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Analysis of the autonomous replication behavior in human cells of the dihydrofolate reductase putative chromosomal origin of replication

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ABSTRACT

Chinese hamster genomic DNA sequences from the region downstream of the dihydrofolate reductase (DHFR) gene reported to contain a chromosomal origin of bidirectional DNA replication (OBR-1) were tested for their ability to support autonomous DNA replication in human cells. A 13.3 kilobase fragment containing OBR-1 and surrounding sequences supported replication in short-term and long-term replication assays, while a 4.5 kb fragment containing OBR-1 did not support substantial replication in either assay. These results are consistent with our previous observations that large fragments of human DNA support replication, while smaller fragments are less efficient. The replication activities of plasmids containing OBR-1 were no greater than those of randomly chosen human fragments of similar size. Furthermore, two-dimensional gel analysis of plasmids containing OBR-1 indicated that initiation does not preferentially occur within the OBR-1 region. These results suggest that in the context of autonomous replication, the DHFR sequences tested do not contain genetic information specifying site-specific replication initiation. Possible implications of these results for chromosomal replication are discussed.

INTRODUCTION

DNA replication in eukaryotic chromosomes is a poorly understood phenomenon. In order to determine which DNA sequences are required for replication initiation in human cells, we developed a genetic assay for autonomous replication (1), a general approach that has been used to isolate origins of replication in other organisms (e.g. 2). We succeeded in obtaining long-term autonomous replication in human cells by using vectors carrying sequences from Epstein-Barr virus (EBV) that provide nuclear retention. This system permitted us to isolate a number of human genomic DNA fragments that conferred efficient once per cell cycle autonomous replication on such vectors (1,3,4). Upon removal of the EBV sequences, the human fragments still replicated in a short-term assay (1,4), indicating that replication activity was inherent to the human sequences and did not require any viral sequences or proteins.

Our autonomous replication system has revealed unexpected features of replication initiation in human cells. We were unable to subclone short sequences that retained replication activity. Instead, replication ability of the human fragments was found to be positively correlated with size of the fragments, with large fragments replicating more efficiently than smaller ones (4). Some fragments replicated better than other fragments of equal size, and DNA from *E.coli* replicated more poorly than human DNA. Both of these observations suggest a role for DNA sequence in replication efficiency. However, all large human fragments tested showed replication activity, suggesting that the sequences needed for replication initiation were very common (4). The location of initiation events on a 20 kb human fragment was determined by Brewer and Fangman 2-D gel electrophoresis (5). This analysis demonstrated that DNA synthesis initiates at multiple sites throughout the human fragment, further suggesting a lack of extensive sequence requirements for initiation. These results contrasted with the results obtained in lower organisms, where specific DNA sequences were found to target initiation (e.g. 6,7).

Our results were reminiscent of the data that had been obtained when DNA was injected into *Xenopus* eggs (8). In that system, all DNA fragments tested replicated, including prokaryotic DNA and fragments of eukaryotic viruses with or without viral origins of replication. Furthermore, once per cell cycle replication control was retained for all these DNA sequences. This data raised the question of whether higher eukaryotic chromosomes would have sequence specific origins of replication.

In the yeast *Saccharomyces cerevisiae* convincing genetic and physical mapping data has been presented supporting the existence of sequence specific replication origins. In this yeast, DNA fragments termed autonomously replicating sequences (ARS) were identified which supported autonomous replication and contained an 11 base pair (bp) core consensus sequence required for replication, as demonstrated by extensive mutational analyses (reviewed in 9). Furthermore, by using 2-dimensional gels, initiation of replication in the vicinity of some ARS sequences was demonstrated in the chromosomes of *S.cerevisiae* (10 –13), corroborating the genetic evidence.

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This evidence from yeast, together with the precedent from prokaryotes and viruses, led to an expectation that mammalian cells, too, might replicate their chromosomes using sequence specific origins. In the search to identify such sequences, physical mapping studies, rather than genetic approaches, have predominated. The most intensively studied region reported to contain a chromosomal origin of replication in mammalian cells is located near the dihydrofolate reductase (*DHFR*) gene in Chinese hamster ovary cells. Several physical mapping techniques have suggested that replication initiates approximately 17 kilobases (kb) downstream from the *DHFR* gene (14−17). The clearest resolution of this site has been reported by Burhans et al. (18). Small replication intermediates, Okazaki fragments, were isolated and hybridized to single-stranded clones spanning the DHFR region. The results suggested that replication began within or near a 450 bp sequence referred to as the origin of bidirectional DNA replication (OBR). This region is now called DHFR OBR-1 (19), because of evidence indicating that more than one OBR may exist downstream of the *DHFR* gene (14,17).

The physical mapping evidence suggesting that replication initiated downstream of DHFR was used to infer that a genetic element specifying replication initiation must exist in this region. The region was sequenced (20) and proteins that bind to it have been isolated (21,22). However, it is important to realize that physical mapping data indicating preferential initiation in a region does not prove that such initiation is being caused by a specific sequence. The region could, for example, be preferentially used for initiation due to aspects of local chromatin structure or transcriptional patterns that are largely independent of the specific sequence in the origin region.

Unlike the situation in *S.cerevisiae*, a genetic analysis of the putative origin sequence demonstrating a series of mutations that inactivate origin function has not been carried out. Furthermore, attempts to observe autonomous replication using DNA fragments from the DHFR region did not succeed for unexplained reasons (18). In addition, 2-dimensional (2-D) gel analysis of the DHFR OBR-1 region in the chromosome has indicated that initiation occurs randomly throughout an area of approximately 55 kb, rather than in one fixed position (23,24), which appears to contradict the previous mapping data obtained with other techniques. Owing to these problems, the existence of a specific sequence that defines the DHFR origin of replication and the concept that specific origin sequences are required for replication initiation in mammalian cells remain controversial.

Because the data from our system did not produce any evidence for highly specific origin sequences, we wanted to test the putative DHFR chromosomal origin of replication to see if it would behave differently in our system than the random DNA sequences we studied previously. In this study, we use our autonomous replication system to evaluate the replication activity of the DHFR OBR-1 domain. DHFR sequences were tested in both short-term and long-term replication assays, and initiation on a plasmid harboring the putative DHFR chromosomal origin of replication was studied by 2-D gel analysis. In addition, small DNA sequences reported by three other groups to contain origins of replication (25−27) were tested for autonomous replication.

MATERIALS AND METHODS

Plasmids

Human DNA fragments 4C, 6A, 12B, and 14B cloned in pNUT and pDONUT have been described (4), as have the plasmids pLIB41 (1,5), pHEBO, pDY$^-$ and p220.2 (1). To construct pOBR-L the 13.3 kb *Kpn*I fragment from plasmid K14A was cloned into the *Kpn*I site of pNUT. K14A, a gift from N. H. Heintz, is plasmid ΔVapm12 (28) with the 13.3 kb DHFR fragment cloned into its *Kpn*I site. The 13.3 kb fragment is derived from the putative DHFR origin region and contains the OBR-1 domain (Fig. 1). pDYOBR-L was constructed by removing the DHFR insert from pOBR-L with *Not*I and cloning it into the *Not*I site of pDonut. pOBR-S was constructed by removing the 4.5 kb *Xba*I fragment which contains OBR-1 from pOBR-L (Fig. 1) and cloning it into the *Xba*I site of pNUT. This same fragment was cloned into the *Xba*I site of pDonut to generate pDYOBR-S. p41OBR-S (Fig. 3C) was created by first changing a *Cla*I site near the center of the pLIB41 human insert to *Not*I by linker insertion. The pDYOBR-S insert was removed with *Not*I and cloned into the new *Not*I site of pLIB41 to generate p41OBR-S. pBR/ORS8 and pBR/ORS12 were obtained from M. Zannis-Hadjopoulos. pmyc(H-P) and pNeo.Myc-2.4 were obtained from H. Ariga and M. Leffak, respectively.

Cell lines

293S cells are a human embryonic kidney cell line (29). 293S/EBNA cells contain an integrated vector that expresses the *EBNA-1* gene from the major immediate early promoter of human cytomegalovirus (30). Both cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Cells were grown at 37°C in a 5% CO_2 incubator.

Replication assays

The short-term replication assay was performed as described (4). Briefly, 5 μgs of plasmid DNA were transfected by calcium phosphate coprecipitation (31) into 293S cells that were approximately 10% confluent in 60-mm dishes. Cells were split 1:4 two days later, and plasmid DNA was collected by Hirt extraction (32) four days after transfection. Half of each sample from one plate of cells was digested with the restriction enzymes *Hin*d III and *Mbo* I (or *Dpn*I), separated on an agarose gel, and transferred to Zetaprobe membrane (Biorad). Membranes were probed with ^{32}P-labeled probe and exposed to X-ray film. Autoradiograms were scanned with a model 300A densitometer from Molecular Dynamics. For the long term assay, 293S/EBNA cells were transfected with 10 μg plasmid DNA per 100-mm dish and grown under hygromycin selection (200 μg/ml) for the first week. Plasmid DNA was extracted 15 days after transfection, cut with *Mbo*I, separated on a 0.54% agarose gel, and analyzed by Southern blotting.

For 2-D analysis (33) cells were treated as in the long-term assay with the exception that they were additionally split into 48 100 mm plates and lysed during active cell growth at 50−75% confluency, 16−17 days post-transfection. DNA isolation for 2-D gels was done as described by Krysan and Calos (5). Typically, recovered plasmid DNA from 12 plates of cells was loaded per gel.

Two-dimensional gel electrophoresis

Gel conditions were as described (5). Briefly, for gels probed with fragments I and II, which are larger than 9 kb, the first dimension was run on 0.28% agarose for 40 h at 25 V at room temperature. The second dimension was run on a 0.58% agarose gel with ethidium bromide (0.3 μg/ml) for 48 h at 35 V at room temperature.

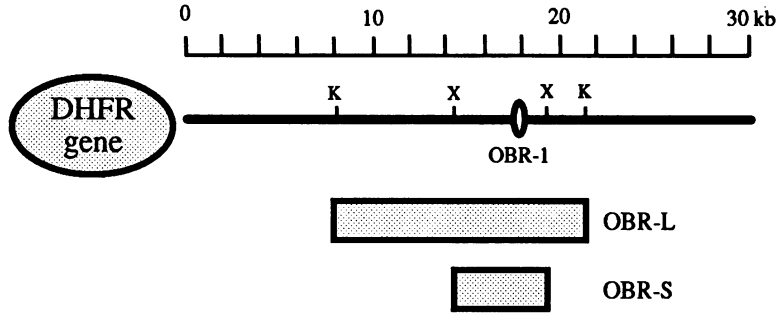

Figure 1. Schematic location of DHFR OBR-1 plasmid inserts. The 450 bp OBR-1 transition point identified by Burhans *et al.* (18) is located approximately 17 kilobases (kb) downstream from the DHFR gene. OBR-L is a 13.3 kb *Kpn*I (K) restriction fragment used as the insert in plasmids pOBR-L and pDYOBR-L. OBR-S is a 4.5 kb *Xba*I (X) restriction fragment used as the insert in plasmids pOBR-S, pDYOBR-S, and p41OBR-S. This fragment is identical to the early-labeled fragment S13X-24 reported by Burhans et al. (15).

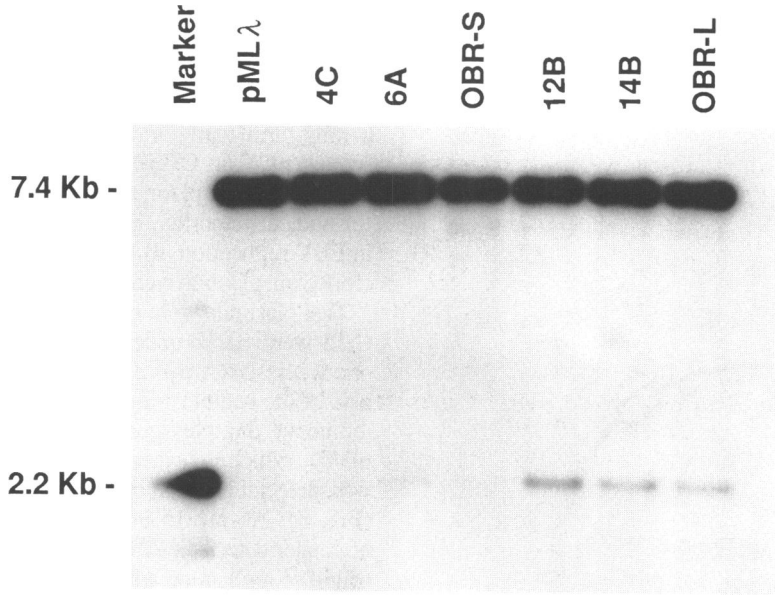

Figure 2. Short-term replication of DHFR OBR-1 containing plasmids. 5 μg of each plasmid were transfected separately into 293S cells and recovered four days later by Hirt extraction. Collected plasmids were digested with *Hin*dIII and *Mbo*I, electrophoresed on an agarose gel, blotted, and probed with the ^{32}P-labeled 2.2 kb *Mbo*I fragment from pMLλ. This fragment can only be generated by plasmids that have replicated at least twice in the human cell host. The probe will hybridize to the 7.4 kb *Hin*dIII fragment from plasmids that have replicated once or less in human cells. The marker lane contains 0.5 ng of pMLl DNA digested with *Sau*3A I and serves as a size marker for the 2.2 kb fragment.

For fragment III, which is 4.5 kb, the first dimension was a 0.35% agarose gel run at 33V for 24 h at room temperature. The second dimension was on a 0.87% agarose gel with ethidium bromide (0.3 μg/ml) run at 75 V for 24 h.

Under both sets of conditions, gels were equilibrated in the second dimension running buffer with ethidium bromide at the temperature of electrophoresis for 1 h prior to separation. The gel buffer in all cases was TBE (108 g TRIZMA base, 54 g boric acid, and 8.4 g EDTA per liter). Gels were blotted and probed as described for replication assays.

RESULTS

Short-term replication of an OBR-1 containing plasmid

To begin analysis of the replication activity of DHFR OBR-1 plasmids, a short-term replication assay was performed. Plasmids pOBR-S and pOBR-L, which both contain DHFR OBR-1 (Fig. 1), were transfected into 293S cells along with plasmids containing human sequences of similar sizes. The backbone vector in each test plasmid is pNUT (4), a prokaryotic vector that contains no viral sequences. Plasmid DNA was extracted four days after transfection, digested with the restriction enzymes *Hin*dIII and *Mbo*I, and analysed by Southern blotting. The probe was a 2.2 kb *Sau*3AI fragment from pNUT. This fragment is generated by *Mbo*I in the test plasmids if they have replicated twice in the host cells, thereby losing their bacterial methylation pattern. In non-replicated DNA the *Sau*3A I probe will hybridize to a 7.4 kb *Hin*dIII fragment.

The plasmid pMLλ (4) (the pNUT vector missing two pUC 19 polylinkers) was transfected as a negative control and replicated to a minimal extent (Fig. 2). Plasmids pNUT4C and pNUT6A contain human sequences of 4 and 6 kb and also replicated inefficiently (Fig. 2). pOBR-S, which contains the 4.5 kb *Xba*I fragment from the OBR-1 region (Fig. 1), replicated

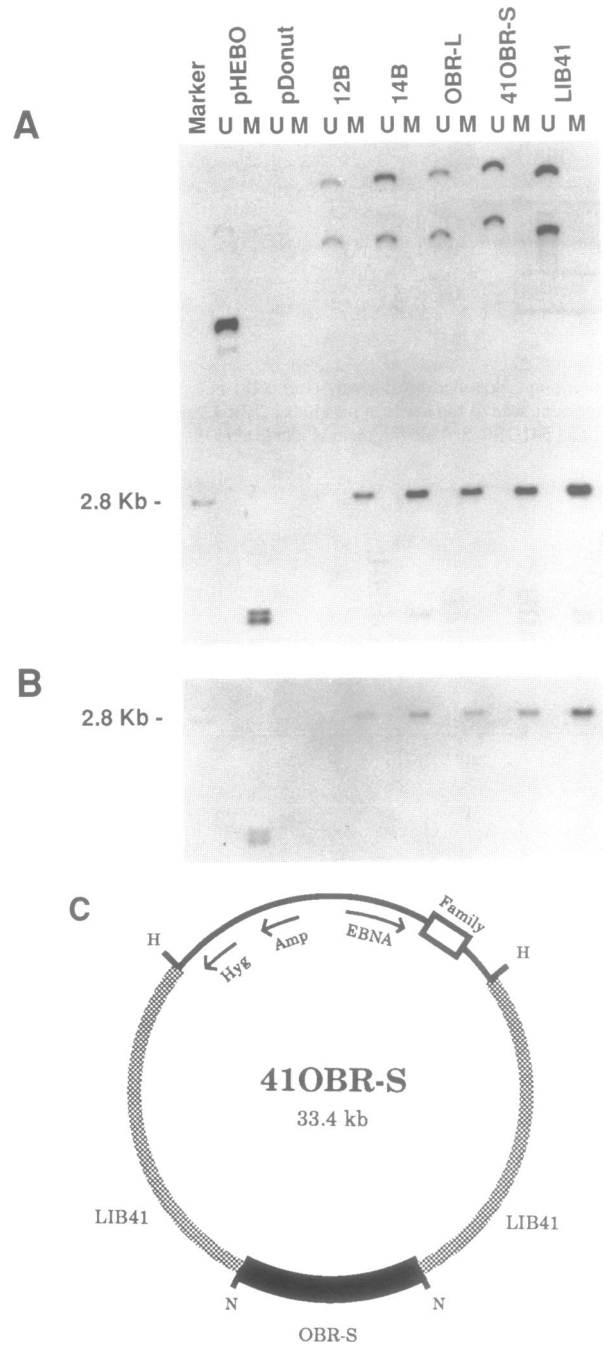

Figure 3. Long-term replication of DHFR OBR-1 containing plasmids. (A) 10 μg of each test plasmid were transfected separately into 293S/EBNA cells and placed under hygromycin selection for one week. 15 days post-transfection plasmid DNA was recovered by Hirt extraction. Half of the sample was digested with *Mbo*I, and equal amounts of uncut (U) and *Mbo*I-digested (M) DNA were electrophoresed, blotted, and probed with ^{32}P-labeled pDY$^-$ DNA. The marker lane contains 0.5 ng of pDY$^-$ plasmid DNA digested with *Sau*3AI to release the 2.8 kb band that is representative of plasmid replication in human cells. (pHEBO is smaller than the other test plasmids and lacks the 2.8 kb band corresponding to the EBNA-1 gene. Hybridization with pDY$^-$ in the *Mbo*I digested lane detects two smaller bands, which are also present in the other plasmids because they also contain these sequences.) (B) Panel B is a shorter exposure of the bottom portion of panel A and is shown to more clearly distinguish the differences in replication copy number between test plasmids. (C) Plasmid p41OBR-S was created by changing a *Cla*I site in the human insert portion of pLIB41 (24) to *Not*I (N) and ligating in the OBR-S insert from pDYOBR-S, represented by the thick black line. H, *Hin*dIII; Hyg, hygromycin resistance gene; Amp, ampicillin resistance gene; EBNA, gene for the EBNA-1 protein of EBV; Family, family of repeats from the EBV origin of replication, *oriP*.

to levels comparable to 4C and 6A. This result illustrated the inability of fragments of this size to sustain an efficient level of autonomous replication, in agreement with our earlier findings (4). Presence of the DHFR OBR-1 on the plasmid did not change the result.

Plasmid pOBR-L was constructed to determine if additional flanking sequences were required for OBR function. pOBR-L contains a 13.3 kb fragment carrying DHFR OBR-1 and its surrounding sequences (Fig. 1). Its ability to replicate was compared to plasmids pNUT12B and pNUT14B, which have human inserts of 12 and 14 kb. The results (Fig. 2) demonstrated that the three plasmids replicated more efficiently than pNUT4C, pNUT6A, and pOBR-S. This result is in agreement with previous findings with this system, in which larger DNA fragments replicate more efficiently than smaller fragments (4). While the OBR-1 domain in pOBR-L replicated efficiently, randomly cloned human fragments of similar size replicated equally well.

Long-term replication of OBR-1 plasmids

We wanted to evaluate the ability of the OBR-1 region, in comparison with random sequences, to maintain replication in a long-term assay in the presence of EBV nuclear retention sequences. The OBR-S and OBR-L fragments were placed in the EBV vector pDonut, which contains the sequences required for nuclear retention, but is deleted for the dyad region involved in EBV replication (4). Human fragments 4C, 6A, 12B, and 14B cloned in pDonut were again used as controls for size.

Test plasmids were transfected into human 293S/EBNA cells. Cells were placed under hygromycin selection for approximately one week. DNA was extracted two weeks after transfection and half of the sample was cut with *Mbo*I, while the other half was left uncut. Samples were analyzed by Southern blots probed with pDY$^-$, which is nearly identical to pDonut. Plasmid replication was detectable as hybridization to the 2.8 kb *Mbo*I fragment (Fig. 3). At day 15 after transfection, unreplicated DNA was no longer detectable. Plasmid pHEBO contains the complete EBV origin of replication without the *EBNA-1* gene and was a positive control for the presence of EBNA-1 in the 293S/EBNA cell line. The uncut pHEBO lane (Fig. 3A) demonstrated that pHEBO was maintained as an extrachromosomal plasmid. The majority of pHEBO DNA was *Mbo*I sensitive, indicating that pHEBO was replicating efficiently. pDonut was the negative control and generated only faint extrachromosomal and 2.8 kb bands (Fig. 3A), verifying its inability to replicate efficiently. For the remaining samples, the presence of signal in the uncut lanes indicated that the plasmids were maintained extrachromosomally.

The pDonut derivatives of 4C, 6A and OBR-S replicated to copy numbers that were only slightly higher than the pDonut alone (data not shown). Therefore, OBR-S over a 15 day period did not replicate more efficiently than random human DNA fragments of similar size. The replication efficiencies for the 12B, 14B and OBR-L fragments cloned in pDonut can be more clearly seen in Fig. 3B, which is a shorter exposure of the bottom portion of Fig. 3A. OBR-L replicated with an efficiency similar to that of 12 and 14B. Considering that the insert size of OBR-L is 13.3 kb, these results indicate that OBR-1 in a larger size context can replicate more efficently than OBR-S (4.5 kb), but copy numbers are comparable to random human sequences of similar size. The long-term assay gives a clearer distinction between small differences in replication efficiency, since the differences accumulate over many cell generations.

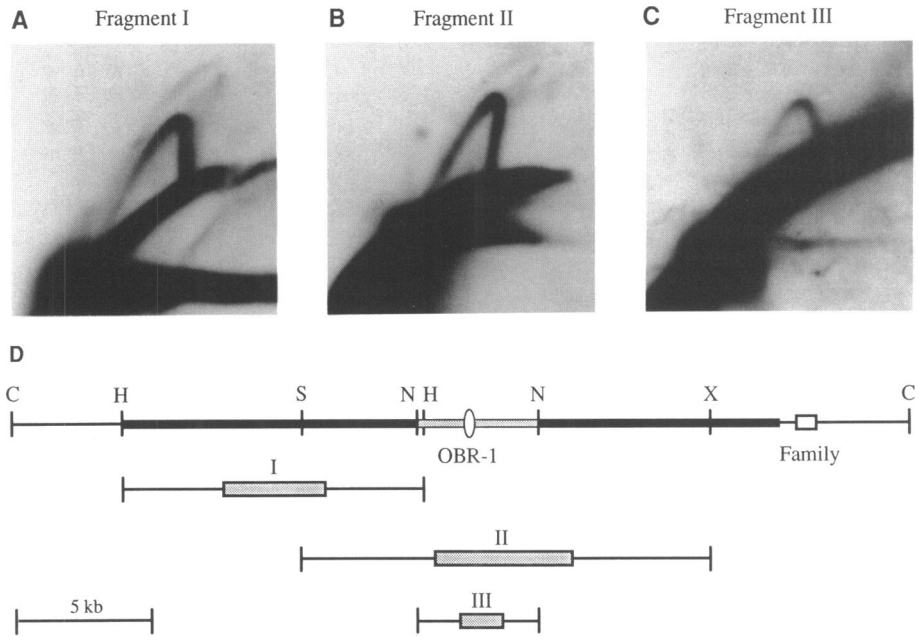

Figure 4. Two-dimensional gel analysis of the autonomously replicating plasmid p41OBR-S in human cells. Transfected DNA was collected from actively growing 293S/EBNA cells and subjected to two-dimensional gel electrophoresis as described in Materials and Methods. (A) Two-dimensional gel from a *Hin*dIII-*Eco*RV digest probed with ^{32}P-labeled fragment I, an 11-kb *Hin*dIII fragment. (B) Two-dimensional gel from an *Xho*I-*Sca*I digest probed with fragment II, a 14.75-kb *Xho*I-*Sca*I fragment. (C) Two-dimensional gel from a *Not*I-*Xba*I-*Xho*I digest probed with fragment III, a 4.5-kb *Not*I fragment. (D) Linear map of p41OBR-S. The thick black line represents the LIB41 human sequences. The thin black line is vector sequence displaying the location of the EBV family of repeats.The thick gray portion in the middle of the LIB41 sequence is the OBR-S insert, with the location of OBR-1 represented by the oval. The three restriction fragments used as probes in A thru C are labeled I to III. The thick gray box in the center third of each fragment designates the fragment's bubble detection zone. C, *Cla*I; H, *Hin*dIII; S, *Sca*I, N, *Not*I; X, *Xho*I.

Because we have found that all large fragments of human DNA mediate replication (4), it was expected that the 13.3 kb fragment of DNA containing OBR-1 would replicate. We have shown using density gradients that replication efficiency per generation of plasmids containing large, random, human fragments is close to 100% (3). Therefore, we could not expect the 13.3 kb fragment to replicate significantly better than large, random, human fragments, even if it contained a specific origin of replication. For the 13.3-kb hamster fragment containing the DHFR OBR-1, our replication assay reveals only that this fragment behaves in the manner expected of a similarly-sized random fragment of human DNA.

Two-dimensional gel analysis of an OBR-1 plasmid

The OBR-1 containing plasmids did not demonstrate replication strength different from random human fragments. To test if OBR-1 had the ability to localize initiation events preferentially over itself, we used the two-dimensional gel electrophoresis technique of Brewer and Fangman (33). This method has been used to map replication initiation in a number of different systems (e.g. 12,13, 23,24,34), including a 20 kb human fragment that replicates autonomously in human cells (5).

Copy number of the plasmid pDYOBR-L was too low to generate strong 2-D gel patterns. We enlarged the size of the mammalian insert to increase its copy number and facilitate 2-D analysis by constructing p41OBR-S (Fig. 3C), which contains the 4.5 kb OBR-S insert in the center of pLIB41. Plasmid pLIB41 replicates autonomously in human cells using multiple sites of initiation (5).

The copy number two weeks after transfection of p41OBR-S, while three times less than pLIB41, was twice as high as that of pDYOBR-L (Fig. 3B) and allowed adequate signal to be obtained with 2-D analysis (Fig. 4). After transfection and replication in human cells for approximately two weeks, p41OBR-S DNA was extracted, digested with *Hin*dIII and *Eco*RV, and probed with an 11 kb *Hin*dIII fragment that covers the left half of the pLIB41 human insert (Fig, 4D, fragment 1). *Eco*RV was used to cut mitochondrial DNA into smaller pieces, so that it would not interfere with the migration of the *Hin*dIII fragment from p41OBR-S. The inner third of all fragments used as probes in Fig. 4D represents the region in which replication bubbles can be unambiguously identified, called the bubble detection zone (5,35). Hybridization with fragment 1 shows the presence of abundant simple Y replication intermediates above the arc of linear DNA molecules (Fig. 4A). Above the simple Y arc, a lighter arc of replication bubbles can be seen. This result indicates that this portion of p41OBR-S replicates the majority of the time by using initiation sites found outside of the *Hin*dIII fragment bubble detection zone and that a minority of initiations occur from within the fragment·s bubble detection zone.

An *Xho*I and *Sca*I digest of p41OBR-S probed with fragment II (Fig. 4D) is shown in Fig. 4B. The bubble detection zone of fragment II covers most of the OBR-S insert. A very strong Y arc with a faint bubble arc above it is detected by the probe. This hybridization pattern is similar to that of fragment I and indicates that most initiation events occur outside of the 4.5 kb OBR-1 domain.

Qualitatively, the ratio of bubble arc to Y arc for fragment II is somewhat less than for fragment I. This characteristic has been seen in repeated trials with these probes. Thus, it is possible that initiation events are occurring less frequently in the OBR-1 domain, especially considering that the bubble detection zone of

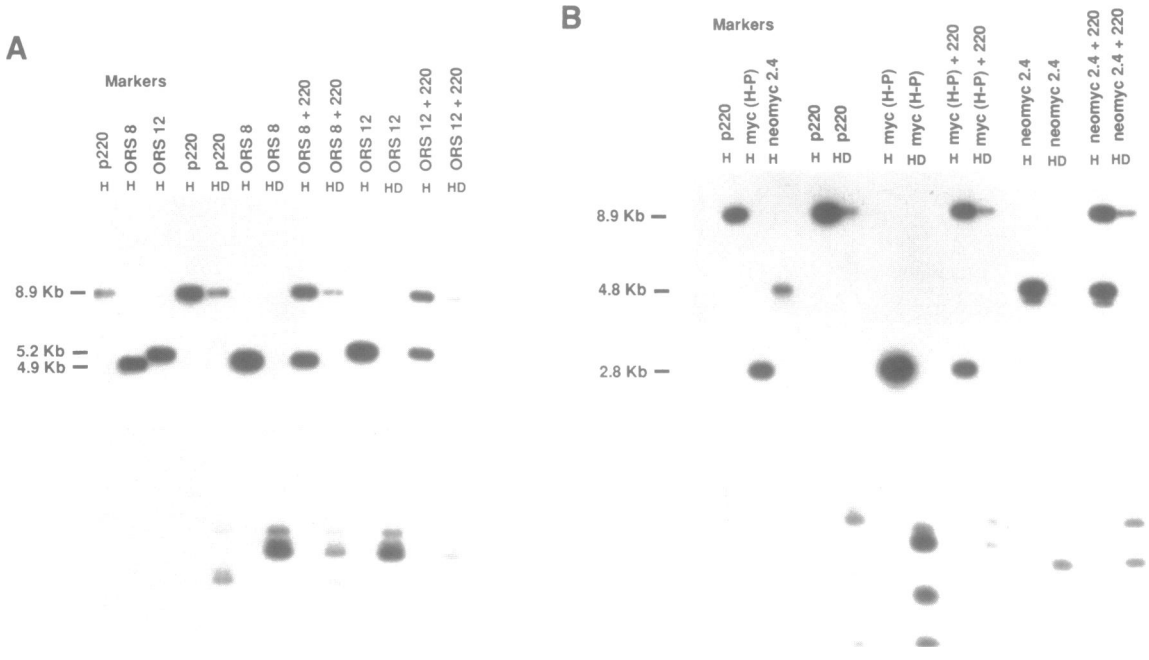

Figure 5. Short-term replication assay of reported origins of replication. A. Replication tests of p220.2, pBR/ORS8, and pBR/ORS12. B. Replication tests of p220.2, pmyc(H-P), and pNeo.Myc-2.4. The presence of a band corresponding to full-length vector in the *Dpn*I lanes indicates replication. The small bands in these lanes represent unreplicated input DNA. 5 μg of vector DNA were used for individual transfections. 3 μg of p220.2 DNA plus an equimolar amount of the test plasmid were used for cotransfections. DNA was introduced into 60mm dishes of 293S cells by calcium phosphate coprecipitation, allowed to replicate for four days, harvested, purified by Hirt extraction, digested with *Hin*dII and *Dpn*I, and run on a 0.7% agarose gel. The gel was blotted and probed with ^{32}P-labelled pBR322 DNA. H = *Hin*dIII; D = *Dpn*I; M = size markers, .5−1 ng of *Hin*dIII digested plasmid indicated.

fragment I is 3.7 kb, whereas that of fragment II is 4.9 kb. Fragment II covers a region that is 32% larger than the comparable region of fragment I, but fails to detect a higher percentage of replication bubbles, despite the presence of OBR-1.

Next, we examined in more detail the initiation events coming from the OBR-1 domain by focusing on the 450 bp region which contains the reported chromosomal Okazaki fragment transition point (18). Fragment III (Fig. 4D) is the 4.5 kb OBR-S insert and was used to probe a *Not*I, *Xba*I, and *Xho*I digest of p41OBR-S DNA (Fig. 4C). (*Xba*I and *Xho*I were used to cut mitochondrial DNA into smaller fragments.) The 450 bp OBR-1 transition point is located to one side of the 1.5 kb bubble detection zone of fragment III. The signal for this digest is not as strong as those shown in Figs. 4A and 4B because of the smaller size of the 4.5 kb restriction fragment being probed. However, the hybridization results clearly identify a simple Y structure without the presence of a bubble arc. Although the simple Y signal is too weak to exclude the presence of a faint bubble arc, it is clear that the predominant mode of replication of fragment III is due to initiation from outside OBR-1. We note that simple Y signals without a bubble arc were consistently seen over the DHFR region in pDYOBR-L, which replicates using the 13.3 kb DHFR fragment in the absence of any human DNA (data not shown), confirming the conclusion that OBR-1 is not a preferred site for initiation.

Testing other putative replication origins

Three other groups have reported the isolation of DNA sequences from mammalian cells that can support autonomous replication when reintroduced into mammalian cells (25−27). These sequences were isolated either by enrichment for newly replicated DNA (25) or by their proximity to a putative origin of replication near the c-*myc* gene (26,27). We have found that a wide variety of large fragments are positive in our autonomous replication system and that in a transient replication assay, there is a correlation between replication activity and large fragment size (1,4). In contrast, the fragments reported to supply replication activity in the other systems are small, ranging from 2.4 kb to 210 bp or less. In order to determine whether these small fragments reported by others had special replication properties, we tested them in a standard transient replication assay.

Four plasmids carrying small inserts reported to mediate replication were tested for replication after transfection into human 293S cells. The plasmids tested were pBR/ORS8 and pBR/ORS12 derived from monkey cells (25) and pmyc (H-P) (26) and pNeo.Myc-2.4 (27) from the region upstream of the human c-*myc* gene. The positive control for the experiments was p220.2 (1), a vector derived from Epstein-Barr virus that replicates once per cell cycle (3). This vector was a suitable control, since it is similar in size to the test vectors and, if the test sequences represent origins of replication, they might be expected to replicate at levels comparable to once per cell cycle. Each plasmid was transfected either alone or in cotransfection with p220.2. After four days in human cells, the vector DNA was recovered, digested with *Hin*dIII to linearize and *Dpn*I to digest unreplicated (retaining bacterial methylation) DNA, run on a gel, and hybridized to a pBR322 probe, which has a similar length of homology with all the plasmids (Fig. 5). The p220.2 control vector showed easily detectable replication (*Dpn*I-resistant DNA) in every case, whether alone or in combination with another plasmid. In contrast, none of the test plasmids showed any measurable replication, either alone or in combination with

p220.2. Each of the plasmids was tested several times with consistent results. We conclude that none of the test plasmids can replicate efficiently in 293S cells.

DISCUSSION

We have determined that the DHFR OBR-1 region replicates autonomously in human cells only as well as random human DNA fragments of similar size.

The inability of the 4.5-kb OBR-1 fragment to replicate in our assay is significant, since if the fragment contained a specific sequence that were being recognized by an initiator protein, it would have been expected to replicate more efficiently than the poor replication of small random fragments. Furthermore, 2-D gel analysis indicates that replication initiates at multiple sites on plasmids containing OBR-1, rather than showing preferential initiation in the OBR-1 region. This finding indicates that, at least in the context of a circular plasmid, there is no special sequence element within the DHFR OBR-1 region that permits it to target initiation events. Instead, the results with OBR-1 plasmids were similar to the dispersed initiation pattern seen with pLIB41(5), which contains a 20-kb random human insert.

If initiation were mediated by specific binding between an initiator protein and an origin of replication sequence, a mammalian origin of replication would be expected to give rise to a specific initiation site on an autonomous replicon. There has been no difficulty detecting the typical 2-D gel pattern signifying specific initiation in mammalian cells when the object of study was an origin of replication with a specific sequence and and an initiator protein that binds it, as in viral origins such as SV40 or EBV (e.g. 5,34).

Our results are consistent with the work of Vaughn et al., who, using the two-dimensional gel technique, demonstrated that initiation took place throughout a region of approximately 55 kb surrounding the DHFR putative origin when situated in the chromosome (23,24), a conclusion that seems to be in conflict with site-specific initiation at OBR-1. Linskens and Huberman have proposed a model (36) that attempts to reconcile the disparate results obtained with the 2-D gel and nascent strand polarity mapping techniques that have been used to map the chromosomal DHFR origin region. The model postulates that 2-D gels may be detecting a series of minibubbles centered around an OBR. We have not performed nascent strand polarity mapping on our plasmids. However, analysis of pausing at replication fork barriers on pLIB41 (5) indicates that this plasmid does not replicate the same way each time. This result would argue against a defined OBR existing on pLIB41 and presumably on the DHFR plasmids as well.

In considering the relevance of our findings to chromosomal replication, several interpretations should be evaluated. It could be argued that our results have no bearing on chromosomal replication because the autonomous replication system fails to model key aspects of chromosomal replication. For example, aspects of small circular molecules could make them initiate replication in a fundamentally different way than do large linear chromosomes. On the other hand, there is no available precedent for this explanation. All other origins tested have functioned and preserved their specificity when cloned on small circles, including those from yeast. In yeast, typically replication mediated by ARS sequences has been found to follow the same mechanism and be similarly affected by mutations whether the ARS is in a plasmid or a chromosomal context (e.g. 12).

Examples have been described in yeast in which certain aspects of replication can differ between the plasmid and chromosome context. In the case of *ARS501*, it has been demonstrated that the timing of replication changes from late to early when the ARS is moved from the telomere vicinity of chromosome V to a circular plasmid (37). However, origin function of the ARS is not abolished, nor is the mechanism of initiation altered. In another example, *HML*-associated ARS elements that functioned on plasmids did not function in a chromosomal context (38). However, we are not aware of any example where a genetically defined origin that functions in the chromosome has failed to replicate in the plasmid context. Therefore, while this situation remains a possibility, it is a speculative one without a specific precedent or rationale.

If the DHFR-mediated replication taking place in the autonomous system bears no resemblance to chromosomal replication, its high efficiency must be explained. In a previous study we demonstrated, using density labeling, that three plasmids carrying different fragments of human DNA in the size range of 14 to 21 kb replicated with a per generation efficiency indistinguishable from that of the total genomic DNA from the same cells (3). In that study we also showed that the plasmids replicated semi-conservatively and once per cell cycle. The high efficiency and controlled nature of our autonomous replication system suggest that it is a good model for understanding how human chromosomes are replicated and that the replication is not due to DNA repair or some aberrant reaction.

It seems likely that the same apparatus which replicates the human chromosomes is responsible for replicating our plasmids. The alternative would be that there is a separate replication system in human cells which is responsible for efficiently replicating extrachromosomal plasmids in a once per cell cycle manner. It is difficult to imagine why human cells would harbor a replication apparatus of this type, since small extrachromosomal plasmids are not believed to be necessary for the normal growth and development of human cells. These arguments favor the interpretation that our results with DHFR OBR-1 are relevant to the chromosome.

It is possible that the hamster DHFR OBR-1 cannot function in human cells because of species differences in replication signals. This explanation seems unlikely because of the close evolutionary relationship between the two species. We have found in this study that the 13.3 kb hamster DHFR fragment mediates replication in human cells with a similar efficiency as human fragments of the same size and uses the same mode of dispersed initiation. These results support the hypothesis that the two mammalian species are similar in their replication mode.

It could be argued that, while the extrachromosomal replication system may be generally valid for studying the sequence requirements for replication, our negative results with the DHFR OBR-1 may have limited relevance because we tested an inadequately sized region of DHFR DNA. It could be argued that sequences outside the 4.5 kb fragment tested are required to engender specific initiation at OBR-1. Alternatively, the context of being embedded within The human pLIB41 fragment could for some reason have adversely affected the site specificity of the 4.5 kb OBR-1 fragment. However, we did perform 2-D analysis on a plasmid replicating with 13.3 kb of DNA from the DHFR region including OBR-1 without any human DNA, and this plasmid did not show specific initiation at OBR-1. Therefore, it would have to be hypothesized that the necessary sequences to activate specific replication lie outside the 13.3 kb region. This

explanation is possible but unprecedented. Transcriptional enhancers can act at a distance of several kb, but they affect the rate rather than the site specificity of the reaction.

Our examination of other sequences reported to mediate specific replication intiation also failed to find evidence that these sequences had any special replication activity. These plasmids were reported to replicate in short-term assays in the original studies using a similar *Dpn*I assay and/or a bromodeoxyuridine substitution assay. Conceivably, our failure to reproduce these results may be due to our use of 293S cells, rather than the HeLa or HL-60 cells used in the original studies. On the other hand, if the putative origins are authentic, they might be expected to replicate in a variety of dividing cells. We have shown, for example, that sequences we isolated using 293S cells also replicate efficiently in lymphoid NC-37 cells and monkey CV-1 cells (4). Furthermore, tests of pBR/ORS12 and pARS65, a plasmid containing a 2.5 kb fragment of mouse DNA reported to replicate autonomously (39), in the cell types where replication was originally reported also failed to detect replication (18). These data confirm our inability to detect efficient replication of small genomic sequences in human cells.

The evidence presented in this paper argues that neither the 13.3 kb region encompassing the DHFR OBR-1 nor the other sequences tested can stimulate site-specific autonomous replication in human cells. These results suggest that if chromosomal replication in higher eukaryotic cells is highly sequence specific, it is encoded in a much more complicated way than in lower organisms. Alternatively, chromosomal replication, like that of our autonomous plasmids, may require only a low level of sequence specificity for initiation.

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