fig. 1*C*). Furthermore, ORS*12* replicated with the highest efficiency of the three and produced at least twice as many copies of progeny DNA in 72 hr (Fig. 1*C*). In Fig. 1 *A* and *C*, the plasmid DNAs were linearized with *Bam*HI prior to *Dpn* I digestion, and both linear (form III) and nicked (form II) plasmids were recovered. The pBR322 *Dpn* I-resistant band (background) in Fig. 1 migrates as form II (indicated by arrowheads), and it represents the majority of the *Dpn* I-resistant pBR322 DNA. Linear pBR/ORS*8* [4845 base pairs (bp)] and ORS*9* (4910 bp) migrate only slightly ahead of nicked pBR322 (4363 bp), resulting in the doublets seen in Fig. 1*C*. In Fig. 1*B* the plasmid DNAs were linearized with *Cla* I, and only form III *Dpn* I-resistant plasmids were recovered, including pBR322 (arrowhead). The frequent finding of *Dpn* I-resistant pBR322 bands has also been reported by others (25).

Table 1. *Dpn* I resistance of ORS plasmid DNA after cotransfection with pBR322 DNA

	CV-1			COS-7		HeLa			
ORS	1	2	3	1	2	1	2	3	4
1	−	−	−	−	−	−	+	−	−
2	−	−	−	−	−	−	+	−	−
3	−	−	−	+	−	−	−	+	−
4	−	−	?	−	?	−	−	−	−
5	+	+	−	?	−	−	−	++	−
6	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−
8	−	++	?	++	+	+++	++	++	+
9	+	++	−	++	+	?	?	++	−
10	−	−	+	+	+	−	−	+	−
11	−	−	+	−	+	−	−	−	−
12	++	+	++	++	?	+	+++	+++	++

+, ++, and +++, *Dpn* I-resistant ORS DNA bands that are approximately two times, two to four times, and greater than four times as strong as pBR322 *Dpn* I-resistant bands, respectively. −, *Dpn* I-resistant ORS bands that are less than two times as strong as those of pBR322. ?, results in which *Dpn* I resistance could not be determined due to poor recovery or loss of sample.

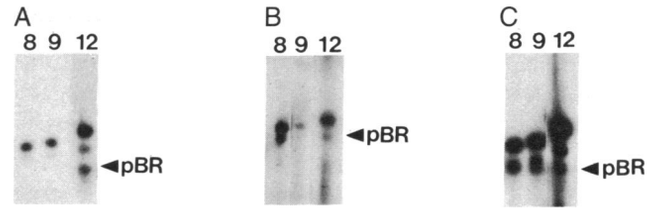

FIG. 1. *Dpn* I resistance of ORS*8*, *-9*, and *-12* plasmids. (*A*) CV-1 monkey cells were cotransfected with 3 μg of pBR/ORS*8* (lanes 8), pBR/ORS*9* (lanes 9), or pBR/ORS*12* (lanes 12) and 3 μg of pBR322. At 72 hr posttransfection the cells were lysed, and the recovered plasmids were linearized with *Bam*HI, then digested with *Dpn* I, electrophoresed on 1.5% agarose, blot-transferred, and probed with pBR322. *Dpn* I-resistant bands are shown. Arrowhead indicates the position of the *Dpn* I-resistant pBR322 band (form II). (*B*) The conditions were the same as in *A*, except that COS-7 cells were used and recovered plasmids were linearized with *Cla* I. Arrow indicates the position of the *Dpn* I-resistant pBR322 band (form III). (*C*) The conditions were the same as in *A* except that HeLa cells were used.

A time course of the replication of pBR/ORS*8*, *-9*, and *-12* in which HeLa cells were cotransfected with 3 μg of pBR/ORS*8*, *-9*, or *-12* and 3 μg of pBR322 is shown in Fig. 2. Plasmid DNA was isolated at intervals, linearized with *Cla* I, and digested with *Dpn* I. Both forms II and III of *Dpn* I-resistant pBR/ORS*8*, *-9*, and *-12* were recovered, and the intensity of these bands increased 5- to 10-fold between 24 and 96 hr (Fig. 2). No pBR322 *Dpn* I-resistant band could be detected in this experiment. To ensure that *Dpn* I-resistant bands (Figs. 1 and 2) were not a result of partial digestion by the enzyme, λ DNA was included in each reaction, and the expected digestion products were verified by ethidium bromide staining (data not shown).

Semiconservative Replication of Transfected Plasmids. Because of the variability in the results of *Dpn* I resistance, we

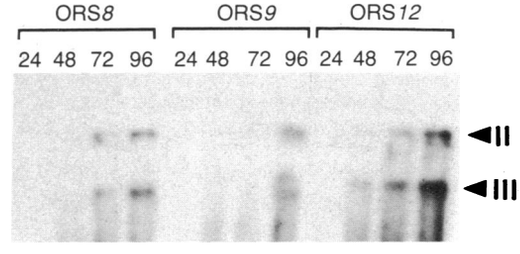

FIG. 2. Time course of appearance of *Dpn* I-resistant bands for ORS*8*, *-9*, and *-12* plasmids. HeLa cells were cotransfected with ORS*8*, *-9*, or *-12* and pBR322 plasmid DNAs as in Fig. 1. At 24, 48, 72, and 96 hr posttransfection, the plasmid DNAs were harvested, treated, and analyzed as in Fig. 1. Arrowheads indicate the position in the gel of *Dpn* I-resistant nicked-circular (II) and linear (III) ORS plasmid DNAs.

used an independent assay for autonomous replication in mammalian cells as a criterion for replication of transfected ORS plasmids. BrdUrd, a heavy analogue of thymidine, when incorporated into replicating DNA, increases the den-

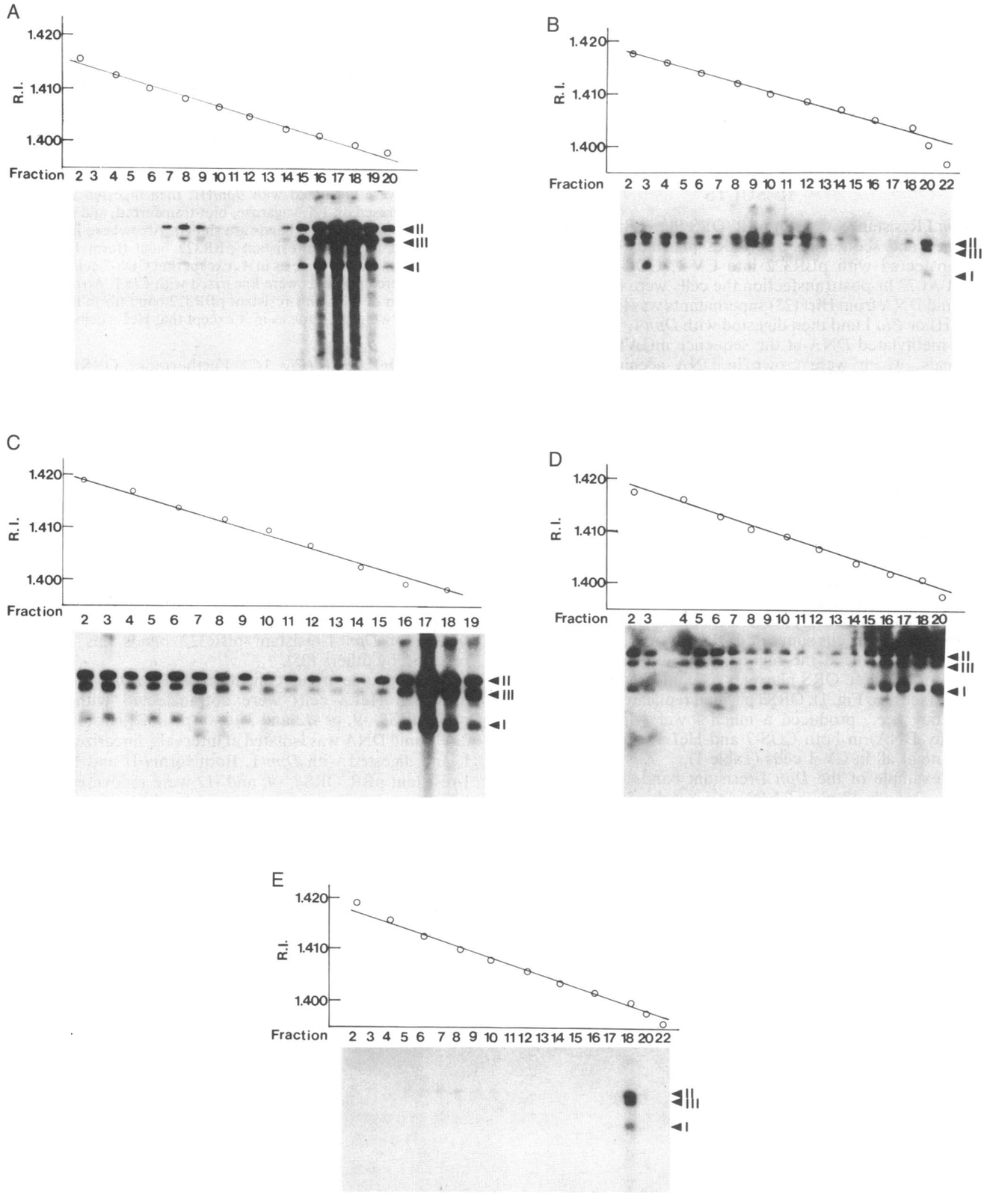

FIG. 3. BrdUrd incorporation in ORS*3*, *-8*, *-9*, and *-12* plasmids. HeLa cells were transfected with ORS*3* (*A*), ORS*8* (*B*), ORS*9* (*C*), ORS*12* (*D*), or pBR322 (*E*) plasmids and incubated at 37°C in medium containing BrdUrd at 12.5 μg/ml. At 24 hr posttransfection the cells were lysed, and the recovered plasmid DNAs were centrifuged to equilibrium on CsCl gradients. Fractions were collected from the bottom of each gradient, and an aliquot from each fraction was subjected to agarose gel electrophoresis and blot-hybridization analysis. The positions of supercoiled (I), nicked (II), and linear (III) plasmid DNAs are indicated by arrowheads. The refractive index (R.I.) of every second fraction was taken for each gradient, and the resulting profiles are plotted above each gel.

sity of the replicated molecule and causes it to band at a density higher than that of unsubstituted (light–light; LL) DNA. One cycle of replication leads to a hybrid density (heavy–light; HL) DNA with only one strand substituted with the BrdUrd, whereas two or more cycles in its presence lead to the production of fully substituted (heavy–heavy; HH) DNA.

The replication of ORS*3*, *-8*, *-9*, and *-12* plasmids was assayed by density-labeling with BrdUrd. The results for pBR/ORS*3*, *-8*, *-9*, and *-12* as well as pBR322 are shown in Fig. 3. For all 5 plasmids, a peak of unreplicated (input) LL plasmid DNA is seen near the top of each gradient (Fig. 3*A*, fractions 17 and 18; Fig. 3*B*, fraction 20; Fig. 3*C*, fraction 17; Fig. 3*D*, fraction 17; Fig. 3*E*, fraction 18). In the pBR322 gradient, virtually all of the plasmid DNA is present in the LL fraction (Fig. 3*E*), whereas in the pBR/ORS*8*, *-9*, and *-12* gradients two additional peaks of HH and HL plasmid DNAs are evident. The DNA centered at fraction 9 for ORS*8* (Fig. 3*B*), fraction 7 for ORS*9* (Fig. 3*C*), and fraction 6 for ORS*12* (Fig. 3*D*) bands at a higher density than LL DNA, as would be expected of HL plasmid DNA; the DNA centered at fraction 3 for ORS*8* (Fig. 3*B*) and ORS*9* (Fig. 3*C*) and fraction 2 for ORS*12* (Fig. 3*D*) bands near the bottom of the gradient with a density shift consistent with that of HH DNA. The distribution of ORS*3* was different and indicated that it underwent only one round of replication in 24 hr, producing only HL DNA (peak fraction 8, Fig. 3*A*). These results are consistent with ORS*3* replication as assayed by *Dpn* I resistance.

EM. ORS*8* and ORS*12* plasmid DNAs isolated from transfected HeLa cells were examined by EM for replicating molecules. The distribution of replicating ORS plasmids versus that of nonreplicating pBR322 is in agreement with that obtained by the previous two methods. Replicating circular (both relaxed and supercoiled) and linear monomers of ORS*8* and ORS*12* plasmid DNAs were observed, whereas only nonreplicating pBR322 molecules were seen. Only plasmids of the expected correct size (4.8 kb for ORS*8*, 5.2 kb for ORS*12*, and 4.3 kb for pBR322) were scored. ORS*12* had 12% (7 out of 60) and ORS*8* had 9% (6 out of 64) of the molecules replicating at various stages, whereas no pBR322 molecules (0 out of 40) with replication bubbles were seen. Examples of supercoiled and relaxed circular ORS*12* plasmids containing a replication bubble at different stages of replication are shown in Fig. 4 *A* and *B*, respectively.

To determine whether initiation of replication occurred within the ORS, replicative intermediates of ORS*12* plasmid DNA isolated from transfected HeLa cells were cleaved with the single-cut restriction enzyme *Apa* I and examined by EM. Of the 30 molecules that were examined, 3 had Y-shaped ends (Fig. 4*C*) indicative of a replication bubble cleaved by *Apa* I, and the rest were linear molecules with no bubbles. *Apa* I cleaves the pBR/ORS*12* once within the ORS at map location 1593, but it has no cleavage site in pBR322 (Fig. 4*D*). We conclude that 3 out of 30 (10%) molecules had replication bubbles within ORS*12* and were cleaved by *Apa* I. This percentage agrees with that of 12% that we previously found for uncut ORS*12* replicative intermediates. We could not perform the same type of experiment with ORS*8* plasmid DNA because it lacks restriction sites that are unique to the ORS*8* sequence. Detailed mapping of the position of the replication bubble in ORS*3*, *-8*, *-9*, and *-12* will be given elsewhere.

DISCUSSION

Using two independent assays of DNA replication, we have shown that four monkey ORS, ORS*3*, *-8*, *-9*, and *-12* (15, 17), when inserted in pBR322, are capable of replicating in mammalian cells, suggesting that they contain mammalian origins of DNA replication. ORS*12* is the most efficient of the four, and it produces the highest copy number of progeny plasmid DNA in all three cell lines that we tested. *Dpn* I-resistant copies of ORS*12* plasmid increased approximately 10-fold over a period of 96 hr, which is at least twice as much as ORS*8* and ORS*9*. ORS*12* consists of 812 bp of monkey ORS, and it contains the highly reiterated α-satellite family; ORS*8* is a unique sequence of 483 bp, and both ORS*3* (1172 bp) and ORS*9* (549 bp) contain the highly reiterated *Alu* family (17). There is no extensive sequence homology between the four ORS, but they all contain inverted repeat sequences (17).

The *Dpn* I-resistant ORS*3*, *-8*, *-9*, and *-12* plasmid DNAs recovered at 72 hr posttransfection represent only a small

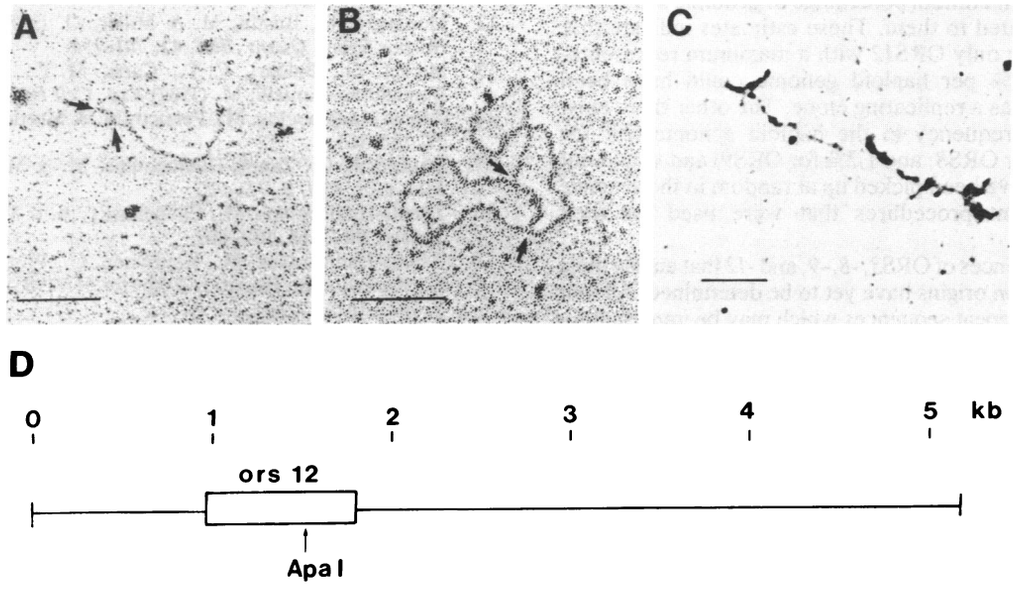

FIG. 4. EM of ORS*12* plasmid DNA isolated from transfected HeLa cells. ORS*12* plasmid DNA recovered from HeLa cells was spread for EM onto parlodion-coated copper grids, and the grids were stained with uranyl acetate and shadowed with platinum/palladium. Supercoiled (*A*) and relaxed circular (*B*) plasmids containing one replication bubble each were observed. Such molecules were linearized with *Apa* I (*C*), which cuts once within ORS*12* (*D*). Arrows indicate replication fork growing points. (Bars = 1 kb.)

fraction of the total DNA recovered for each plasmid. This suggests that the ORS plasmids may be undergoing controlled replication, as is characteristic of mammalian replicons that initiate once per cell cycle. In contrast, when CV-1 and COS-7 cells were transfected with SV40 DNA, the majority of the viral DNA recovered 72 hr posttransfection was *Dpn* I-resistant, which reflects the uncontrolled replication of SV40 (data not shown). When a time course of pBR/ORS*8*, *-9*, and *-12* replication was carried out, there was an approximate doubling of the intensity of the *Dpn* I-resistant DNA every 24 hr, as would be expected if the copy number of these plasmids in each cell cycle was controlled. The replication of the four ORS plasmids was found to be semiconservative by density-labeling with BrdUrd. Thus ORS*3*, *-8*, *-9*, and *-12* have biological activity that enables their pBR322 derivative plasmids to replicate in mammalian cells in a seemingly controlled manner.

Of the two methods used to assay DNA replication in this paper, the *Dpn* I resistance method gave the most variable results. *Dpn* I resistance has been commonly used to detect the replication of SV40 and polyoma viruses, which undergo multiple rounds of replication per S phase (18, 19, 26). Mammalian origins, however, are normally expected to initiate only one round of replication per cell cycle. ORS*3*, *-8*, *-9*, and *-12* have a seemingly reduced ability to undergo multiple rounds of DNA replication when they are cloned in pBR322 and transfected into mammalian cells. This results in a *Dpn* I-resistant band of much lower intensity than those produced by either SV40 or polyoma. This band cannot always be distinguished, either qualitatively or quantitatively, from a background *Dpn* I-resistant band contributed by pBR322 alone. Thus, we found it necessary to repeat these assays in order to identify those ORS that consistently produced *Dpn* I-resistant bands with intensities above background. In contrast, the assay of BrdUrd incorporation of the transfected ORS gave consistent results.

We have previously reported data showing that at least 12 of the ORS do not represent a random sample of the monkey genome but rather a unique subset of DNA sequences (17). In this study we have identified at least 4 of the 12 ORS (ORS*3*, *-8*, *-9*, and *-12*) that can support plasmid replication in mammalian cells. Based upon our previous estimates of the molecular abundance of each of the 4 replicating ORS (17), we estimated the maximum percentage of genomic sequences that could be related to them. These estimates indicate that of the four clones only ORS*12* with a maximum representation of about 24% per haploid genome could have been randomly picked as a replicating clone. The other three exist in much lower frequency in the haploid genome (2% for ORS*3*; 0.004% for ORS*8*; and 1.2% for ORS*9*) and would not be expected to have been picked up at random in the absence of the enrichment procedures that were used for their isolation (15, 17).

The exact sequences of ORS*3*, *-8*, *-9*, and *-12* that enable them to act as replication origins have yet to be determined. All four contain inverted repeat sequences which may be important for origin function. *Alu* sequences, which have been found by Johnson and Jelinek (25) to enable pBR322 derivatives (BLUR 8) to replicate in COS-7 cells, are present in ORS*3* and ORS*9* but not in ORS*8* or ORS*12*. As those authors suggest, *Alu* sequences may only function as replication origins in the presence of the SV40 T antigen. In another report, the 4.3-kb fragment containing the replication origin associated with the amplified domain of the Chinese hamster ovary cell line, CHOC 400, does not hybridize with *Alu* family repeats (10). When we looked at the replication of BLUR 8 plasmid in HeLa cells using the *Dpn* I assay, we found only weak *Dpn* I-resistant bands in comparison to those produced by pBR/ORS*8* and pBR/ORS*12* in the same experiment (data not shown). Therefore, in our system, *Alu* sequences are neither necessary nor sufficient for replication. The construction of a series of deletion mutants will be required to dissect the sequence requirements for ORS replication function.

Finally, it is interesting to speculate on the fact that ORS replication was most efficient in HeLa cells in comparison to that in CV-1 or COS-7 cells. It is conceivable that HeLa cells produce initiator proteins either in addition to or in higher concentration than those present in the quasi-normal CV-1 cells and in COS-7 cells. These proteins may recognize replication origins derived from monkey cells because of sequence and/or structural homologies that exist among the replication origins in the two species.

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