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Replication Timing Control Can Be Maintained in Extrachromosomally Amplified Genes

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Extrachromosomal elements are common early intermediates of gene amplification in vivo and in cell culture. The time at which several extrachromosomal elements replicate was compared with that of the corresponding amplified or unamplified chromosomal sequences. The replication timing analysis employed a retroactive synchrony method in which fluorescence-activated cell sorting was used to obtain cells at different stages of the cell cycle. Extrachromosomally amplified Syrian hamster CAD genes (CAD is an acronym for the single gene which encodes the trifunctional protein which catalyzes the first three steps of uridine biosynthesis) replicated in a narrow window of early S-phase which was approximately the same as that of chromosomally amplified CAD genes. Similarly, extrachromosomally amplified mouse adenosine deaminase genes replicated at a discrete time in early S-phase which approximated the replication time of the unamplified adenosine deaminase gene. In contrast, the multicopy extrachromosomal Epstein-Barr virus genome replicated within a narrow window in late S-phase in latently infected human Raji cells. The data indicate that localization within a chromosome is not required for the maintenance of replication timing control.

The mammalian genome consists of an estimated 6×10^9 bp of DNA which typically replicate within approximately 8 h in most somatic cells. Since the elongation rate is roughly 2 kb/min, complete synthesis requires initiation to begin at roughly 10,000 DNA replication origins. Electron microscopic (20) and biochemical (4, 36) evidence which supports the existence of such origins has now been obtained.

The time at which DNA synthesis initiates from each origin appears to be programmed and is reproducible from cell cycle to cell cycle (19, 32, 33). While the mechanisms responsible for replication timing control are not understood, it is clear that altering gene position can dramatically alter replication timing. For example, naturally occurring chromosome translocation in mammalian cells and in vitro gene repositioning in yeast cells can convert an early replicating region to a late replicating region and vice versa (5, 13). The effect of chromosome position on replication timing has been interpreted to indicate that the time at which a sequence replicates is dictated by its distance from the closest origin, by the existence of *cis*-acting sequences or structures which influence when an origin fires, or by a combination of the two (5, 13).

An extreme case of repositioning of genomic information occurs in the production of extrachromosomal elements during gene amplification. Studies employing transfected (6, 29, 30) and endogenous (21, 41) genes reveal that acentric extrachromosomal elements can be produced by a mechanism which deletes the corresponding chromosomal sequences. These elements contain 100 to >1,000 kb of DNA flanking the amplification target gene (37) and have been demonstrated by cytogenetic (9) and biochemical (3, 7) methods to replicate once per cell cycle.

While the program which ensures that DNA is replicated only once per S-phase appears to be preserved in extrachromosomal-amplification intermediates, the regulation of timing control in such structures is only beginning to be explored. Interestingly, the extrachromosomal elements containing dihydrofolate reductase (DHFR) genes in the mouse cell line 3T6R50 appear to replicate throughout S-phase (34), while the chromosomally located single-copy and amplified DHFR genes in a variety of mouse, human, and hamster cell lines generally replicate in a discrete interval confined to the first half of S-phase (10, 19, 24).

The difference in replication timing of extrachromosomal and chromosomal DHFR amplicons raises the possibility that either the recombination events which generate extrachromosomal amplicons sever the connection between replication origins and adjacent timing control sites or that timing control is an intrinsic property of sequences confined within chromosomes. To investigate these issues further, we have determined the replication times of two extrachromosomal amplicons harboring either CAD genes in CHO cells or adenosine deaminase (ADA) genes in mouse cells and the Epstein-Barr virus (EBV) episome in human Raji cells. All three extrachromosomal elements replicate within narrow windows of S-phase: CAD and ADA in early S-phase and the EBV episome in late S-phase. The CAD extrachromosomal amplicons also replicate, within experimental error, at the same time in S-phase as the corresponding chromosomally amplified genes, and extrachromosomal ADA amplicons replicate at approximately the same time as the unamplified wild-type gene. The results show that replication timing control can be preserved in extrachromosomal structures and thus that chromosomal association is not required for the correct temporal regulation of DNA replication. Furthermore, if *cis*-acting timing control sequences exist, they are contained in both the extrachromosomal CAD and ADA amplicons.

MATERIALS AND METHODS

Cell lines. The growth conditions for cell lines B54, T5S13, and C5R0.5 were as previously described (7). Raji cells containing the EBV episome were grown in RPMI containing 10% fetal bovine serum. The mouse cell lines Cl-1D and

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B1-50, containing wild-type and amplified ADA genes, respectively, were kindly provided by R. Kellems and were maintained as described elsewhere (42). The Cl-1D and B1-50 cell lines are derived from a mouse cell line which lacks endogenous thymidine kinase activity [42]; therefore, in order to perform the experiments described in this paper, the herpes simplex virus thymidine kinase gene was introduced into these cells by calcium phosphate coprecipitation, and stable transformants were selected by growth in HAT (hypoxanthine-aminopterin-thymidine) medium (31). One clone from each cell line which incorporated [^{3}H]-thymidine at approximately the same level as wild-type mouse cells was used in these experiments (7a).

Labeling and separation of cells into cell cycle fractions. The methods for labeling cells with 5-bromo-2′-deoxyuridine (BrdU), fractionating cells on the cell sorter, and quantitating the incorporated label were previously described by Gilbert and Cohen (16). Cultures at 90% confluence were trypsinized and plated in fresh medium within 24 to 48 h of analysis to maximize the number of cells in S-phase. This procedure occasionally resulted in populations depleted in G_1-phase cells at the time of analysis (e.g., see Fig. 1, cell line B5-4). However, independent experiments which resulted in different fluorescence-activated cell-sorting profiles generated replication timing profiles which were experimentally indistinguishable. In order to distinguish replicated from unreplicated DNA, the DNA was density labeled by incubating the cells for 90 min in 30 μM BrdU in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The cells were trypsinized, washed in phosphate-buffered saline containing 1 mM EDTA, and fixed in 70% ethanol. The fixed cells could be stored refrigerated for up to 3 days. Cells were prepared for sorting by being washed in phosphate-buffered saline and were suspended at 3×10^6/ml in an aqueous solution of the DNA-specific stain chromomycin A3 (CA3) (20 μg/ml) containing 3 g of $MgCl_2 \cdot 6H_2O$ per liter. Cells were sorted on the Salk Institute cell sorter adjusted to sort CA3-stained cells by using fluorescent signals from nuclei excited at a wavelength of 457 nm at a flow of 2,000 cells per s. Approximately 0.5×10^6 to 1×10^6 cells were collected in each window. To evaluate the accuracy of sorting, an aliquot of each window was restained in CA3 and again analyzed on the cell sorter.

Isolation and separation of replicated and unreplicated DNAs. DNA was isolated from the collected cells as follows. The cells were pelleted at 2,000 rpm in a microfuge, and the pellet was lysed by the addition of 0.5 ml of lysis buffer (1% sodium dodecyl sulfate, 100 mM Tris [pH 8], 200 mM EDTA, 100 μg of proteinase K per ml) and incubated at 37° for 4 to 6 h. The lysate was extracted once with 1 volume of phenol-chloroform and once with chloroform and then ethanol precipitated with 0.2 M NaCl. The DNA was suspended in 100 μl of TE (100 mM Tris [pH 8], 1 mM EDTA) containing 25 μg of RNase per ml and incubated at 37°C for 30 min. The DNA was added to 1 ml of a solution of CsCl in TE (58% [wt/wt]; refractive index = 1.4052) and sheared by passage 20 times through a 27-gauge needle. The sheared DNA was placed in a 5-ml quick-seal centrifuge tube, and additional CsCl solution was added to fill the tube. Gradients were centrifuged in a VTi65 rotor (Beckman) at 45,000 rpm for 16 h at 20°C. Fractions were collected through a 22-gauge needle inserted into the bottom of the tube; 22 to 24 aliquots were collected at 6 drops per fraction, and the refractive index was read on every other fraction.

Hybridization analysis of gradient fractions. Aliquots (125 μl) of each fraction were denatured in 0.3 N NaOH at 60°C for 15 min, allowed to cool, and then neutralized by the addition of 1 volume of 2 M ammonium acetate. The samples were applied to Hybond N membrane (Amersham) by using a slot blot apparatus (Schleicher & Schuell). The membranes were baked and hybridized as described elsewhere (26) with nick-translated or random-primed probes. The following fragments were used as probes: for CAD, the 8.6-kb *Bam*HI fragment 102 (27); for ADA, the 1.4-kb *Eco*RI fragment derived from cosmid E8 (1); for EBV, the 2.3-kb *Bam*HI-*Sac*I fragment of the W fragment (2); and for *ras*, a 1-kb Kirsten-*ras* fragment (12). The amounts of heavy-light (HL) and light-light DNA were quantitated on autoradiographs with a Helena Laboratories Quick Scan densitometer using exposures in the linear range of the film.

RESULTS

Strategy for replication timing analysis. The retroactive synchrony method was used to determine replication time, since this method measures replication in exponentially growing cultures and does not employ cytotoxic agents to induce cell synchrony (15, 16). In this procedure, cells are exposed briefly to a nontoxic concentration of BrdU to label DNA which is being replicated at the time of incubation (see Materials and Methods). DNA replicated during this period will incorporate BrdU unifililarly and can be distinguished from unreplicated DNA by CsCl density gradient centrifugation. Each cell population is then fractionated according to the DNA content of individual cells by using the cell sorter.

Most of the experiments reported below were performed with cells sorted into six fractions using equally spaced sort windows over the range of CA3 fluorescence corresponding to cells spanning the extremes of the cell cycle from G_1(2N) to G_2+M(4N). Thus, the first window contains a mixture of cells containing 1.0 to ~1.17 times the diploid content. Window 1 should, therefore, contain a continuum of cells ranging from those in G_1 to those which have replicated as much as 17% of their DNA. Similarly, window 2 contains cells which have replicated from 17 to 34% of their DNA, and so on. The retroactive synchrony method has the capacity to reveal two facets related to DNA replication: (i) it indicates how broad a period of S-phase is required for the replication of a DNA sequence, and (ii) it approximates the time of replication by revealing the fraction of the genome which has replicated at the time BrdU-labeled DNA complementary to the probe is first detected. Since replication timing is inferred from DNA content determined directly by the sorter, it is possible to compare the replication timing data obtained from different cell lines which may differ in their cell cycle kinetics.

Representative DNA histograms obtained from four of the cell lines analyzed are shown in Fig. 1, in which the CA3 fluorescence is plotted on a linear scale. These histograms reveal that the growth conditions employed generate a substantial number of cells in S-phase in each cell line. In some experiments, we observed a relative depletion of cells in G_1 (e.g., see cell line B5-4). The replication timing of each gene was determined in several independent experiments performed at different times, and different flow histograms were occasionally obtained in independent experiments involving the same cell line; however, dissimilar histograms did not produce significant differences in the replication timing of any of the genes analyzed.

A typical example of the quality of the preparative cell fractionation achievable with the cell sorter is shown in Fig. 2A. This population of 10^7 cells was divided into six win-

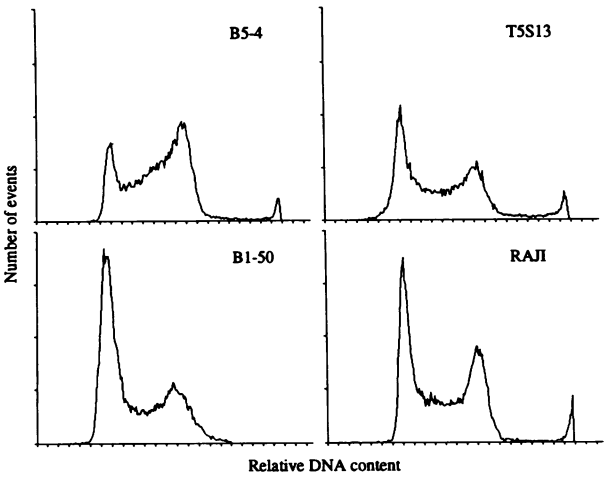

FIG. 1. Flow histograms of representative cell lines. Cells grown as described in Materials and Methods were briefly labeled with BrdU, fixed, and then incubated with the DNA-specific stain CA3. The stained cells were then fractionated on the basis of their DNA content by using the cell sorter. The relative DNA contents of individual cells as measured by total CA3 fluorescence are displayed in a linear scale on the abscissa, and the numbers of cells are shown on the ordinate. Approximately 30,000 cells were analyzed for each cell line.

dows on the basis of DNA content as described above. Each fraction, or window, was then resorted to determine the extent of contamination with adjacent fractions. This analysis revealed that the purity of cells in a given window was greater than 95%.

The sensitivity of the replication timing measurements is partially determined by the amount of BrdU-labeled DNA in each CsCl gradient, and this in turn is related to the number of S-phase cells in the corresponding sort window. We therefore attempted to include approximately similar numbers of S-phase cells in each sample analyzed by CsCl gradient fractionation. This necessitated estimating the proportion of cells in S-phase in each sort window. This was done by fitting the experimentally determined flow histogram data points to the theoretical cell cycle distribution according to the method of Dean and Jett (11; Fig. 2B). We then compensated for the low proportion of S-phase cells in fractions 1 and 6 by applying DNA from a proportionately greater number of cells derived from these fractions to the respective CsCl gradients.

Analysis of replication timing of extrachromosomally and chromosomally amplified CAD genes. The retroactive synchrony method was used to determine the approximate time of replication of the CAD gene in three cell lines. B5-4 is a Syrian hamster kidney cell line in which approximately 100 copies of the CAD gene are located at a single chromosomal site near the position of the resident gene (39, 40). C5R0.5 and T5S1-3 are CHO cell lines containing the transfected cloned Syrian hamster CAD gene amplified exclusively in either extrachromosomal or chromosomal sites, respectively (7, 38). The analyses shown in Fig. 3A to C and Table 1 reveal two facets about the replication of the CAD gene in each of these cell lines: (i) replication occurs in a narrow window of S-phase, and (ii) in all cases, the CAD sequences replicate in the first half of S-phase. The replication timing determined here is similar to that determined previously for the wild-type CAD gene in CHO cells by other methods (32).

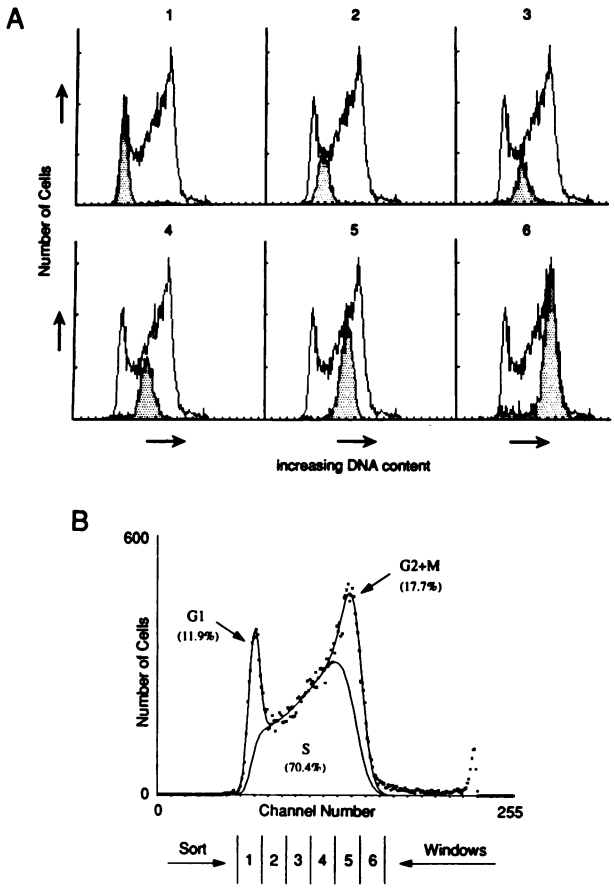

FIG. 2. (A) Resorting of cells reveals the purity of each fraction. Exponentially growing B5-4 cells were labeled with BrdU, stained with CA3, and sorted into six windows based on DNA content. An aliquot of each fraction was resorted to determine the percent contamination of a given window by cells of higher or lower DNA content. The shaded area under the curve represents the reanalysis of individual windows. (B) Cell cycle analysis of B5-4 cells. Cell cycle analysis of the DNA distributions was done with software developed by one of us (J.T.) and based on the mathematical model of Dean and Jett (11). The histogram was then divided into six equally spaced windows by setting a lower limit at the left side of the G_1 distribution and an upper limit at the right side of the G_2+M distribution. The total width of the distribution was then divided by 6 to obtain the width for each sort window. In general, window 1 contains mainly G_1 cells, windows 2 to 5 contain S-phase cells, and window 6 contains mainly G_2+M cells. In some experiments, a small number of early and late S-phase cells were present in windows 1 and 6, respectively. In such cases, the proportions of S-phase cells in these windows were estimated as described in the text to enable application of proportionately more sample to the corresponding CsCl gradient. This enabled each CsCl gradient to contain roughly equivalent quantities of DNA obtained from S-phase cells.

To ensure that HL DNA was actually present in all of the CsCl gradients, the slot blots were stripped of CAD probe and rehybridized with blur 8, a human *alu* sequence (23) which also detects CHO *alu*-like sequences. Substantial hybridization was detected in all windows, indicating that these sequences replicated throughout S-phase (data not shown). To detect the replication of sequences present at lower abundance than the amplified CAD genes in C5R0.5, the same slot blots employed as described above were

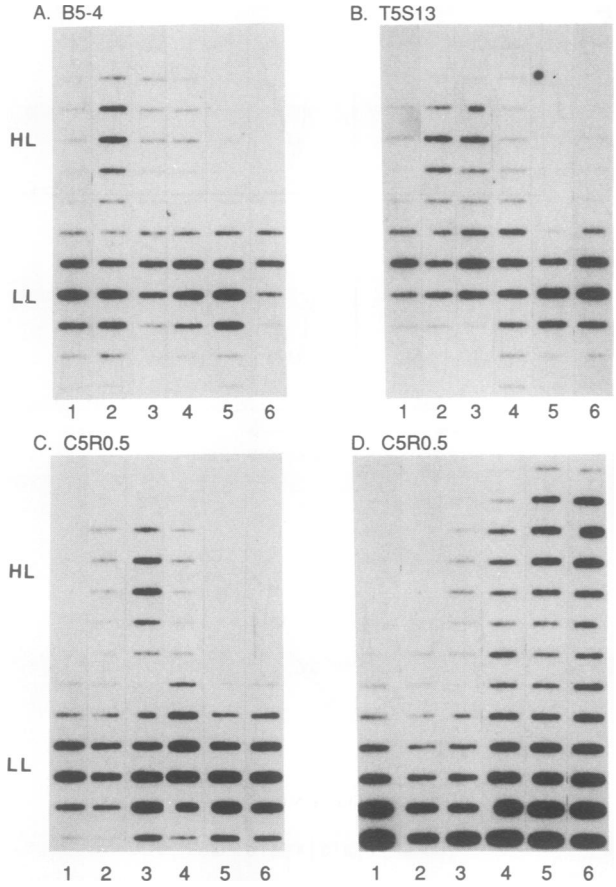

FIG. 3. Replication times of chromosomally and extrachromosomally located CAD genes. DNA was isolated from BrdU-labeled cells which had been sorted into six windows and was then fractionated on a CsCl gradient and blotted as described in Materials and Methods. The positions of HL and light-light (LL) DNAs are shown on the side, and the windows of the cell cycle are indicated below the lanes. The blots were hybridized with a CAD-specific probe (A, B, and C). The blot containing the sorted C5R0.5 cells (C) was stripped of the CAD probe and rehybridized with a *ras* probe (D). The B5-4 cell line contains approximately 100 copies of the CAD gene located on a single chromosome. T5S13 and C5R0.5 contain approximately 250 copies of chromosomally and extrachromosomally located CAD genes, respectively. The density of the gradient fractions was determined with a refractometer, and differences in the positions of the fractions containing HL DNA reflect differences in the starting density of the CsCl.

stripped of hybridized sequences and rehybridized with a Kirsten (k)-*ras* probe which detects the *ras* proto-oncogene. This gene was chosen because it is amplified approximately 10-fold in CHO cells, but the amplified copies are inactive and have previously been shown to replicate late in S-phase (8, 22). Consistent with previous observations, Fig. 3D clearly shows that the k-*ras* probe detects HL DNA only in the last three sorted windows. This result emphasizes that sufficient DNA was present in this experiment to have enabled detection of a small fraction of late-replicating CAD sequences. We conclude, therefore, that the replication of the vast majority, if not all, of extrachromosomally amplified CAD genes in this CHO cell line is confined to the first half of S-phase. Thus, the timing characteristics of chromosomally and extrachromosomally amplified CAD genes are the same within the experimental error of the methods employed.

Preservation of timing control in extrachromosomally amplified ADA genes. The results described above contrasted significantly with those obtained previously with the DHFR extrachromosomal elements in mouse cells (34). Since the CAD extrachromosomal elements arose in an experiment involving transfection of a cloned CAD gene (7, 28, 38), we compared the replication timing of the wild-type ADA gene in mouse Cl-1D cells and in a derivative, B1-50, which contains 5,000 ADA genes localized exclusively in heterogeneously sized extrachromosomal elements (42). The amplicon in B1-50 cells is an approximately 460-kb circular element organized as an imperfect inverted repeat (26a) which contains a single site at which DNA synthesis initiates bidirectionally (5a). Figure 4 and Table 1 show that the wild-type ADA gene replicates at a discrete time in the first half of S-phase in Cl-1D cells and that the 5,000 amplicons in B1-50 replicate in a very narrow window of early S-phase which is the same, within experimental error, as that observed for the single-copy wild-type gene. Similar results were obtained after hybridization of slot blots with several different probes spanning the ADA amplicon (data not shown).

Late replication of EBV episome in Raji cells. Genes within the CAD and ADA episomes must be expressed in order for the cell to survive under the selective conditions imposed. The early replication timing observed in these extrachromosomal elements is not unexpected, since transcriptionally active chromosomal regions also tend to replicate early in S-phase (19). Since transcriptionally inert chromosomal sequences tend to replicate late in S-phase (19), it was of interest to examine the replication timing of an episome in which most of the sequences within it are known to be

TABLE 1. Replication times of amplified genes[a]

Cell line	Gene	No. of copies	Location	% HL DNA/window[b]					
				1	2	3	4	5	6
C5R0.5	CAD (t)	100	E	<2	23	20	9	<2	<2
T5S13	CAD (t)	100	C	10	60	29	<2	<2	<2
B54	CAD	100	C	<2	33	24	<2	<2	<2
C5R0.5	*ras*	10	C	<2	<2	16	22	27	31
B1-50	ADA	5,000	E	<2	23	23	<2	<2	<2
Cl-1D	ADA	2	C	<2	<2	26	10	<2	<2
Raji	EBV	60	E	<2	<2	9	24	13	<2

[a] Autoradiograms from experiments performed as described in the text and shown in the figures were quantitated by using a densitometer; exposures in the linear range of the film were scanned. The data shown are from a single experiment, although two or three experiments were performed on each cell line. The patterns observed for all experiments were the same within experimental error. t, transfected; E, extrachromosomal; C, chromosomal.

[b] Column numbers refer to the sorted fractions of cells as described in the text and in the legend to Fig. 1. Percentages were calculated as HL/(HL + light-light).

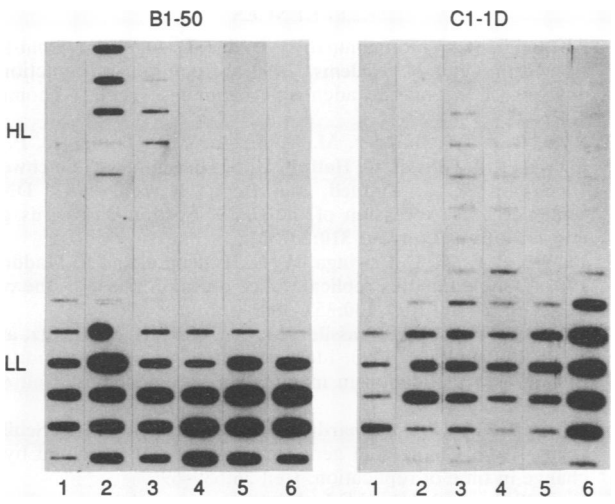

FIG. 4. Replication times of wild-type and extrachromosomally amplified ADA genes. DNA was prepared from sorted B1-50 and Cl-1D cells and was processed as described in the legend to Fig. 2 and in Materials and Methods. The blots were hybridized with an ADA-specific probe. The B1-50 and Cl-1D cells used in these experiments are clones containing a transfected viral thymidine kinase gene (7a). Cl-1D contains the wild-type ADA gene, and the B1-50 cell line contains a 5,000-fold amplification of the ADA gene. LL, light-light DNA.

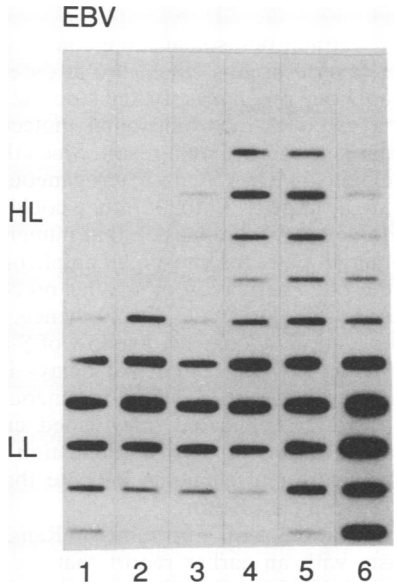

FIG. 5. Replication time of multicopy EBV episome in human Raji cells. Raji cells were labeled and sorted, and the isolated DNA was processed as described in Materials and Methods and the legend to Fig. 2. The blots were hybridized with an EBV-specific probe. LL, light-light DNA.

transcriptionally repressed. One example is provided by the EBV episome in latently infected human Raji cells, since only limited expression of the 172-kb episome is observed (2). The EBV plasmid is maintained at approximately 60 copies per cell and is replicated by the cellular machinery initially bidirectionally starting at *ori*P, but then a barrier encountered by one fork results in the remainder of replication occurring unidirectionally (14). To detect EBV-specific HL DNA, we used a 2.3-kb *Bam*HI-*Sac*I fragment derived from the *Bam*HI W fragment which is within 10-kb of *ori*P in the direction of replication (2). Figure 5 and Table 1 clearly show that replication of the EBV plasmid occurs in late S-phase.

DISCUSSION

This report demonstrates that the same control of replication timing exhibited by chromosomally located genes can be maintained in extrachromosomally amplified genes. This conclusion is based on three observations. First, extrachromosomally amplified CAD and ADA genes replicate within a limited number of windows of S-phase. Similar discrete replication times have been reported for many single-copy genes (19, 32). Second, the time of replication of both of these extrachromosomal amplicons is confined to early S-phase, as it is for the corresponding single-copy ADA and CAD (32) genes and for chromosomal amplicons of the CAD gene. Third, the general correlation that transcriptionally active regions replicate earlier in S-phase than inactive regions appears to be preserved in this analysis of three extrachromosomal replicons.

Replication timing control was found to be the same for the Syrian hamster CAD gene when the gene was amplified within a chromosome near its native site or in extrachromosomal or chromosomal sites subsequent to its introduction as a cosmid into the heterologous CHO nuclear environment by gene transfer. The CAD amplicon in the CHO clones analyzed here is approximately 260 kb and consists entirely of five Syrian hamster CAD cosmids (7, 11a). Recent data indicate that a site of bidirectional DNA synthesis is located within 15 kb of the 5′ end of the Syrian hamster CAD gene used to produce the CHO transformants analyzed here (24a). Thus, if *cis*-acting elements are required for the correct control of replication timing, they are located in the available CAD cosmid and are tightly linked to the CAD gene.

We have recently mapped a site of bidirectional initiation of DNA synthesis in the ADA amplicon to within 30 kb of the probes utilized in these studies (5a). The initiation of DNA synthesis occurs from this site within 15 min of the transition from G_1- to S-phase. A similar result was obtained for the CAD amplicon in both C5R0.5 and B5-4 cells (24a). These data further emphasize that both the CAD and ADA episomes initiate their replication at early times in S-phase.

The narrow replication window of the three extrachromosomal amplicons studied here contrasts with the apparent lack of replication time control in the extrachromosomally amplified murine DHFR genes in 3T6R50 cells (34). The apparent lack of timing control in these extrachromosomal amplicons is intriguing, since the single-copy DHFR gene has been observed to replicate in a narrow window either early (19, 24, 25) or late (19) in S-phase. There are several possible reasons for these differences. First, the use of a chemical synchrony method by Tlsty and Adams (34) may have influenced the replication of the extrachromosomal elements. We note that our results using retroactive synchrony generated data and conclusions similar to those reported in two independent analyses which employed centrifugal elutriation to ascertain replication timing patterns of the CAD and k-*ras* genes (22, 32). Second, since extrachromosomal elements can be generated by a process which involves chromosome breakage and removal of sequences from the native site (41), it is conceivable that *cis*-acting elements involved in timing control may have been removed

during formation of the DHFR amplicon in 3T6R50 cells. Third, if the extrachromosomal amplicon is very large, it may contain several origins which fire at different times in S-phase. If only one origin is activated stochastically in each S-phase in a given extrachromosomal molecule, a broad replication timing pattern should result. Since the extrachromosomal elements in 3T6R50 are heterogeneously sized and since some are as large as 3 Mb (35), they could span several replicons. However, we emphasize that numerous analyses reveal that large extrachromosomal amplicons are often formed by the oligomerization of smaller precursors which each contain the same origin (7, 37) and, hence, are expected to initiate replication in a narrow window of S-phase. While Tlsty and Adams (34) suggested that delays in replication may be generated because of difficulties encountered in the resolution of newly replicated intertwined circular molecules, our observations of narrow replication windows for three extrachromosomal elements indicate that this is not likely to be a significant factor.

The late replication of EBV episomes in Raji cells reported here contrasts with an earlier report that these molecules replicate in early S-phase (18). In those experiments, replication time was determined in cells subjected to synchronization by a double-thymidine block. Although the impact of this treatment on the maintenance of the EBV plasmid in a latent state has not been investigated, this treatment has been observed to cause virus activation (17). It is conceivable that the earlier study actually measured the replication time of EBV genomes in producer cells in the population. By contrast, we measured the replication of latent EBV genomes under conditions in which virus activation should not have occurred.

The 5,000 extrachromosomal ADA amplicons in B1-50 cells replicate in a window of S-phase which is similar to that of the diploid ADA locus in wild-type Cl-1D cells. The synchrony of replication exhibited by these amplicons is remarkable in light of the fact that they contribute an additional 5,000 replication origins to the ADA genome. If all of these origins are functional, this represents an increase of up to 50% in the total number of replication origins contained in the entire mouse genome. Furthermore, the ADA amplicons of B1-50 cells increase the cell genome size by almost 40% (i.e., $5{,}000 \times 460$ kb $= 2.3 \times 10^6$ kb of DNA). This suggests that if *trans*-acting factors are required to maintain replication timing control, either they are not limiting in these mouse cells or their synthesis is increased to compensate for the increased origin or DNA burden or both. If replication timing is dictated solely by DNA primary sequence, then the synchrony detected here is not surprising, since molecular analyses of the ADA amplicon reveal that most, if not all, ADA amplicons in B1-50 cells have a common molecular organization (26a). The observation that these highly amplified extrachromosomal molecules exhibit a replication program indistinguishable from that of the corresponding wild-type chromosomal gene suggests the utility of such systems in dissecting the molecular mechanisms contributing to replication timing control.

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REFERENCES

1. **Al-Ubaidi, M., V. Ramamurthy, M. Maa, D. Ingolia, J. Chinsky, B. Martin, and R. Kellems.** 1990. Structural and functional analysis of the murine adenosine deaminase gene. Genomics **7**:476–485.
2. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnell, and B. G. Barrell.** 1987. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
3. **Barker, P. E., H. L. Drwinga, W. N. Hittleman, and A. Maddox.** 1980. Double minutes replicate once during S phase of the cell cycle. Exp. Cell Res. **130**:353–360.
4. **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.
5. **Calza, R. E., L. A. Eckhardt, T. DelGiudice, and C. L. Schildkraut.** 1984. Changes in gene position are accompanied by a change in time of replication. Cell **36**:689–696.

5a. **Carroll, S., et al.** Unpublished data.

6. **Carroll, S., M. DeRose, P. Gaudray, C. Moore, D. Needham-VanDevanter, D. Von Hoff, and G. Wahl.** 1988. Double minute chromosomes can be produced from precursors derived from a chromosomal deletion. Mol. Cell. Biol. **8**:1525–1533.
7. **Carroll, S., P. Gaudray, M. De Rose, J. Emery, J. Meinkoth, E. Nakkim, M. Subler, D. Von Hoff, and G. Wahl.** 1987. Characterization of an episome produced in hamster cells that amplify a transfected CAD gene at high frequency: functional evidence for a mammalian replication origin. Mol. Cell Biol. **7**:1740–1750.

7a. **Carroll, S., and G. Wahl.** Unpublished data.

8. **Chattopadhyay, S. K., E. H. Chang, M. R. Lander, R. W. Ellis, E. M. Scolnick, and D. R. Lowy.** 1982. Amplification and rearrangement of onc genes in mammalian species. Nature (London) **296**:361–364.
9. **Cowell, J. K.** 1982. Double minutes and homogenously staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. **16**:21–59.
10. **D'Andrea, A. D., U. Tantravahi, M. Lalande, M. A. Perle, and S. A. Latt.** 1983. High resolution analysis of the timing of replication of specific DNA sequences during S phase of mammalian cells. Nucleic Acids Res. **11**:4753–4774.
11. **Dean, P. N., and J. H. Jett.** 1974. Mathematical analysis of DNA distributions from flow microfluorometry. J. Cell Biol. **60**:523.

11a. **Draper, B. W., M. L. DeRose, and G. M. Wahl.** Unpublished data.

12. **Ellis, R. W., D. DeFeo, T. Shis, M. A. Gondo, H. A. Young, N. Tsuchida, D. R. Lowry, and E. M. Scholnick.** 1981. The p-21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrated genes. Nature (London) **292**:506–511.
13. **Ferguson, B. M., B. J. Brewer, A. E. Reynolds, and W. L. Fangman.** 1991. A yeast origin of replication is activated late in S phase. Cell **65**:507–515.
14. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. Cell **58**:527–535.
15. **Gilbert, D. M.** 1986. Temporal order of replication of Xenopus laevis 5S ribosomal RNA genes in somatic cells. Proc. Natl. Acad. Sci. USA **83**:2924–2928.
16. **Gilbert, D. M., and S. N. Cohen.** 1987. Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. Cell **50**:59–68.
17. **Hampar, B.** 1979. Activation of the viral genome in vitro, p. 283–295. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, New York.
18. **Hampar, B., T. Tanaka, M. Nonoyama, and J. G. Derge.** 1974. Replication of the resident repressed Epstein-Barr virus genome during the early S phase (S-1 period) of nonproducer Raji cells. Proc. Natl. Acad. Sci. USA **71**:631–633.
19. **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.
20. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanisms of DNA replication in mammalian chromosomes. J. Mol. Biol. **32**:327–341.
21. **Hunt, J. D., M. Valentine, and A. Tereba.** 1990. Excision of N-myc from chromosome 2 in human neuroblastoma cells containing amplified N-myc sequences. Mol. Cell. Biol. **10**:823–829.
22. **Iqbal, M. A., J. Chinsky, V. Didamo, and C. L. Schildkraut.** 1987. Replication of proto-oncogenes early during S phase in mammalian cell lines. Nucleic Acids Res. **15**:87–103.
23. **Jelink, W. R., T. P. Toomey, L. Leinwand, C. H. Duncan, P. A. Biro, P. V. Chardary, S. M. Weissman, C. M. Rubin, C. M. Houck, P. L. Deininger, and C. W. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. USA **77**:1398–1402.
24. **Kellems, R. E., M. E. Harper, and L. M. Smith.** 1982. Amplified dihydrofolate reductase genes are located in chromosome regions containing DNA that replicates during the first half of S-phase. J. Cell Biol. **92**:531–539.
24a. **Kelly, R., and G. M. Wahl.** Unpublished data.
25. **Mariani, B. D., and R. T. Schimke.** 1984. Gene amplification in a single cell cycle in Chinese hamster ovary cells. J. Biol. Chem. **259**:1901–1910.
26. **Meinkoth, J. M., and G. M. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**:267–284.
26a. **Nonet, G. H.** Unpublished data.
27. **Padgett, R., G. M. Wahl, and G. Stark.** 1982. Structure of the gene for CAD, the multifunctional protein that initiates UMP synthesis in Syrian hamster cells. Mol. Cell. Biol. **2**:293–301.
28. **Robert de St. Vincent, B., S. Delbruck, W. Eckhart, J. Meinkoth, L. Vitto, and G. M. Wahl.** 1981. The cloning and reintroduction into animal cells of a functional CAD gene, a dominant amplifiable genetic marker. Cell **27**:267–277.
29. **Ruis, J. C., and G. M. Wahl.** 1988. Formation of an inverted duplication can be an initial step in gene amplification. Mol. Cell. Biol. **8**:4302–4313.
30. **Ruis, J. C., and G. M. Wahl.** 1990. Chromosomal destabilization during gene amplification. Mol. Cell. Biol. **10**:3056–3066.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Taljanidisz J., J. Popowski, and N. Sarkar.** 1989. Temporal order of gene replication in Chinese hamster ovary cells. Mol. Cell. Biol. **9**:2881–2889.
33. **Ten Hagen, K. G., D. M. Gilbert, H. F. Willard, and S. N. Cohen.** 1990. Replication timing of DNA sequences associated with human centromeres and telomeres. Mol. Cell. Biol. **10**: 6348–6355.
34. **Tlsty, T. D., and P. Adams.** 1990. Replication of the dihydrofolate reductase gene on double minute chromosomes in a murine cell line. Exp. Cell. Res. **188**:164–168.
35. **van der Bliek, A. M., C. R. Lincke, and P. Borst.** 1988. Circular DNA of 3T6R50 double minute chromosomes. Nucleic Acids Res. **16**:4841–4851.
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. **Wahl, G. M., S. Carroll, and B. Windle.** *In* Gene amplification in mammalian cells: techniques and applications, in press. Marcel Dekker, Inc., New York.
38. **Wahl, G. M., B. Robert de Saint Vincent, and M. L. DeRose.** 1984. Effect of chromosomal position on amplification of transfected genes in animal cells. Nature (London) **307**:516–520.
39. **Wahl, G. M., L. Vitto, R. A. Padgett, and G. R. Stark.** 1982. Single copy and amplified CAD genes in Syrian hamster chromosomes localized by a highly sensitive method for in situ hybridization. Mol. Cell. Biol. **2**:308–319.
40. **Wahl, G. M., L. Vitto, and J. Rubnitz.** 1983. Co-amplification of rRNA genes with CAD genes in *N*-(phosphonacetyl)-L-aspartate-resistant Syrian hamster cells. Mol. Cell. Biol. **3**:2066–2075.
41. **Windle, B., B. W. Draper, Y. Yin, S. O'Gorman, and G. M. Wahl.** 1991. A central role for chromosome breakage in gene amplification, deletion formation and amplicon integration. Genes Dev. **5**:160–174.
42. **Yeung, C., D. Ingolia, C. Bobonis, B. Dunbar, M. Riser, M. J. Sciliano, and R. E. Kellums.** 1983. Selective overproduction of adenosine deaminase in cultured mouse cells. J. Biol. Chem. **258**:8338–8345.