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Autonomous DNA Replication in Human Cells Is Affected by the Size and the Source of the DNA

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We previously developed short-term and long-term assays for autonomous replication of DNA in human cells. This study addresses the requirements for replication in these assays. Sixty-two random human genomic fragments ranging in size from 1 to 21 kb were cloned in a prokaryotic vector and tested for their replication ability in the short-term assay. We found a positive correlation between replication strength and fragment length, indicating that large size is favored for efficient autonomous replication in human cells. All large fragments replicated efficiently, suggesting that signals which can direct the initiation of DNA replication in human cells are either very abundant or have a low degree of sequence specificity. Similar results were obtained in the long-term assay. We also used the same assays to test in human cells a random series of fragments derived from *Escherichia coli* chromosomal DNA. The bacterial fragments supported replication less efficiently than the human fragments in the short-term and long-term assays. This result suggests that while the sequence signals involved in replication in human cells are found frequently in human DNA, they are uncommon in bacterial DNA.

In this study we investigated the genetic requirements for origin of replication function in human cells. The study utilizes a system we have developed that allows us to test fragments of DNA for autonomous replication when introduced into human tissue culture cells in both long- and short-term assays. This system should ultimately permit us to deduce the DNA sequence requirements for autonomous replication. It is likely that this information will be relevant for chromosomal replication as well.

Over the course of many cell divisions, extrachromosomal vectors are lost from the nuclei of mammalian cells unless they have a means of nuclear retention. The long-term replication assay that we developed overcomes the problem of plasmid loss by using a replication-defective vector derived from Epstein-Barr virus (EBV) that permits linked sequences to be retained in human cells (22). When random human DNA was cloned into this vector and introduced into human cells, we found that a large number of fragments could confer replication ability on the vector in a long-term assay. The resulting plasmids were stably maintained for at least 2 months in human cells.

The fragments could also provide for replication in our short-term assay (22). In this assay, the fragments are moved to a prokaryotic vector that lacks all viral sequences, and DNA is harvested 4 days after transfection, since the plasmids have no means of nuclear retention. These experiments indicated that the human DNA fragments that we had isolated contained sequences involved in the initiation of DNA replication in human cells. Subsequent studies have shown that, like the chromosomes, these plasmids undergo replication once per cell cycle in human cells (unpublished data). This characteristic makes the autonomously replicating system a reasonable model for chromosomal replication.

The human genomic fragments that mediated replication in the previous study (22) averaged at least 12 kb in size, even though the average fragment insert in the starting library was 6 kb. This result suggested that large fragments replicate more efficiently than small fragments. This conclusion was reinforced by our inability to subclone the replication activity present in large fragments (unpublished data). In this study we performed our replication assays on a random collection of human genomic fragments chosen on the basis of size alone. These experiments allow us to test whether large fragments are favored for replication. In addition, the experiments provide information about the fraction of genomic fragments that is positive in the assay, which bears on the frequency of replication initiation signals in the genome. We also compared the short- and long-term assays by using these randomly chosen fragments. A parallel study is presented that uses genomic DNA derived from *Escherichia coli* instead of human cells to determine whether this DNA can support replication in human cells. The data we have obtained indicate that replication in human cells, both in the preference for large size and in the apparently low level of sequence specificity, demonstrates features distinct from those which have been found in bacteria, plasmids, and viruses.

MATERIALS AND METHODS

Plasmids. p220.2, pDY$^-$, and pHEBO are EBV-based plasmids that have been described previously (22). pLIB16 and pLIB41 are plasmids based on pDY$^-$ that contain human DNA fragments (22). The plasmid pMLλ was created by cloning a 2.2-kb *Sau*3A fragment from the bacteriophage λ genome into pML, a 2.9-kb derivative of pBR322 which was isolated from the plasmid pJYM (24) by digestion with *Bam*HI. Two copies of the bacteriophage λ fragment were inserted in a head-to-tail arrangement into the *Bam*HI site to bring the total size of pMLλ to 7.4 kb. pNUT-*lac* is a 9.0-kb plasmid based on pMLλ. pNUT-*lac* contains a 1.6-kb *Hin*dIII spacer fragment which was derived from the *lacI* and *lacZ* coding regions and was isolated from the plasmid pUCR (13) and cloned into the unique *Hin*dIII site of pMLλ. *Not*I linkers were added to create two *Not*I sites flanking the two *Hin*dIII sites created by the insertion of the spacer

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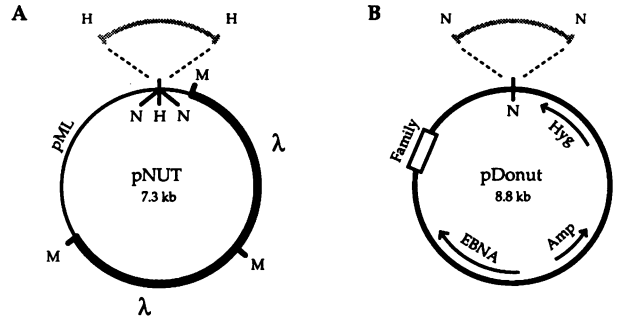

FIG. 1. Plasmids. (A) pNUT is a completely prokaryotic plasmid which contains two copies of a 2.2-kb *Mbo*I fragment isolated from the bacteriophage λ genome. This λ fragment serves as a marker fragment in the short-term replication assay in human cells (see text). Random genomic DNA from a *Hin*dIII partial digest was cloned into the unique *Hin*dIII site of pNUT. The two *Not*I sites which closely flank the *Hin*dIII site allow these inserts to be easily removed. The plasmid pMLλ is identical to pNUT, except that it does not contain the two *Not*I sites. (B) pDonut is identical to the previously described plasmid pDY$^-$, except that a *Not*I site has been added adjacent to the unique *Hin*dIII site of pDY$^-$. Genomic inserts in pNUT were isolated as *Not*I fragments and cloned into the *Not*I site of pDonut. H, *Hin*dIII; N, *Not*I; M, *Mbo*I; λ, 2.2-kb *Mbo*I fragment of bacteriophage λ DNA; pML, a derivative of pBR322; Family, family of repeats from EBV *oriP*; EBNA, gene for EBNA-1 of EBV; Amp, bacterial ampicillin-resistance gene; Hyg, gene for hygromycin resistance in human cells. The grey lines represent random genomic DNA inserts.

fragment. pNUT (Fig. 1A) is a 7.3-kb plasmid which is identical to pNUT-*lac*, except that it does not contain the 1.6-kb *Hin*dIII spacer fragment. Randomly selected inserts of both human and *E. coli* DNA, ranging from 1 to 25 kb in size, were cloned into the pNUT vector. Total genomic DNA was isolated from either human 293S cells or *E. coli* strain 295, a derivative of MC1061. Genomic DNA was partially digested with *Hin*dIII and run on a SeaPlaque agarose gel (FMC Bioproducts, Rockland, Maine). Gel slices corresponding to several different size ranges were excised and ligated with *Hin*dIII-digested pNUT DNA. The vector pDonut (Fig. 1B) was created from pDY$^-$ by the insertion of a *Not*I linker adjacent to the unique *Hin*dIII site of pDY−. A subset of human and *E. coli* DNA fragments was moved from pNUT to pDonut by digestion with *Not*I and ligation into the unique *Not*I site of pDonut. The plasmid pON405 is an 8.3-kb vector in which the immediate early promoter and enhancer of murine cytomegalovirus drive the expression of the *E. coli* gene for β-galactosidase (25).

Cell lines. The cell line 293S is a derivative of the human embryonic kidney cell line 293 (32). The cell line 293S/EBNA was created by calcium phosphate transfection (37) of 293S with a vector that expresses *EBNA-1* from the major immediate early promoter of human cytomegalovirus (34) and contains the neomycin-resistance gene. A clonal isolate of the transfected cells was grown in the presence of 200 μg of neomycin per ml for 4 weeks. The resulting cells have at least one integrated copy of the *EBNA-1* expression unit. CV-1 is an African green monkey kidney fibroblast cell line (19). Cell lines containing EBV plasmids were created from CV-1 by transfection of 10 μg of plasmid DNA by using calcium phosphate coprecipitation which was followed by selection in medium containing hygromycin at a concentration of 200 μg/ml. NC-37 is a human lymphoid cell line (6). Cell lines containing EBV plasmids were created from NC-37 by electroporation (4) with 30 μg of the plasmids p220 and pDY$^-$ and 60 μg of the plasmids pLIB16 and pLIB41 which was followed by selection in medium containing 300 μg of hygromycin per ml. More plasmid DNA was used for pLIB16 and pLIB41 to compensate for the reduced electroporation efficiency of large plasmids. NC-37 cells were grown in RPMI medium, and 293S, 293S/EBNA, and CV-1 cells were grown in Dulbecco modified Eagle medium. Both media contained 10% fetal calf serum, penicillin, and streptomycin, and all cells were grown at 37°C in a 5% CO_2 incubator.

Short-term replication assay. 293S cells were grown to 80 to 90% confluency and split 1:20 into 60-mm dishes containing 5 ml of fresh medium and grown for 24 h. Cells were transfected with 5 μg of plasmid DNA by using calcium phosphate coprecipitation. One day posttransfection, the growth medium was replaced with fresh medium. Two days posttransfection, plates were split 1:4 into 60-mm dishes. Four days posttransfection, plasmid DNA was recovered by the method of Hirt (15). One-half of the sample extracted from a single 60-mm dish was digested with *Hin*dIII and *Mbo*I, electrophoresed on a 0.65% agarose gel, and transferred to a Zetaprobe membrane (Bio-Rad, Richmond, Calif.) by using 0.4 N NaOH as the transfer buffer. Filters were probed with the ^{32}P-labeled 2.2-kb λ fragment from pNUT. Autoradiograms were scanned with the Molecular Dynamics 300A computing densitometer by using volume integration for quantification of all bands.

Long-term replication assay. 293S or 293S/EBNA cells (80 to 90% confluent) were split 1:10 into 100-mm dishes containing 10 ml of fresh medium, and cells were grown for 24 h. Cells were then cotransfected with 9.5 μg of test plasmid and 0.3 μg of pON405 by calcium phosphate coprecipitation. Approximately 3 days following transfection, cells were stained for β-galactosidase activity with the fluorogenic substrate fluorescein di-β-galactopyranoside (Molecular Probes, Eugene, Ore.) and sorted on the fluorescence-activated cell sorter (FACS) as previously described (30). The same β-galactosidase level was used as the FACS gate for every sample. This gate determined whether a cell was selected by the FACS for propagation; cells at or above this set level of β-galactosidase expression were sorted into nonselective media, while those cells below this cutoff were discarded. This gate was used to sort cells with the highest β-galactosidase expression levels, a group which made up approximately 10% of the total transfected population and was completely nonoverlapping with the distribution of endogenous β-galactosidase activity demonstrated by untransfected 293S or 293S/EBNA cells. Cells were sorted directly into 60-mm dishes containing 2 ml of fresh medium and 3 ml of conditioned medium and grown continuously in culture without hygromycin selection. At 25 days posttransfection, plasmid DNA was isolated by Hirt extraction and run both uncut and digested with *Mbo*I on a 0.575% agarose gel. Blotting was performed as described for the short-term assay. Filters were probed with ^{32}P-labeled pDY$^-$ DNA, and autoradiograms were scanned and quantitated as with the short-term assay.

RESULTS

Replication of human sequences in the cell lines NC-37 and CV-1. We previously developed a long-term replication assay in the human fibroblast cell line 293S (22). By using this protocol, human DNA fragments were isolated that could serve as functional origins of replication (22). Two

vectors recovered from such a selection, pLIB16 and pLIB41, contain human DNA fragments of 14 and 20 kb, respectively. To ensure that the ability of these vectors to replicate autonomously was not limited to 293S cells, we tested the abilities of pLIB16 and pLIB41 to replicate autonomously in two other cell lines. These two cell lines, NC-37 and CV-1, do not produce the adenovirus-transforming genes that are present in 293S cells. Furthermore, NC-37 is from a different tissue type and CV-1 is from a different species.

An EBV-immortalized human lymphoid cell line, NC-37, was electroporated with the plasmids p220.2, pDY$^-$, pLIB16, and pLIB41. The cells were placed under hygromycin selection and grown continuously in culture for 52 days, at which time plasmid DNA was isolated by Hirt extraction (15). Equal amounts of recovered extract for each plasmid were run on an agarose gel, both uncut and treated with the restriction enzyme *Mbo*I. *Mbo*I only cuts DNA which has completely lost its bacterial methylation pattern, so that only plasmid DNA molecules that have replicated at least twice in NC-37 cells will be susceptible to digestion with *Mbo*I. Such plasmids will release a 2.8-kb *Mbo*I fragment found in the pDY$^-$ vector backbone of each plasmid. The gel was blotted and then probed with ^{32}P-labeled pDY$^-$ DNA. The resulting autoradiogram is shown in Fig. 2A. NC-37 contains endogenous copies of EBV that appear upon hybridization with pDY$^-$, as demonstrated by the two lanes on the blot containing Hirt extract from untransfected NC-37 cells grown in nonselective conditions. This endogenous signal gave rise to a high-molecular-weight band in the uncut lane and a 2.5-kb band in the *Mbo*I lane in every sample, which serve as a measure of gel loading. pDY$^-$ showed no *Mbo*I band at 2.8 kb, indicating that this plasmid did not replicate. However, the plasmids p220.2, pLIB16, and pLIB41 were all completely digested by *Mbo*I, indicating that they had replicated in the NC-37 cells. Their uncut samples ran in agreement with their respective uncut markers, showing that the plasmids in NC-37 were of the correct size and were extrachromosomal.

The plasmids p220.2, pDY$^-$, pLIB16, and pLIB41 were also tested in CV-1, a monkey fibroblast cell line which does not contain endogenous copies of EBV. CV-1 cells were transfected by using calcium phosphate coprecipitation. Cells were propagated for 40 days under hygromycin selection and then harvested and blotted as with NC-37. The resulting autoradiogram is shown in Fig. 2B. The result with CV-1 was the same as with the cell lines 293S and NC-37: pDY$^-$ did not replicate, while p220.2, pLIB16, and pLIB41 all replicated autonomously as extrachromosomal plasmids. These results indicate that the human sequences we have cloned provide for replication in a variety of cell lines. The experiments were not done in such a way that we could establish whether replication of the human sequences was significantly more efficient in some cell lines than in others.

Short-term replication of human DNA fragments. In order to rigorously determine whether fragment size affected replication efficiency, a collection of 62 random human genomic fragments was made. The fragments ranged in size from 1 to 21 kb and were chosen on the basis of size alone, without prior selection for replication ability. The fragments were cloned into the prokaryotic vector pNUT (Fig. 1A), so that the replication abilities of a large set of fragments over a broad size range could be examined in a short-term assay in the absence of EBV sequences. Each experiment consisted of a set of separate transfections of 5 μg of purified plasmid DNA into 293S cells. 293S cells were used because we

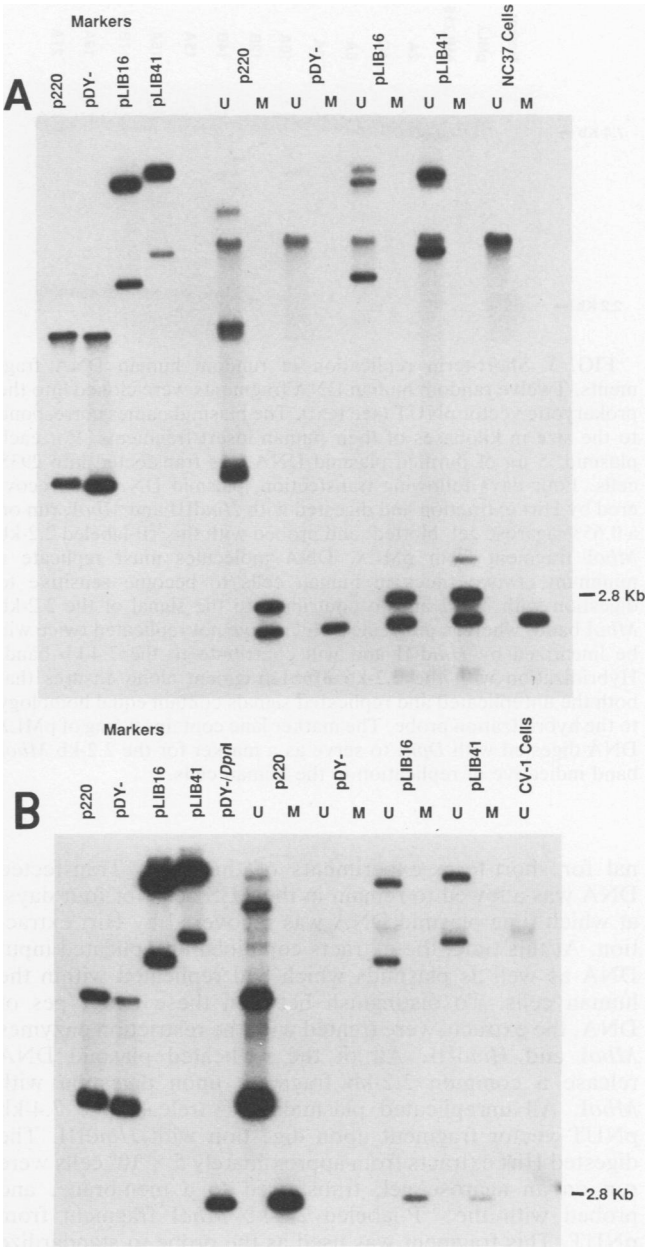

FIG. 2. Replication of human sequences in NC-37 and CV-1. NC-37 (A) or CV-1 (B) cells were transfected with the designated plasmids, placed under hygromycin selection, and passaged for 52 (NC-37) or 40 (CV-1) days. The Hirt extract from approximately 10^6 cells was run uncut (lanes U) or digested with *Mbo*I (lanes M) on an agarose gel, blotted, and probed with ^{32}P-labeled pDY$^-$ DNA. The marker lanes for both panels A and B contain 0.5 ng of uncut plasmid DNA. In panel B, the lane pDY$^-$/*Dpn*I contains 0.5 ng of pDY$^-$ plasmid DNA cut with *Dpn*I to release the 2.8-kb *Mbo*I fragment indicative of replication. Lanes in panel A containing Hirt extract from NC-37 cells show hybridization signals from endogenous EBV genomes (see text). Bands indicative of plasmid dimer formation can be observed in the uncut Hirt extract lanes for the plasmids p220.2 (A and B), pLIB16 (A), and pLIB41 (A).

previously established that plasmids replicating with human sequences follow correct once-per-cell-cycle control in these cells and because of their high transfection efficiency with calcium phosphate coprecipitation, generating sufficient sig-

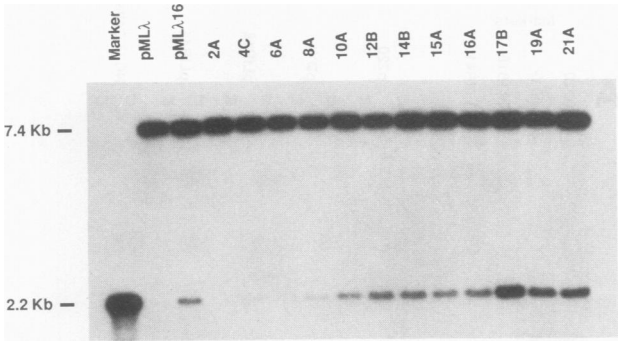

FIG. 3. Short-term replication of random human DNA fragments. Twelve random human DNA fragments were cloned into the prokaryotic vector pNUT (see text). The plasmid names correspond to the size in kilobases of their human insert fragments. For each plasmid, 5 μg of purified plasmid DNA was transfected into 293S cells. Four days following transfection, plasmid DNA was recovered by Hirt extraction and digested with *Hin*dIII and *Mbo*I, run on a 0.65% agarose gel, blotted, and probed with the ^{32}P-labeled 2.2-kb *Mbo*I fragment from pMLλ. DNA molecules must replicate a minimum of two times in human cells to become sensitive to digestion with *Mbo*I and to contribute to the signal of the 2.2-kb *Mbo*I band, whereas molecules which have not replicated twice will be linearized by *Hin*dIII and will contribute to the 7.4-kb band. Hybridization with the 2.2-kb *Mbo*I fragment alone ensures that both the unreplicated and replicated signals contain equal homology to the hybridization probe. The marker lane contains 0.5 ng of pMLλ DNA digested with *Dpn*I to serve as a marker for the 2.2-kb *Mbo*I band indicative of replication in the human cells.

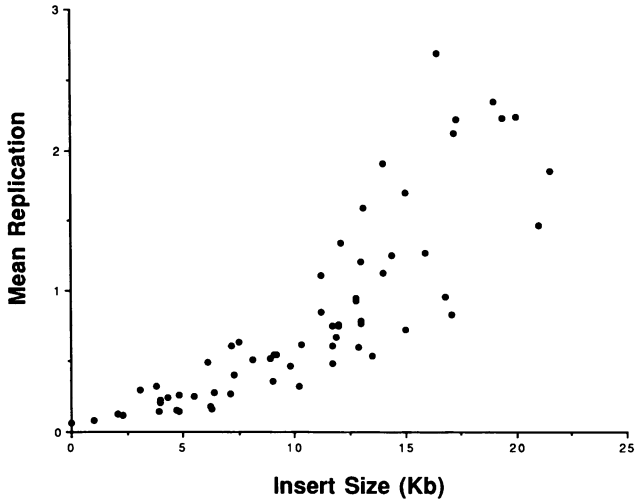

FIG. 4. Survey of the short-term replication abilities of 62 random human DNA fragments. Three trials of the short-term replication assay were carried out on each of 62 random human DNA fragments cloned into the prokaryotic plasmid pNUT (see text). In each trial, the amount of replicated signal from each plasmid containing a random human DNA fragment was normalized to the amount of signal produced by the plasmid pMLλ16. The mean value of normalized replication from these three trials is plotted versus the size in kilobase pairs of the random human DNA inserts.

nal for short-term experiments of this type. Transfected DNA was allowed to remain in the 293S cells for four days, at which time plasmid DNA was recovered by Hirt extraction. At this time, the extracts contained unreplicated input DNA as well as plasmids which had replicated within the human cells. To distinguish between these two types of DNA, the extracts were treated with the restriction enzymes *Mbo*I and *Hin*dIII. All of the replicated plasmid DNA release a common 2.2-kb fragment upon digestion with *Mbo*I. All unreplicated plasmid DNA release the 7.4-kb pNUT vector fragment upon digestion with *Hin*dIII. The digested Hirt extracts from approximately 5×10^6 cells were run on an agarose gel, transferred to a membrane, and probed with the ^{32}P-labeled 2.2-kb *Mbo*I fragment from pNUT. This fragment was used as the probe to standardize the hybridization, so that both the unreplicated input DNA present in the 7.4-kb *Hin*dIII band and the 2.2-kb *Mbo*I band resulting from the replicated DNA molecules would contain equal homology to the probe. The plasmids pMLλ and pMLλ16 were included in each experiment as controls. pMLλ is effectively the vector pNUT containing no human DNA insert and served as a negative control (Fig. 1A). pMLλ16 contains the 14-kb human fragment from pLIB16 (22) and served as a positive control. An autoradiogram resulting from one such experiment is shown in Fig. 3. This experiment involved 12 of the 62 human inserts cloned into pNUT, selected to represent the entire size range studied. The names of the plasmids correspond to the size in kilobase pairs of their human DNA inserts. The gel loadings were adjusted so that each sample lane contained approximately equal amounts of unreplicated input DNA, as judged by the amount of hybridization signal from the 7.4-kb band. The plasmid pMLλ showed very little replication activity. The intensity of the 2.2-kb *Mbo*I band for each plasmid containing a human insert indicated that there was a relationship between the amount of replication signal produced and the size of the human DNA insert, with plasmids containing larger inserts yielding more *Mbo*I-sensitive DNA.

The replication efficiency in these short-term assays was quantitated by densitometry. The autoradiogram from each experiment was scanned by using a Molecular Dynamics scanning densitometer, and for each plasmid the amounts of signal in both the unreplicated (7.4-kb *Hin*dIII) and the replicated (2.2-kb *Mbo*I) bands were determined. The proportion of replicated plasmid was calculated by dividing the value of the replicated signal (*Mbo*I band) by the total signal (*Hin*dIII and *Mbo*I bands). The resulting value for each plasmid was then normalized to the amount of replication generated by the pMLλ16 sample on the same autoradiogram. In this way, pMLλ16 could serve as a standard of comparison between experiments.

A survey of the replication efficiency of the 62 plasmids containing random human DNA inserts was performed, with three separate experimental trials of each plasmid. In each trial, the replication activity of the human fragment in pNUT was normalized to the activity of pMLλ16. The mean value of the three trials was calculated for the 62 inserts and plotted versus insert fragment size in Fig. 4. From this data, there appeared to be a continuum of replication activity, generally correlating with the size of the human fragment. Progressively larger human DNA fragments provided increasing amounts of replication. This finding indicated that the sequence signals necessary for a fragment to promote replication in the short-term assay were abundant in human DNA.

Short-term replication of bacterial DNA. To test whether replication signals were specific to human DNA, we compared the replication ability of DNA isolated from bacteria with that of human DNA in the short-term replication assay. Ten random *E. coli* DNA fragments, ranging in size from 1 to

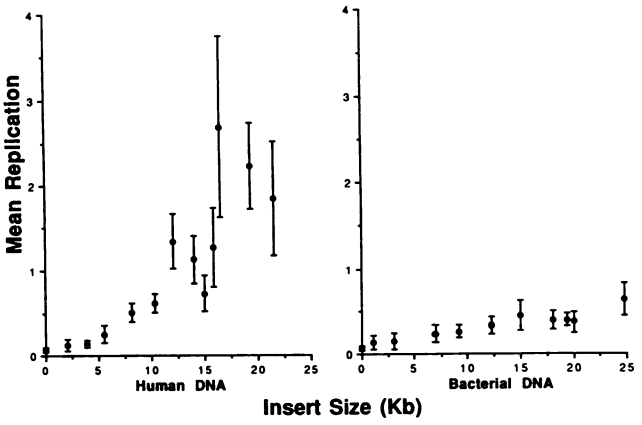

FIG. 5. Comparison of the short-term replication abilities of human and bacterial DNA fragments. Twelve human and 10 bacterial random DNA inserts were cloned into the prokaryotic vector pNUT. A total of 10 experimental trials of the short-term replication assay in human 293S cells were performed on each plasmid (see text). The resulting autoradiograms from each trial (see Fig. 3) were scanned and quantitated via laser densitometry. In each trial, the amount of replicated signal was normalized to the amount of signal produced by a previously studied 14-kb human DNA fragment contained in the plasmid pMLλ16. The mean values of normalized replication were calculated for each plasmid and plotted versus insert size for both human and bacterial DNA inserts. The error bars represent 95% confidence intervals from a two-tailed *t* test.

25 kb, were cloned in pNUT. These plasmids were assayed for short-term replication ability as described above for the constructs containing random human DNA fragments. Each plasmid containing a bacterial DNA insert was tested in 10 separate trials, with the amount of replication signal normalized to that of pMLλ16 in each trial. In parallel, the 12 human fragments shown in Fig. 3 were also run in 10 trials of the short-term replication assay. The mean values of normalized replication for the 10 trials are plotted versus insert fragment size for both human and bacterial DNA inserts in Fig. 5. The error bars on each point represent 95% confidence intervals from a two-tailed *t* test. The data in Fig. 5 illustrate that the human DNA fragments in pNUT were noticeably more efficient than the *E. coli* DNA fragments in the short-term assay. The *E. coli* fragments showed an effect of insert size on replication ability, with some larger inserts performing more efficiently than smaller ones, although this effect was less pronounced than that seen with human DNA. Nevertheless, bacterial DNA fragments generally promoted significantly less replication than their human DNA counterparts.

Long-term replication in 293S cells. We wished to test the replication abilities of the same human and bacterial DNA fragments in a long-term replication assay. To achieve this end, the 12 human and 10 bacterial DNA fragments whose short-term replication efficiencies were plotted in Fig. 5 were moved into the vector pDonut (Fig. 1B). The vector pDonut is essentially identical to the vector pDY$^-$, with the addition of a single *Not*I site. pDonut provides the EBV components necessary for nuclear retention in human cells. This vector thus allows us to test the abilities of the human and bacterial DNA fragments to provide for long-term replication in human cells. Each of the DNA fragments was released from the short-term assay vector pNUT by digestion with *Not*I and was cloned into *Not*I-digested pDonut.

The resulting plasmids were separately transfected into human 293S cells and grown without selection for 25 days. Plasmid DNA was then Hirt extracted from the cells and analyzed by Southern blotting to determine the copy number of each plasmid. We were able to avoid growing the cells under drug selection by using a FACS in our transfection protocol. This approach also served as a means of standardizing for transfection efficiency.

The FACS-transfection protocol (30) was performed by cotransfecting 293S cells with test plasmid (a human or bacterial DNA fragment in pDonut) and with the plasmid pON405, which expresses the *E. coli* gene for β-galactosidase in human cells. Three days following transfection, cells were trypsinized and stained intracellularly for β-galactosidase activity with the fluorogenic substrate fluorescein di-β-galactopyranoside. Intracellular β-galactosidase cleaves fluorescein di-β-galactopyranoside to release fluorescein, which can be detected and quantitated by the FACS. Cells which demonstrated a high level of fluorescein after staining with fluorescein di-β-galactopyranoside contained a high level of β-galactosidase and therefore must have received transfected plasmid DNA. Cells with high levels of β-galactosidase activity were sorted by the FACS into nonselective media and propagated in culture, while all other cells were discarded. We determined that the level of β-galactosidase activity derived from cotransfection with pON405 correlated with the amount of test plasmid that was transfected into the cells (data not shown).

Equal amounts of Hirt extract for each plasmid were run as both uncut and *Mbo*I-digested samples on an agarose gel, which was then blotted and probed with ^{32}P-labeled pDY$^-$ DNA. The four resulting autoradiograms, containing the 10 bacterial and 12 human DNA fragments in pDonut, are shown in Fig. 6. The uncut lanes demonstrated that the plasmids were extrachromosomal and of the correct size, while the *Mbo*I-digested lanes verified that all of the plasmid DNA had replicated in the human cells. Each blot represents a set of plasmids whose transfections were sorted at the same time on the FACS, along with the control plasmids pLIB16 and pDonut. These samples were propagated simultaneously and were blotted together on the same hybridization filter.

The long-term assay measured plasmid copy number 25 days after transfection into human cells. The blots in Fig. 6 show that the plasmids containing larger human DNA inserts produced higher plasmid copy numbers in 293S cells. The bacterial DNA inserts, on the other hand, did not produce detectable plasmid copy numbers, regardless of insert size.

Long-term replication in 293S/EBNA cells. The sorting of transfected cells by using the FACS and propagation in nonselective media overcame the necessity of these plasmids to express the gene for hygromycin resistance. However, the assay still required that each plasmid express the gene encoding the EBV protein EBNA-1, which is necessary for the nuclear retention of these plasmids. Without production of the EBNA-1 protein, plasmids would be lost from the cell nucleus and would not appear in Hirt extracts of the cell population at day 25, regardless of their ability to replicate. Consequently, the possibility remained that the plasmids carrying bacterial DNA fragments had failed to produce appreciable plasmid copy numbers because they had inhibited expression of the *EBNA-1* gene.

To address this possibility, we performed the long-term replication assay with the cell line 293S/EBNA. This cell line is a derivative of 293S and contains a stably integrated copy of the *EBNA-1* gene. Plasmids containing random DNA inserts transfected into this cell line no longer require

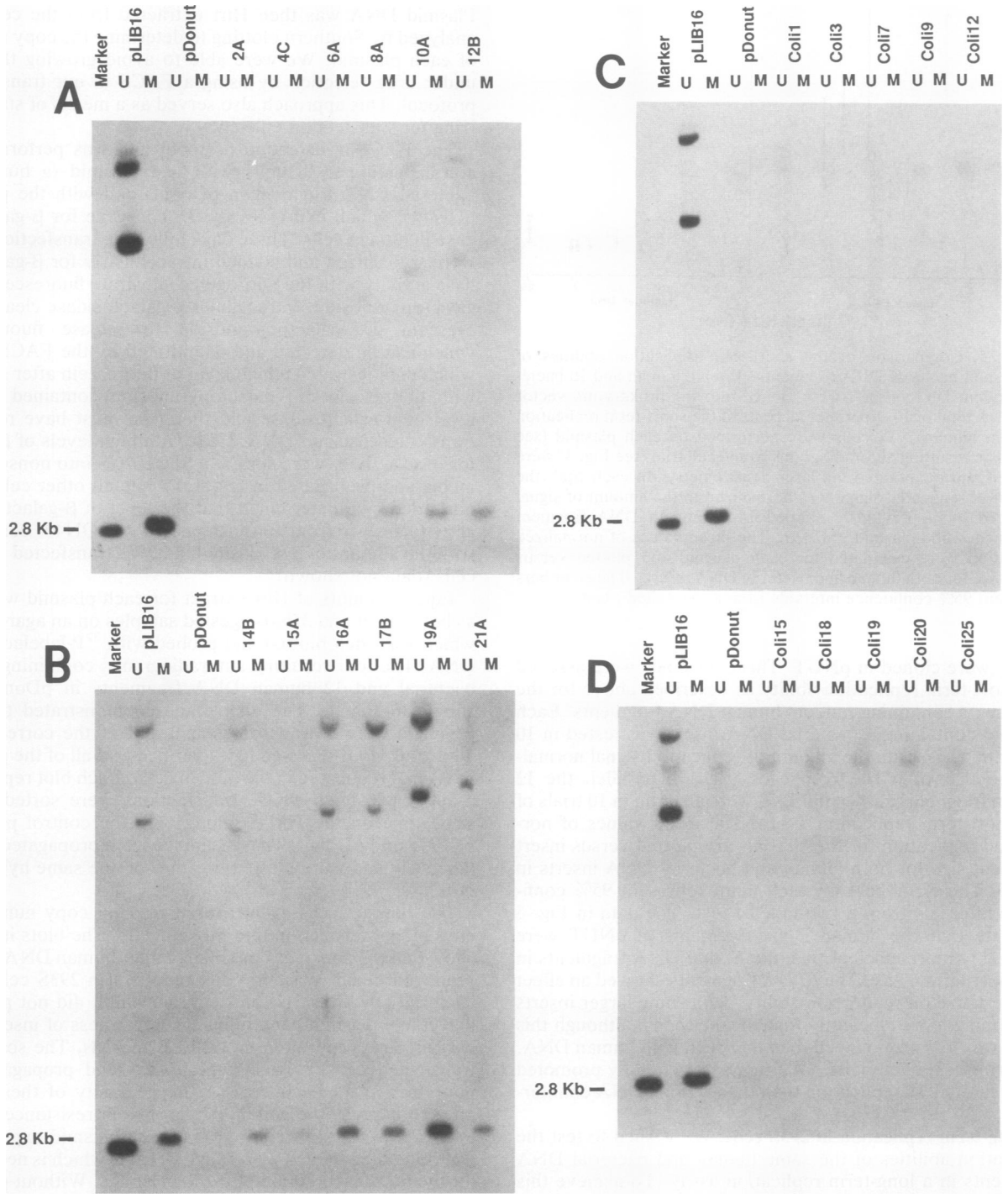

FIG. 6. Long-term replication assay with 293S cells. Each blot represents a set of plasmids containing either human or bacterial DNA inserts cloned into the vector pDonut and the control plasmids pLIB16 and pDonut. The names of the plasmids represent the size of the DNA insert in kilobase pairs. Panels A and B contain the plasmids with human DNA inserts of 2 to 12 and 14 to 21 kb, respectively, and panels C and D contain the plasmids with bacterial DNA inserts of 1 to 12 and 15 to 25 kb, respectively. For each sample, 9.5 μg of test plasmid was cotransfected via calcium phosphate coprecipitation with 0.3 μg of the nonreplicating vector pON405, which expresses the *E. coli* β-galactosidase gene. Three days following transfection, cells were stained for β-galactosidase activity, and cells with high levels of β-galactosidase activity (those cells which must have received plasmid DNA during the transfection) were sorted into nonselective media by using the FACS. Cells were propagated continuously in culture, and at 25 days posttransfection, plasmid DNA was recovered by Hirt extraction. Equal amounts of Hirt extract were run on an agarose gel as both uncut (lanes U) or *Mbo*I-digested (lanes M) samples. The gels were blotted, and filters were probed with ^{32}P-labeled pDY$^-$ DNA. The marker lane on each blot contains 1 ng of pDY$^-$ plasmid DNA digested with *Dpn*I to release the 2.8-kb *Mbo*I fragment indicative of replication of the test plasmids in human cells.

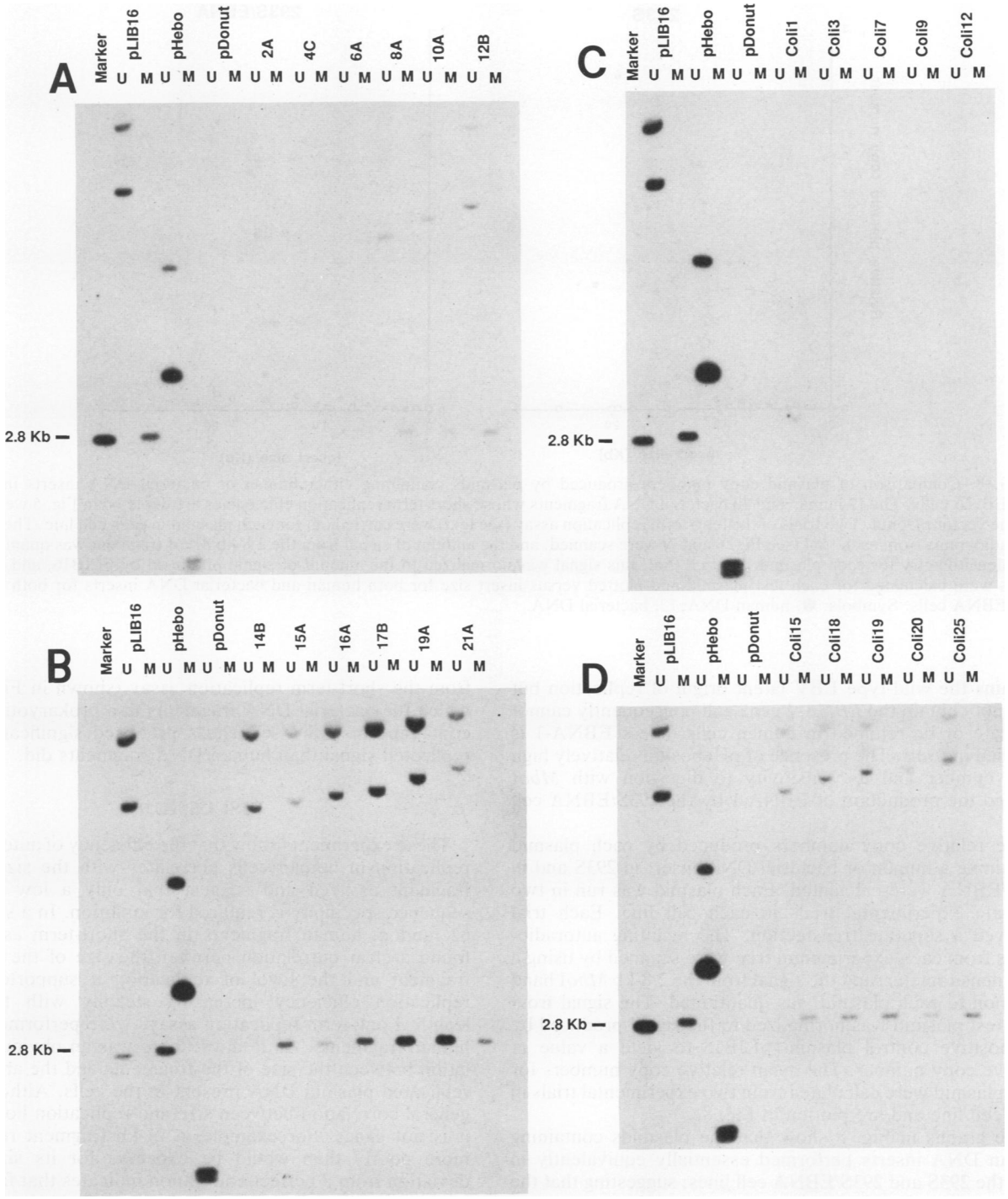

FIG. 7. Long-term replication assay with 293S/EBNA cells. The long-term replication assay was carried out as described for 293S cells in the legend to Fig. 6. The names of the plasmids represent the size of the DNA insert in kilobase pairs. Panels A and B contain the plasmids with human DNA inserts of 2 to 12 and 14 to 21 kb, respectively, and panels C and D contain the plasmids with bacterial DNA inserts of 1 to 12 and 15 to 25 kb, respectively. The plasmid pHEBO, which does not contain the *EBNA-1* gene, was included in each experiment as a control to verify the expression of EBNA-1 in *trans* by the 293S/EBNA cells. The 2.8-kb *Mbo*I fragment, indicative of replication in human cells, is derived from the *EBNA-1* gene, so this fragment is not found in pHEBO. U, Uncut samples; M, *Mbo*I-digested samples.

expression of the *EBNA-1* gene carried in *cis*. The 12 human and 10 bacterial DNA fragments in pDonut were transfected into 293S/EBNA cells. The cells were then sorted by using the FACS, propagated in culture, harvested, and blotted as described above for the long-term replication experiments in 293S. The results of these experiments are shown in Fig. 7. The plasmid pHebo was added as an additional control in each experiment performed in 293S/EBNA. This plasmid

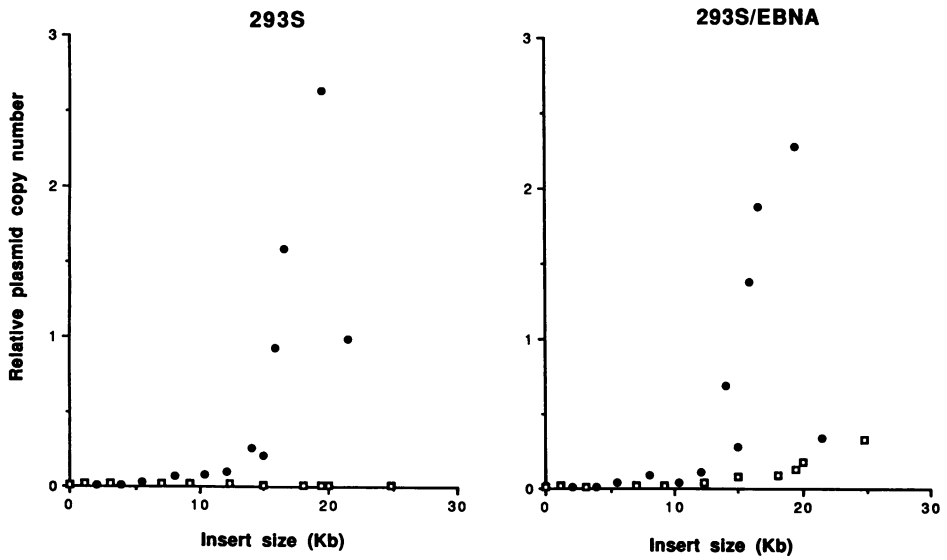

FIG. 8. Comparison of plasmid copy numbers produced by plasmids containing either human or bacterial DNA inserts in 293S or 293S/EBNA cells. The 12 human and 10 bacterial DNA fragments whose short-term replication efficiencies are depicted in Fig. 5 were moved into the vector pDonut. Two trials of the long-term replication assay (see text) were carried out for each plasmid in each cell line. The resulting autoradiograms from each trial (see Fig. 6 and 7) were scanned, and the amount of signal from the 2.8-kb *Mbo*I fragment was quantitated via laser densitometry for each plasmid. In each trial, this signal was normalized to the amount of signal produced by pLIB16, and the mean values were calculated for each test plasmid and plotted versus insert size for both human and bacterial DNA inserts for both 293S and 293S/EBNA cells. Symbols: ●, human DNA; □, bacterial DNA.

contains the wild-type EBV latent origin of replication but does not contain the *EBNA-1* gene and consequently cannot replicate or be retained in human cells unless EBNA-1 is supplied in *trans*. The presence of pHebo at a relatively high copy number and its sensitivity to digestion with *Mbo*I verified the production of EBNA-1 by the 293S/EBNA cell line.

The relative copy numbers produced by each plasmid containing a human or bacterial DNA insert in 293S and in 293S/EBNA were calculated. Each plasmid was run in two separate experimental trials in each cell line. Each trial involved a separate transfection. The resulting autoradiograms from each experimental trial were scanned by using a laser densitometer, and the signal from the 2.8-kb *Mbo*I band common to each plasmid was quantitated. The signal from each test plasmid was normalized to the signal produced by the positive control plasmid pLIB16 to yield a value of relative copy number. The mean relative copy numbers for each plasmid were calculated from two experimental trials in each cell line and are plotted in Fig. 8.

The graphs in Fig. 8 show that the plasmids containing human DNA inserts performed essentially equivalently in both the 293S and 293S/EBNA cell lines, suggesting that the further addition of the *EBNA-1* gene product in *trans* did not enhance or alter the ability of these inserts to produce their characteristic plasmid copy numbers. By contrast, the plasmids containing bacterial DNA inserts produced plasmid copy numbers above background in 293S/EBNA cells, compared with their performance in 293S cells. This observation supports the hypothesis that the bacterial DNA fragments interfered with the expression of the *EBNA-1* gene from the pDonut vector. Nevertheless, the relative plasmid copy numbers produced by the plasmids containing bacterial DNA inserts in 293S/EBNA cells were noticeably lower than the copy numbers produced by the plasmids containing the human DNA inserts. This result parallels the observation from the short-term replication assay (shown in Fig. 5), in which the bacterial DNA fragments in a prokaryotic vector containing no EBV sequences produced significantly less replicated signal than human DNA fragments did.

DISCUSSION

These experiments show that the efficiency of autonomous replication in human cells correlates with the size of the fragment assayed and suggest that only a low level of sequence specificity is required for initiation. In a survey of 62 random human fragments in the short-term assay, we found a clear correlation between the size of the inserted fragment and the level of replication it supported, with replication efficiency increasing steadily with fragment length. Long-term replication assays were performed on 12 human fragments. At 25 days, there was an obvious correlation between the size of the fragments and the amount of replicated plasmid DNA present in the cells. Although the general correlation between size and replication holds true, it is not exact. For example, a 21-kb fragment replicates more poorly than would be expected for its size. This deviation from a perfect correlation indicates that factors in addition to size determine replication efficiency. The sequence composition of the fragments must also play a role.

The validity of the long-term assay in cells other than 293S cells was verified by testing two of the plasmids in human lymphoid NC-37 cells and in monkey CV-1 fibroblasts. The finding that plasmids containing human sequences were able to replicate autonomously in both of these cell lines indicates that this behavior is likely to be generalizable to a wide variety of mammalian cells and cannot be attributed to any particular property of 293S cells, such as presence of transforming genes from adenovirus.

The ability of all the large fragments tested to replicate raises the possibility that the level of sequence specificity

required for replication initiation might be low. To gain further information about this question, we tested 10 random fragments derived from the *E. coli* chromosome covering the same size range as the human fragments. In both the short-term and the long-term assays, the *E. coli* fragments replicated more poorly than similarly sized human fragments. It is likely that the bacterial fragments interfered with gene expression in *cis* on the pDonut vector in a way that human fragments did not. Poor transcription might also provide an explanation for the poor replication behavior of the bacterial sequences. An association between replication and transcription has been suggested by the close linkage of transcriptional signals and origins of replication in many viruses (3, 5, 20) and by the general correlation between transcriptional activity and early replication in mammalian cells (8, 11).

The dependence of replication efficiency on fragment size that we have documented in this study has not been seen in studies of origins of replication in prokaryotes and viruses. Typically, origins of replication have been identified as specific sequences of approximately 50 to 250 bp which can be inactivated by mutation. An isolated origin of replication can support the replication of linked sequences, regardless of the size of the plasmid. For example, *oriC*, which is responsible for the replication of the entire *E. coli* chromosome, works equally well supporting the replication of a small plasmid (31). Similarly, the origins of replication from simian virus 40, EBV, and other viruses and plasmids can support autonomous replication of small plasmid vectors (24, 33). In addition, the *ARS* sequences from the yeast *Saccharomyces cerevisiae*, which have in some cases been associated with yeast chromosomal origins (17), can replicate as small plasmids with no apparent requirement for large size (14, 29). By contrast, our findings may indicate a need for a more extensive expanse of DNA to obtain efficient initiation in human cells.

Autonomous replication of vectors that are based on small fragments of genomic DNA in mammalian cells has been reported (7, 18, 27). However, these results have not been reproducible in the hands of other investigators (2).

Our data suggest that only a low degree of sequence specificity is required to initiate DNA replication in human cells. The fact that all large human DNA fragments tested showed efficient replication indicates that replication signals must exist on average at least every 10 kb in human DNA. This result is in contrast with the results observed in organisms with small genomes, whose origins have been shown to involve very specific DNA sequences. These sequences, which are recognized by one or more specific proteins, are required for initiation and localize the start of DNA synthesis to the origin region.

Organisms with larger genomes appear to use different strategies. In the yeast *S. cerevisiae*, a specific sequence is involved in encoding origins of replication, but the sequence is only 11 bp long (29). Flanking sequences affect origin function, but these sequences vary from one origin to another, implying that there are many ways to fulfill the sequence requirements for origin function. Initiation appears to be localized to the vicinity of the 11-bp consensus sequence (1, 16). In the yeast *Saccharomyces pombe* there may not be a consensus sequence required for autonomous replication (26).

Higher eukaryotic cells may follow this apparent trend away from highly specified origins of replication. DNA injected into unfertilized *Xenopus laevis* eggs replicates without regard to sequence composition (10, 28). While evidence has been presented that a specific origin of replication is associated with the Chinese hamster dihydrofolate reductase gene (2, 9, 12, 23, 35), recent results obtained by using two-dimensional gel techniques appear to be in contradiction to these findings and suggest instead that initiation is dispersed over a region of at least 30 kb (36). Our data are more consistent with relaxed sequence requirements for initiation. Specific initiation on the chromosomes, if it exists, may be determined by other factors such as chromatin configuration or attachment to the nuclear matrix. These features may, in turn, be determined by the transcriptional activity of the sequence. A relaxed sequence specificity for initiation on our plasmids is supported by two-dimensional gel analysis (1, 21). These studies are consistent with dispersed initiation throughout the plasmid rather than a fixed initiation point. The connection between the effects seen on autonomous replicons and chromosomal replication remains to be determined. At this stage of our knowledge, the possibility must be considered that initiation of replication in higher eukaryotic cells may not have the strong sequence requirements that are characteristic of the smaller genomes of bacteria, plasmids, and viruses.

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REFERENCES

1. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on ARS plasmids in *S. cerevisiae*. Cell **51**:463–471.
2. **Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis.** 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell **62**: 955–965.
3. **Cheng, L., and T. J. Kelly.** 1989. Transcriptional activator nuclear factor I stimulates the replication of SV40 minichromosomes in vivo and in vitro. Cell **59**:541–551.
4. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
5. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell **52**:635–638.
6. **Durr, F. E., J. H. Monroe, R. Schmitter, K. A. Traul, and Y. Hirshaut.** 1970. Studies on the infectivity and cytopathology of Epstein-Barr virus in human lymphoblastoid cells. Int. J. Cancer **6**:436–449.
7. **Frappier, L., and M. Zannis-Hadjopoulos.** 1987. Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. Proc. Natl. Acad. Sci. USA **84**:6668–6672.
8. **Goldman, M. A., G. P. Holmquist, M. C. Gray, L. A. Caston, and A. Nag.** 1984. Replication timing of genes and middle repetitive sequences. Science **224**:686–692.
9. **Handeli, S., A. Klar, M. Meuth, and H. Cedar.** 1989. Mapping replication units in animal cells. Cell **57**:909–920.
10. **Harland, R. M., and R. A. Laskey.** 1980. Regulated replication of DNA microinjected into eggs of X. laevis. Cell **21**:761–771.
11. **Hatton, K. S., V. Dhar, E. H. Brown, M. A. Iqbal, S. Stuart, V. T. Didamo, and C. L. Schildkraut.** 1988. Replication program of active and inactive multigene families in mammalian cells. Mol. Cell. Biol. **8**:2149–2158.
12. **Heintz, N. H., and J. L. Hamlin.** 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. USA **79**:4083–4087.
13. **Heinzel, S. S., P. J. Krysan, M. P. Calos, and R. B. DuBridge.** 1988. Use of simian virus 40 replication to amplify Epstein-Barr virus shuttle vectors in human cells. J. Virol. **62**:3738–3746.

14. **Hieter, P., C. Mann, M. Snyder, and R. W. Davis.** 1985. Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. Cell **40**:381–392.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. **26**:365–369.
16. **Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. El-Assouli, and L. R. Davis.** 1987. The *in vivo* replication origin of yeast 2μm plasmid. Cell **51**:473–481.
17. **Huberman, J. A., J. Zhu, L. R. Davis, and C. S. Newlon.** 1988. Close association of a DNA replication origin and an *ARS* element on chromosome III of the yeast *Saccharomyces cerevisiae*. Nucleic Acids Res. **16**:6373–6384.
18. **Iguchi-Ariga, S. M. M., T. Okazaki, T. Itani, M. Ogata, Y. Sato, and H. Ariga.** 1988. An initiation site of DNA replication with transcriptional enhancer activity present upstream of the c-*myc* gene. EMBO J. **7**:3135–3142.
19. **Jensen, F. C., A. J. Girardi, R. V. Gilden, and H. Koprowski.** 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **52**:53–59.
20. **Kelly, T. J., M. S. Wold, and J. Li.** 1988. Initiation of viral DNA replication. Adv. Virus Res. **34**:1–42.
21. **Krysan, P. J., and M. P. Calos.** 1991. Replication initiates at multiple locations on autonomously replicating plasmid in human cells. Mol. Cell. Biol. **11**:1454–1463.
22. **Krysan, P. J., S. B. Haase, and M. P. Calos.** 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. **9**:1026–1033.
23. **Leu, T. H., and J. L. Hamlin.** 1989. High resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation. Mol. Cell. Biol. **9**:523–531.
24. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
25. **Manning, W. C., and E. S. Mocarski.** 1988. Insertional mutagenesis of the murine cytomegalovirus genome: one prominent α gene (ie2) is dispensable for growth. Virology **167**:477–484.
26. **Maundrell, K., A. Hutchison, and S. Shall.** 1988. Sequence analysis of ARS elements in fission yeast. EMBO J. **7**:2203–2209.
27. **McWhinney, C., and M. Leffak.** 1990. Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-myc gene. Nucleic Acids Res. **18**:1233–1242.
28. **Mechali, M., and S. Kearsey.** 1984. Lack of specific sequence requirement for DNA replication in Xenopus eggs compared with high sequence specificity in yeast. Cell **38**:55–64.
29. **Newlon, C. S.** 1988. Yeast chromosome replication and segregation. Microbiol. Rev. **52**:568–601.
30. **Nolan, G. P., S. Fiering, J.-F. Nicolas, and L. A. Herzenberg.** 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc. Natl. Acad. Sci. USA **85**:2603–2607.
31. **Oka, A., K. Sugimoto, and M. Takanami.** 1980. Replication origin of the *Escherichia coli* K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. Mol. Gen. Genet. **178**:9–20.
32. **Stillman, B. W., and Y. Gluzman.** 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. **5**:2051–2060.
33. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5**:410–413.
34. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.
35. **Vassilev, L. T., W. C. Burhans, and M. L. DePamphilis.** 1990. Mapping an origin of DNA replication at a single-copy locus in exponentially proliferating mammalian cells. Mol. Cell. Biol. **10**:4685–4689.
36. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell **61**:1075–1087.
37. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. Cell **16**:777–785.