Nucleic Acids Research, Vol. 19, No. 18 5053–5058

Replication control of autonomously replicating human sequences

Steven B.Haase and Michele P.Calos*
Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA

Received April 19, 1991; Revised and Accepted June 19, 1991

ABSTRACT

Three autonomously replicating plasmids carrying human genomic DNA and a vector derived from Epstein-Barr virus were studied by density labelling to determine the number of times per cell cycle these plasmids replicate in human cells. Each of the plasmids replicated semi-conservatively once per cell cycle. The results suggest that these human autonomously replicating sequences undergo replication following the same controls as chromosomal DNA and represent a good model system for studying chromosomal replication. We also determined the time within the S phase of the cell cycle that three of the plasmids replicate. Centromeric alpha sequences, which normally replicate late in S phase when in their chromosomal context, were found to replicate earlier when they mediate replication on an extrachromosomal vector. Reproducible patterns of replication within S phase were found for the plasmids, suggesting that the mechanism specifying time of replication may be subject to experimental analysis with this system.

INTRODUCTION

An unknown mechanism ensures that all the DNA in a human cell replicates exactly once during the S, or DNA synthesis, phase of the cell cycle. Furthermore, many sequences have a characteristic time of replication within S phase, but how replication timing is determined is also currently obscure. Insight into these mechanisms could be gained by using an autonomously replicating system based on human genomic sequences that retained normal replication control. This type of system would offer the possibility of working with completely defined sequences which could be easily manipulated.

To address questions related to human DNA replication, we developed an autonomously replicating system based on human genomic DNA sequences. We obtained such a system by taking advantage of vectors derived from Epstein-Barr virus (EBV). EBV vectors from which part of the origin of replication has been deleted are defective for replication (1). However, we have shown that in the absence of replication they still permit linked DNA to be retained in the nuclei of human cells for an extended period of time (2). By cloning fragments of human genomic DNA into such vectors, we obtained plasmids that could replicate and be maintained in human cells for a prolonged period. The human fragments were necessary for replication, since the vector alone could not replicate in a long-term assay. Also, the human fragments conferred replication ability in the absence of all viral sequences when tested in a short-term assay (2, 3).

This system has the potential to serve as a convenient model for approaching many questions pertaining to replication in human cells. A critical step in evaluating the ability of the system to help understand the control of DNA replication was to establish whether or not our system was subject to once per cell cycle regulation. In order to shed light on the mechanism controlling time of replication, we determined the time within S phase that our plasmids replicated. We included a plasmid replicating autonomously with a human fragment whose replication timing in its chromosomal context was known, to determine whether the fragment would retain its characteristic time of replication. We also included a plasmid that has been shown previously to replicate without the use of a specific initiation site (4).

METHODS

Plasmids and probes

The plasmids p220.2, pLIB16, pLIB41, and pDY$^-$ are described elsewhere (2, 5). The plasmid pCOS4A (a gift of Hunt Willard, unpublished) carries 40 kb of alpha repeat DNA from human chromosome 17. pCOS4A was partially digested with *Eco*RI. Fragments containing alpha repeats from the partial digest were cloned into the *Eco*RI site of pUCR (6). One such plasmid containing a 16.2 kb alpha fragment was named pUCRalpha. The fragment containing the alpha repeats was removed from the pUCR vector by digestion with *Bam*HI and cloned into the unique *Bam*HI site of pDY$^-$ to form pDYAL. The Alu probe was obtained by digesting pBLUR 8 (7) with *Bam*HI and gel purifying the 300 bp Alu fragment. The alpha probe was obtained by digesting pMGB7, which contains alpha repeats from human chromosome 7 (8), with *Hin*dIII and *Msp*I and gel purifying the 2.7 kb alpha fragment.

Tissue culture and labelling

The cell line 293S (9) is a derivative of the human embryonic kidney cell line 293 (10). Cells were grown in Dulbecco's

* To whom correspondence should be addressed

modified Eagle Medium supplemented with 10% fetal calf serum and with penicillin and streptomycin. Cells were incubated at 37°C in a 5% CO_2 incubator. Media selective for the hygromycin resistance gene also contained hygromycin B (Calbiochem-Behring, La Jolla, CA) at a concentration of 200 μg/ml. Before labelling with 5-bromo-2'-deoxyuridine (BrdUrd), cells were split into non-selective medium. Log phase cells were labelled by the addition of 30 μg/ml BrdUrd to the culture medium.

Construction of cell lines

Cell lines were constructed by transfection of plasmid DNA into 293S cells using calcium phosphate coprecipitation (11). 10 μg of plasmid DNA were used per 100 mm dish seeded with 2×10^6 cells. Transfected cells were allowed to grow to confluence and then were split into selective media containing 200 μg/ml hygromycin. The resulting populations were continually passaged in the presence of hygromycin selection.

Fluorescence activated cell sorter (FACS) analysis and sorting

Cell cycle analysis and sorting was done by the method of Gray and Coffino (12). BrdUrd labelled cells were trypsinized and fixed in 70% ethanol for 30 minutes at 4°C. Cells were then stained in 50 μg/ml chromomycin A3 (Sigma) for 1 hour at 4°C. Cell density was adjusted to 5×10^6 cells/ml before sorting. Stained cells were analyzed on a Becton Dickinson Facstar using a 495 nM long pass filter. Cells were sorted into 1.9 ml microcentrifuge tubes at a flow rate of 4000 cells/second. Aliquots of sorted fractions were reanalyzed by removing 50 μl aliquots of cells and staining for 15 minutes in 300 μl of chromomycin A3 before reanalysis by FACS.

DNA isolation and separation of BrdUrd substituted DNA

Genomic DNA was extracted by incubating in extraction buffer (0.5 M EDTA, 0.5% sarcosyl, 100 μg/ml proteinase K) at 65°C for 30 minutes. Cells were sequentially extracted with phenol, phenol-chloroform, and chloroform. DNA was then precipitated with ethanol. Pellets were resuspended in 20 μg/ml RNAse and digested with *Bam*HI for 2 hours at 37°C before loading onto density gradients. BrdUrd substituted DNA was separated by isopycnic centrifugation in Cs_2SO_4 density gradients. Labelled DNA was loaded onto Cs_2SO_4 gradients, the refractive index was adjusted to 1.3710, and the gradients were spun at 30,000 rpm in a Beckman VTi 80 for > 48 hours. Fractions were collected from the bottom of each gradient tube using a peristaltic pump.

Blotting, hybridization, and quantitative analysis

Aliquots of the gradient fractions were denatured by adding 0.1 volume of 4N NaOH. Samples were then immobilized on a Zeta Probe (Bio-Rad, Richmond, CA) blotting membrane using a slot blot apparatus and 0.4N NaOH as the transfer buffer. Membranes were prehybridized in 2×SSPE [20×SSPE is 174 g NaCl, 27.6 g $NaH_2PO_4\cdot H_2O$, and 7.4 g EDTA in 1.0 liter of H_2O (pH 7.4)], 1% sodium dodecyl sulfate (SDS), 0.5% (w/v) powdered non-fat milk, and 100 μg/ml denatured salmon sperm DNA at 65°C for 6 hours. Blots were hybridized in fresh prehybridization buffer for at least 12 hours at 65°C with probes prepared by random priming. Membranes were sequentially washed at room temperature for 15 minutes each in: 2×SSC (20×SSC is 175 g NaCl and 88 g sodium citrate dehydrate in 1.0 liter of H_2O), 0.1% SDS, 0.5×SSC, 0.1% SDS, 0.1×SSC, 0.1% SDS. A final wash was done in 0.1×SSC, 1% SDS at 65°C for 30 minutes. Blots were then exposed to X-ray film using intensifying screens at −70°C. In order to reprobe, blots were stripped in 0.4 N NaOH for 1 hour at room temperature, followed by an incubation in 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2) and 1% SDS for 1 hour at 65°C. Band intensities were quantitated using a Molecular Dynamics 300A computing laser densitometer.

RESULTS

Cell lines

The four cell lines used in this study carry autonomously replicating extrachromosomal plasmids. The plasmid p220.2 is a fully replication competent, stable EBV vector carrying the EBV origin of latent replication, *oriP*, and the *EBNA-1* gene (5). The three remaining plasmids are based on a replication-defective EBV vector, pDY^-. The plasmid pDY^- a derivative of p220.2, contains a deletion in the EBV origin of replication rendering it unable to replicate (1, 2). The plasmids pLIB16 and pLIB41 contain 14 kb and 20 kb fragments of human genomic DNA cloned into the pDY^- vector (2). The plasmid pDYAL contains approximately 16 kb of alpha repeated sequences from human chromosome 17 cloned into pDY^-. The replication of these three plasmids is dependent on their human inserts. All four plasmids carry the gene for hygromycin resistance.

Populations of 293S cells into which these plasmids had been transfected were selected and passaged for one month in the presence of hygromycin. Plasmid DNA was then isolated by Hirt extraction (13) and run uncut on a 0.6% agarose gel. As demonstrated by Figure 1, the plasmids are maintained in human cells as extrachromosomal circular molecules. Average copy numbers range from approximately 3 to 100, as determined by densitometric analysis compared against known quantities of plasmid DNA.

Once per cell cycle analysis

To determine the number of times the plasmids replicated per cell cycle, we employed a density shift assay. Cells carrying autonomously replicating plasmids were labeled in media

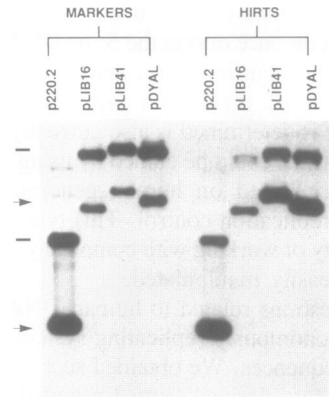

Figure 1. Cell lines carrying plasmids. 293S cells were transfected independently with the plasmids p220.2, pLIB16, pLIB41, and pDYAL. Established populations carrying the plasmids were subjected to Hirt extraction to isolate low molecular weight DNA. Hirt extracts from approximately 3×10^6 cells (Lanes 5–8) and 1 ng of marker DNA (Lanes 1–4) were run uncut on a 0.6% agarose gel. The gel was blotted and hybridized with radioactively labelled pDY^-. Arrows indicate the positions of supercoiled plasmid. Dashes indicate the positions of nicked circle.

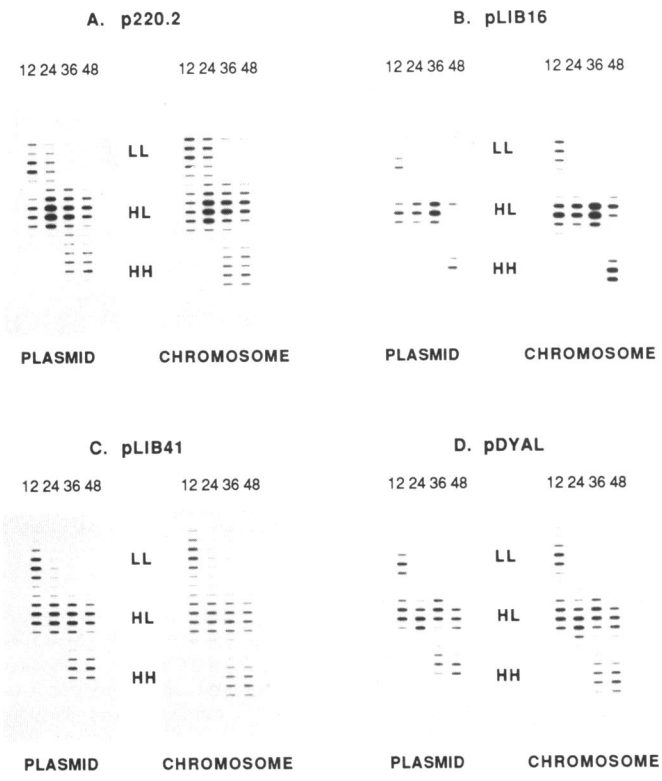

Figure 2. Once per cell cycle analysis. Logarithmically growing cell lines carrying the plasmids were labelled with 30 μg/ml BrdUrd for the number of hours indicated. Filters were first probed with pDY⁻ (left side, plasmid), which hybridizes only to a common plasmid backbone shared by each plasmid. Blots were then stripped and reprobed with the human Alu sequence isolated from pBLUR8 (17) (right side, chromosome). The pLIB16 sample was reprobed with human alpha repeats isolated from p*cos*4A instead of Alu, because the human insert in the pLIB16 contains an Alu repeat. **LL**, light-light DNA; **HL**, heavy-light DNA; **HH**, heavy-heavy DNA.

Table 1. Densitometric analysis of slot blots. Band intensities from autoradiograms were quantitated using a Molecular Dynamics 300A computing laser densitometer. Values are stated as a percentage of the total amount of signal in each lane. Plasmid values represent blots probed with pDY⁻. Chromosome values represent the same blot reprobed with Alu or alpha sequences. The results of two experiments are shown for p220.2, pLIB41, and pDYAL. Three experiments are shown for pLIB16.

		12 hr			24 hr			36hr			48 hr		
Plasmid	**Probe**	**LL**	**HL**	**HH**	**LL**	**HL**	**HH**	**LL**	**HL**	**HH**	**LL**	**HL**	**HH**
pLIB16	plasmid	34	65	1	4	95	1	1	99	1	1	34	66
	chromosome	29	71	0	2	98	0	0	99	1	0	34	66
	plasmid	50	50	0	7	91	2	1	93	6	2	58	40
	chromosome	60	40	0	5	94	1	1	96	3	1	82	17
	plasmid	60	39	1	10	90	0	0	98	2	0	88	12
	chromosome	73	25	2	1	98	1	1	94	5	0	81	19
pLIB41	plasmid	48	51	0	7	90	2	2	69	29	1	55	43
	chromosome	53	46	1	14	77	9	7	60	33	5	52	43
	plasmid	52	48	0	1	99	0	4	76	20	1	72	27
	chromosome	59	40	1	2	97	1	7	85	8	7	59	33
pDYAL	plasmid	38	61	0	5	92	3	2	74	24	1	60	38
	chromosome	39	61	0	4	92	4	2	74	24	2	59	39
	plasmid	58	41	1	11	86	3	5	69	26	2	67	31
	chromosome	57	42	1	27	69	4	11	62	27	6	69	25
p220.2	plasmid	49	51	0	10	90	0	1	82	18	2	66	33
	chromosome	55	44	1	17	82	0	3	79	19	3	69	28
	plasmid	45	54	1	6	94	0	2	89	9	4	86	10
	chromosome	55	45	1	17	82	1	5	81	14	7	73	20

containing the heavy nucleotide analog 5-bromodeoxyuridine (BrdUrd) for 12, 24, 36 and 48 hours. After the labelling period cells were immediately harvested and their total DNA was isolated. Five to 20 ug of total DNA for each sample were digested with *Bam*HI and loaded on Cs_2SO_4 density gradients to separate replicated from unreplicated DNA. Each gradient was divided into approximately 22 fractions which were then loaded onto a slot blot. The slot blots were probed first with the pDY⁻ vector to determine the replication pattern of the autonomous plasmids. By probing with pDY⁻, any cross- hybridization to chromosomal DNA was eliminated. As an internal control, the blots were then stripped and reprobed with a chromosomal repeat sequence (Alu or alpha) to determine the pattern of replication of the chromosome.

Results of this analysis are shown in Figure 2. The plasmids based on human sequences (pLIB16, pLIB41, and pDYAL), as well as the EBV plasmid p220.2, exhibited similar replication patterns (Figure 2, left side of each panel). The 12 hour and 24 hour time points showed light-light (LL) and heavy-light (HL) labelling and no significant heavy-heavy (HH labelling, indicating that one or fewer rounds of replication occurred during these time periods. The 36 hour and 48 hour time points showed plasmid DNA moving into the HL and HH regions, indicating one or more rounds of replication. The cell cycle time for 293S cells was determined to be approximately 20 hours by a doubling time analysis (data not shown). Therefore, the pattern of labelling observed is consistent with replication once per cell cycle.

The patterns of replication of the control chromosomal sequences are similar to those seen for their respective plasmid

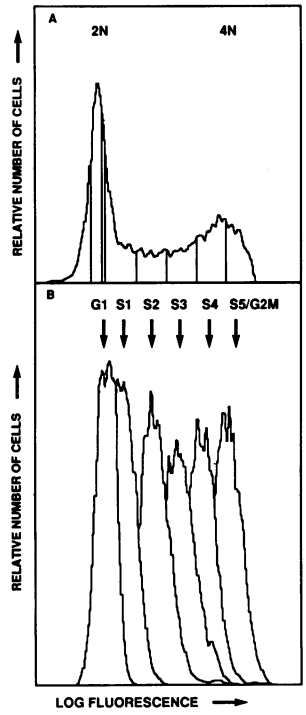

Figure 3. Retroactive synchrony. **A.** Logarithmically growing cell lines carrying test plasmids were labeled for 3 hr with BrdUrd, fixed in 70% ethanol, stained in chromomycin A3, and analyzed using the FACS. In the resulting histogram, relative number of cells is plotted against DNA content. Cells were sorted in the windows shown. **B.** Aliquots from sorted populations were restained and reanalyzed. The sorted subpopulations represent synchronous cells in six different portions of the cell cycle.

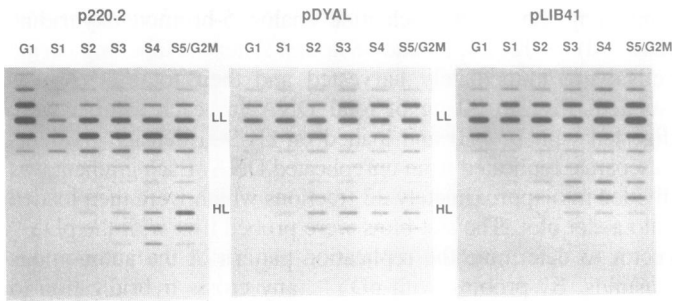

Figure 4. Timing of replication slot blots. Total DNA was extracted from each of the cell cycle fractions shown in Figure 3. The DNA from 250,000 cells was digested with *Bam*HI and spun on Cs_2SO_4 gradients. Each gradient was fractionated into approximately 22 fractions. One half of each gradient fraction was transferred to a nylon membrane using a slot blot apparatus. The slot blots were then probed with radioactively labeled pDY$^-$ to detect the pattern of replication for each of the plasmids. The positions of heavy-light (HL) DNA, representing replicated DNA, and light-light (LL) DNA, representing unreplicated DNA, are indicated.

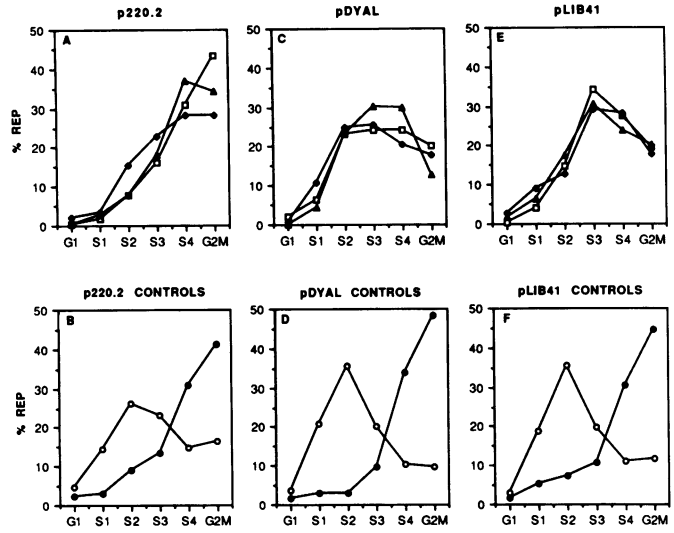

Figure 5. Timing of replication summary. Timing experiments were performed as described in Materials and Methods. Slot blots for each plasmid were first hybridized with the plasmid backbone pDY$^-$ to examine the timing of replication of plasmid sequences. As a control, blots were stripped and rehybridized with human alpha sequences from chromosome 7 (8). As a further control, the blots were stripped and rehybridized with human Alu sequences. HL and LL DNA from each slot blot were quantified by scanning laser densitometry. The replication value reported for each cell cyle fraction is a percentage of the total amount of replication observed for all of the fractions within a single experiment. The total amount of replication observed is equal to the sum of the raw replication values for each cell cycle fraction. The raw replication value for each cell cycle fraction equals the amount of replicated DNA divided by the total amount of DNA [HL/(LL+HL)]. The results of three experiments for p220.2, pDYAL, and pLIB41 are shown in panels A, C and E respectively. The values for the controls for each plasmid are shown directly below in panels B, D and F. The values shown are the average of three experiments for each plasmid. Open circles, Alu sequences; filled circles, alpha sequences.

sequences (Figure 2, right side of each panel). One or fewer rounds of replication are seen in the 12 hour and 24 hour time points, and one or more rounds are seen in the 36 hour and 48 hour time points. Since the chromosomes are known to replicate once per cell cycle, this result confirms our interpretation that the plasmids are also replicating once per cell cycle. Table 1 summarizes the quantitative data obtained by densitometer tracings of several experimental trials and further supports the conclusion that all four plasmids replicate once per cell cycle.

Timing of replication

To investigate the timing of replication of our plasmids within S phase, we used a procedure for the retroactive synchrony of cells (14). Logarithmically growing cells carrying pLIB41, pDYAL, or p220.2 were pulse labelled for 3 hours with BrdUrd. This time is much shorter than the time of S phase in these cells, which was estimated to be approximately 8–10 hours. After labelling, the cells were fixed in ethanol and stained for DNA content with chromomycin A3. Chromomycin stained cells were analyzed by using a fluorescence activated cell sorter (FACS) and sorted into subpopulations representing synchronous cells in six different portions of the cell cycle (Figure 3).

Genomic DNA was isolated from each of the sorted fractions and run on Cs_2SO_4 density gradients. Density gradients were fractionated and slot blotted as described earlier. Slot blots were probed first with the pDY$^-$ vector to determine the timing of replication of the autonomous plasmids. Figure 4 shows a representative blot for each of the three plasmids. As an internal control, the slot blots were stripped and re-probed with two chromosomal sequences which replicate at known times of replication. For this purpose we used an alpha repeat sequence from the centromeric region of chromosome 7. Under the hybridization conditions used, the alpha repeats from chromosome 7 do not cross-hybridize with the alpha repeats from chromosome 17 which are present on the pDYAL plasmid (data not shown). Centromeric regions have been shown to replicate late in S phase (15, 16). In addition, each blot was probed with an Alu repeat sequence (7). We have found Alu sequences to replicate throughout S phase, presumably by virtue of their highly dispersed locations. Alu sequences do not hybridize to sequences on any of the plasmids used in this timing study (data not shown). Reprobing each blot with alpha sequences and with Alu sequences and observing the correct timing of replication for these repeat sequences verified that each sample was correctly fractionated. The slot blots were quantified by scanning laser densitometry. The fraction of DNA replicated during each part of the cell cycle was calculated by dividing the amount of replicated DNA by the total DNA [HL/(HL+LL)]. These values were normalized by expressing them as a percentage of the total amount of replication observed in each blot. Results are shown in Figure 5.

As Figure 5 (b, d and f) indicates, the replication of the control (chromosomal) alpha sequences was confined to the latter part of the S phase, as expected. Figure 5 (b, d and f) also shows the timing of replication of the Alu repeat, which reproducibly replicates throughout S phase with a peak in S2. These results demonstrate that we successfully fractionated the cell cycle into several sequential portions. The plasmid pLIB41 (Figure 5e) showed a peak of replication during S3. This pattern is similar, but slightly later than that of the dispersed Alu repeat. The plasmid pDYAL (Figure 5c) replicated predominantly during S2, S3, and S4 and contrasted with the chromosomal alpha sequences, with their more pronounced pattern of late replication. The EBV vector p220.2 (Figure 5a) replicated preferentially in the latter half of S, in a pattern similar to the chromosomal alpha sequences. These patterns were reproducible over several trials, as shown in Figure 5.

DISCUSSION

Once per cell cycle control

This work suggests that the autonomously replicating human sequences we have isolated may provide a good model system for examining the once per cell cycle control of replication and the timing of replication. The experiments establish that the four plasmids we examined undergo autonomous replication following once per cell cycle control. The absence of vector DNA labelled at the heavy-heavy density before the completion of one cell cycle argues that once per cell cycle replication of the vectors is taking place. Appearance of the same pattern when the samples are probed with chromosomal sequences suggests that the plasmids are subject to the same once per cell cycle control as the chromosomes.

The labelling pattern we observed due to incorporation of BrdUrd into plasmid DNA is also consistent with semi-conservative DNA replication. The patterns of BrdUrd labelling observed for the plasmids are characteristic of normal replicative rather than repair replication. This evidence confirms and extends the conclusions we were able to draw from a previous study (2). In that work autonomous replication was evidenced by long-term retention of plasmid DNA and appearance of *Mbo*I-sensitive vector DNA, i.e. DNA which had lost its bacterial methylation pattern on both strands. Taken together, these results confirm that we have cloned sequences that can mediate autonomous replication in human cells. Further proof is the observation of replication intermediates of pLIB41 on two dimensional gels (4). Moreover, these human sequences and many others have been shown to replicate in short-term replication assays in the absence of all viral sequences (2, 3). Further characterization of these autonomously replicating plasmids should shed considerable light on the nature of replication initiation in human cells and the mechanism of once per cell cycle control.

It has been observed that although *Xenopus* eggs appear able to replicate virtually any introduced DNA sequence, replication is restricted to once per cell cycle (17). Our results reinforce the conclusion that a eukaryotic cell can replicate any sequence containing appropriate signals for initiation in a once per cell cycle manner, unless signals exist for overcoming this regulation and/or regulating copy number independently, as is the case for most viruses and plasmids. An exception is latent Epstein-Barr virus, which appears to replicate once per cell cycle (18). Our data indicates that p220.2, an EBV vector carrying *oriP*, also replicates once per cell cycle. Once per cell cycle replication of vectors based on EBV has also recently been reported by others (19).

Two types of models have been considered to provide the mechanism for once per cell cycle control. To prevent reinitiation, some method of marking a stretch of DNA as having already replicated is required. This marking could take the form of signals which block reinitiation at specific sites or of more global mechanisms that would mark the DNA in a more continuous fashion. For example, binding of a protein to a specific DNA sequence has been proposed as the mechanism to control putative once per cell cycle control of the replication of BPV (20). Alternatively, a global system may exist that marks all newly replicated DNA. The hemimethylation of newly replicated DNA has been raised as a possible method of marking replicated DNA (21). It has been proposed that DNA in *Xenopus* eggs may be marked by a positive 'licensing factor' that is inactivated by initiation events and excluded from the nucleus until nuclear membrane breakdown at mitosis (22). Using a two-dimensional gel technique, we have shown (4) that initiation of replication on pLIB41 occurs at multiple locations on the plasmid. Since we show here that pLIB41 replicates once per cell cycle, it appears that once per cell cycle control can be maintained without a requirement for initiation from specific origin sequences.

Timing of replication

It has been shown that DNA replication in eukaryotes proceeds in a temporal order during S phase. Chromosomal sequences in both yeast (23) and mammalian cells (24, 25) have been shown to replicate at specific times in S, although the mechanism which maintains the control of replication timing has yet to be established. Two types of models may be advanced to explain the differential timing of replication of various parts of the genome. There may be specific DNA signals in the vicinity of origins that govern the time at which they initiate. Conversely, replication timing may be determined more generally by context.

For example, replication timing of a given region of DNA may be influenced by the state of transcription, the chromatin configuration, or chromosomal location, independent of any signals that specifically control replication timing. Several reports show a correlation between early replication and gene expression. Transcriptionally active regions usually replicate early, while inactive areas frequently replicate late (24, 25). However, the correlation between expression and early replication is imperfect, and a cause and effect relationship has not been established between the two phenomena. Cytogenetic evidence has shown that large chromosomal units spanning many replicons seem to initiate replication simultaneously. This data may indicate that chromosomal location or higher order nuclear organization may influence timing of replication (26). Also, it is possible that chromatin structure, such as the highly condensed heterochromatin characteristic of the alpha repeat sequences of mammalian centromeres, may be a factor in determining the late replication of these regions.

Several aspects of our data bear on these questions. We have found that alpha DNA repeats, which are known to replicate late in their normal centromeric DNA context (15, 16), do not retain this replication time when they mediate replication on the extrachromosomal vector pDYAL. This result suggests that late time of replication may be not be specified by the same signals that allow this DNA to replicate. It is possible that a specific configuration adopted by the alpha sequences in the chromosomes is responsible for late replication, and that this configuration is unrelated to the signals that trigger replication *per se*. The loss in pDYAL of the pronounced late replication observed for the chromosomal alpha sequences could be accounted for by the much longer stretches of alpha sequences (hundreds or thousands of kb) present in human centromeres, as opposed to the approximately 16 kb of alpha DNA on the plasmid. Perhaps relatively long stretches of alpha repeats are required to adopt a configuration that could determine late replication. Apparently, the vector backbone does not prevent late replication, since p220.2 was seen to replicate late in S phase. It will be interesting to learn whether vectors will replicate later in S as increasing amounts of alpha DNA are added to the plasmid.

It is unknown whether long stretches of chromosomal alpha repeats at human centromeres are replicated from internal sites of initiation or by forks that initiate in neighboring regions. That the alpha sequences in pDYAL can mediate replication suggests that human centromeres may be replicated from initiation sites within the alpha repeats.

The timing of replication of the pLIB41 human sequence in its chromosomal context is currently unknown. Therefore, in this case we do not know if the replication timing shown by the sequence on an autonomous replicon is consistent with that of the chromosomal sequence.

It has been proposed that the timing of replication of a sequence may merely be a function of its proximity to a site of initiation. The timing of replication of any particular sequence might be based on the amount of time required for a replication fork to move from the replication origin to the sequence, and thus replication timing would be a function of the distance of the region from a site of initiation. Regions in close proximity to sites of initiation would be replicated early and those located at a greater distance would be replicated late. However, our results argue that additional factors must be involved in determining late replication. Because of the relatively small size of the plasmids in our system, proximity to sites of initiation is not expected to be a relevant factor in determining the replication timing of sequences on the vector. All three of the plasmids in this study exhibit the ability to replicate in the latter half of S phase, with p220.2 being the most late. The Alu controls show that our assay is able to identify sequences replicating in the first half of S phase. Therefore, the existence of late replicating plasmids suggests that either initiation or elongation can be restricted to the latter half of S phase in mammalian cells, and that this restriction may be a determinant of late replication.

The very late replication observed for p220.2 conflicts with a report of the timing of whole Epstein-Barr virus. It has been reported that EBV (180 kb) replicates early in S phase (27), whereas we have observed that p220.2, a small vector derived from EBV (9 kb), replicates later in S phase. This result raises the possibility that size of autonomously replicating vectors may play a role in controlling their time of replication. Alternatively, it is possible that other aspects of EBV viral sequences such as their pattern of gene expression are responsible for this change in replication timing.

We have the ability to manipulate autonomously replicating vectors in terms of their size, sequence composition, and patterns of gene expression. Therefore, by using autonomously replicating model systems, it may ultimately be possible to establish the cause and effect relationships between these parameters and the control of replication.

ACKNOWLEDGEMENTS

We thank Patrick Krysan, Scott Heinzel, Dave Gilbert, Garry Nolan, and Steve Fiering for helpful discussions. We also thank Paul Raju for FACS assistance. This work was supported by grants from the National Cancer Institute and the American Cancer Society.

REFERENCES

1. Reisman,D., Yates,J. and Sugden,B. (1985) *Mol. Cell. Biol.* **5**, 1822–1832.
2. Krysan,P.J., Haase,S.B. and Calos,M.P. (1989) *Mol. Cell. Biol.* **9**, 1026–1033.
3. Heinzel,S.S., Krysan,P.J., Tran,C.T. and Calos,M.P. (1991) *Mol. Cell. Biol.* **11**, 2263–2272.
4. Krysan,P.J. and Calos,M.P. (1991) *Mol. Cell. Biol.* **11**, 1462–1472.
5. DuBridge,R.B., Tang,P., Hsia,H.C., Leong,P.M., Miller,J.H. and Calos,M.P. (1987) *Mol. Cell. Biol.* **7**, 379–387.
6. Heinzel,S.S., Krysan,P.J., Calos,M.P. and DuBridge,R.B. (1988) *J. Virol.* **62**, 3738–3746.
7. Rubin,C.M., Houck,C.M., Deininger,P.L., Freidman,T. and Schmid,C.W. (1980) *Nature (London)*, **284**, 372–374.
8. Waye,J.S., England,S.B. and Willard,H.F. (1987) *Mol. Cell Biol.* **7**, 349–356.
9. Stillman,B.W. and Gluzman,Y. (1985) *Mol. Cell. Biol.* **5**, 2051–2060.
10. Graham,F.L., Smiley,J., Russell,W.C. and Nairn,R. (1977) *J. Gen. Virol.* **36**, 59–72.
11. Wigler,M., Sweet,R., Sim,G.K., Wold,B., Pellicer,A., Lacy,E., Maniatis,T., Silverstein,S. and Axel,R. (1979) *Cell* **16**, 777–785.
12. Gray,J.W. and Coffino,P. (1979) *Methods Enzymol.* **58**, 233–248.
13. Hirt,B. (1967) *J. Mol. Biol.* **26**, 365–369.
14. Gilbert,D.M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2924–2928.
15. Taylor,J.H. (1960) *J. Biophys. Biochem. Cytol.* **7**, 455–464.
16. Lima-de-Faria,A. and Jaworska,H. (1968) *Nature* **217**, 138–142.
17. Harland,R.M. and Laskey,R.A. (1980) *Cell*, **21**, 761–771.
18. Adams,A. (1987) *J. Virol.* **61**, 1590–1594.
19. Yates,J.L. and Guan,N. (1991) *J. Virol.* **65**, 483–488.
20. Roberts,J.M., and Weintraub,H. (1986) *Cell*, **46**, 741–752.
21. Taylor,J.H. (1984) *Molec. Cell. Biochem.* **61**, 99–109.
22. Blow,J.J. and Laskey,R.A. (1988) *Nature* **332**, 546–548.
23. Fangman,W.L., Hice,R.H. and Chlebowicz-Siedziewska,E. (1983) *Cell*, **32**, 831–838.
24. Goldman,M.A., Holmquist,G.P., Gray,M.L., Caston,L.A. and Nag,A. (1984) *Science* **224**, 686–692.
25. Hatton,K.S., Dhar,V., Brown,E.H., Iqbal,M.A., Stuart,S., Didamo,V.T. and Schildkraut,C.L. (1988) *Mol. Cell. Biol.* **8**, 2149–2158.
26. Hand,R. (1978) *Cell* **15**, 317–325.
27. Hampar,B., Tanaka,A., Nonoyama,M. and Derge,J.G. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 631–633.