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Position Effects on the Timing of Replication of Chromosomally Integrated Simian Virus 40 Molecules in Chinese Hamster Cells

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Simian virus 40 (SV40) DNA molecules chromosomally integrated at different sites in three Chinese hamster lung fibroblast lines replicated during the middle portion of S phase but not precisely at the same time in all three cell lines. The time of replication was unrelated to the presence of T antigen or to its relative activity in promoting SV40 replication. SV40 sequences and chromosomal DNA sequences adjacent to the SV40 insert in one cell line expressing a temperature-sensitive T antigen showed a T-antigen-independent difference in replication timing from the homologous, allelic locus not linked to SV40. Our results indicate that the timing of replication of these integrated SV40 molecules is dependent upon the site of integration and is not determined by the level of T antigen replication-promoting activity.

In eucaryotic cells, replication of chromosomal DNA proceeds according to a specific temporal order (11, 17, 22, 23). Not only is the sequence of replication of chromosomal genes maintained from one cell generation to the next (2, 16, 31, 36, 37), but the initiation of later stages of DNA synthesis appears to be dependent upon completion of earlier stages (16). DNA replication in eucaryotic cells can be regulated by changes in the number, location, and timing of initiation events. Moreover, the genes within a DNA segment encompassing several hundred kilobases can replicate late and sequentially in one tissue type and early and coordinately in another tissue type (3, 9, 18). In general, genes that replicate late in S phase are transcriptionally inactive, while actively transcribed genes are often early replicating (15, 18). Changes in the expression of genes either in different tissue types (18) or in response to experimentally manipulated factors, such as 5-azacytidine treatment (33) or cell fusion (10, 35), are often accompanied by changes in the timing of replication.

Chinese hamster fibroblasts have been described as a semipermissive environment for simian virus 40 (SV40) DNA replication (24). Work by Marchionni and Roufa (27) suggests that although lytic replication is repressed, T-antigen-dependent programming of the timing of SV40 replication can occur when the virus is integrated into hamster chromosomes. In Chinese hamster cells transformed with an SV40 *tsA* mutant, the timing of replication of integrated SV40 molecules, which was determined by using mitotic selection to retroactively synchronize cells labeled with bromodeoxyuridine (BUdR) in various intervals of S phase, was dependent upon the activity of the temperature-sensitive T antigen; SV40 molecules replicated at different times in different cell lines at the nonpermissive temperature but at similar times, and always early, at the permissive temperature (27). Potentially, such T-antigen-dependent programming of the timing of replication could provide the opportunity to reversibly modify the timing of replication of cloned gene sequences linked to SV40 and to examine the effects of such changes on gene expression.

Prior to carrying out experiments of this type, we reexamined the influence of T antigen on the timing of replication of SV40 in Chinese hamster cells by centrifugal elutriation of BUdR-labeled cells. We found that SV40 and the SV40-linked chromosomal sequences replicated earlier than homologous, unlinked sequences, but that this difference was not dependent upon T-antigen activity. Moreover, SV40 molecules integrated at unique sites in three different cell lines did not replicate at precisely the same time in these different cell lines, and their timing of replication did not correlate with the amount of replication-promoting activity of T antigen.

MATERIALS AND METHODS

Cell culture and labeling. Chinese hamster cell lines CHLA209L5 and CHLA209SL1 were the gift of Donald Roufa. Cell lines SV15 and CHL (a nontransformed line of similar origin) were the gift of Robert Martin. Transformation of these cell lines with SV40 and SV40 *tsA209* was done by Martin and Chou (28) and Tenen et al. (38). CCL16 is an American Type Culture Collection (ATCC) cell line. All cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum and 5% CO_2 at the indicated temperatures. All density labeling was done in the presence of 5 μg of 5-fluorodeoxyuridine (Sigma Chemical Co.) and 30 μg of BUdR (Sigma) per ml for 2 h. In the process of carrying out these experiments, it was discovered that while CHLA209L5 and CHLSV15 were capable of being carried indefinitely at 39.5°C, the cell line CHLA209SL1 was not capable of sustained growth at 39.5°C. Growth of CHLA209SL1 continues normally at 39.5°C for about three generations, at which time the cells enlarge and stop dividing, and dead cells (as measured by acridine orange-ethidium bromide staining) accumulate. Transformation with an adenovirus early-region construct led to survival of CHLA209SL1 at 39.5°C (D. M. Gilbert, Ph.D. thesis, Stanford University, Stanford, Calif., 1989).

Soft-agar assays. Soft-agar assays were done by mixing 10^6 cells per 100-mm dish in 2.5 ml of 2× growth medium with an equal volume of 0.66% Bacto-Agar (Difco Laboratories) in water and overlaying this mixture onto 100-mm dishes with 5 ml of 1% Bacto-Agar in growth medium. Colonies were picked with the end of a Pasteur pipette and replated.

DNA isolations, Southern blotting, and hybridizations. DNA isolations were done as described by Gilbert and

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Cohen (14). Southern blots (26) were done following electrophoresis in 0.7% agarose gels. Probes were labeled by nick translation (26). The SV40 DNA used as a probe in this report was purchased from Bethesda Research Laboratories (BRL; catalog no. 5251SA). Purified inserts were used for probes v-*fes* (ATCC collection of oncogenes and proto-oncogenes; catalog no. 41014) and v-Ki-*ras* (Oncor; catalog no. P2145).

Genomic DNA libraries. Genomic DNA (100 μg) from each of the three cell lines was digested with *Bgl*II and size fractionated by electrophoresis in low-melting-point agarose gels, and DNA fragments roughly the size of the SV40-containing *Bgl*II restriction fragment were isolated as described by Kendall and Cohen (21). This DNA was ligated into the *Bam*HI ends of λ bacteriophage EMBL3 (Stratagene; catalog no. EM10) arms and packaged by using the Gigapack-Plus packaging kit (Stratagene; catalog no. GP10-P). Libraries were screened with an SV40 probe, and purified phage containing SV40 were cut with *Sal*I to release the *Bgl*II insert with some bits of EMBL3 sequences. These fragments were then restriction mapped, and certain fragments were subcloned into pBR322 plasmids (26). Complete restriction maps and available probes are documented in Gilbert (Ph.D. thesis, Stanford University, 1989).

Carcinogen amplification of SV40. Cells were plated in 60-mm dishes and allowed to attach to the plates overnight. Half of the cells were then shifted to 39.5°C, and the indicated amounts of dimethylbenzanthracene (Sigma) or hydroxyurea (Sigma) were added for the described amounts of time. DNA was extracted as described by Gilbert and Cohen (14), and total nucleic acid from each preparation was determined by A_{260} measurements. Equal optical density units (approximately 4 μg) were then loaded on slot blots as described by Brown et al. (4) and hybridized to an SV40 probe; 1/10 of each preparation was loaded on a duplicate slot blot and hybridized to pCHOR32, a probe that contains a hamster *Alu*-like sequence (7, 19). Densitometric scanning was done as described by Gilbert and Cohen (14), and the relative amount of SV40 amplification was expressed as SV40/*Alu* for each sample.

Immunofluorescence and FACS analysis of intracellular T antigen. Cells were prepared and stained for fluorescence microscopy as described by Lebkowski et al. (25) with a monoclonal antibody to T antigen (Oncogene Sciences; catalog no. DPO2) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody (Sigma). For fluorescence-activated cell sorting (FACS) determination of relative concentrations of intracellular T antigen, we used a staining protocol described previously (D. M. Gilbert, Ph.D. thesis, Stanford University, 1989).

Centrifugal elutriation. Elutriations were done with a Beckman JE6B rotor, spun at 2,000 rpm at 8°C. When elutriating CHLA209L5.3 grown at 39°C, two elutriation chambers were used; all other cell lines were elutriated with a single chamber. CHLA209L5.3 cells (3×10^8) grown at 33°C, 4×10^8 CHLA209L5.3 cells grown at 39.5°C, 2.5×10^8 CHLA209SL1.7 cells, and 2×10^8 CHLSV15 cells were loaded at a pump speed of 9.5, 11, 12, or 12 ml/min, respectively; the pump speed was increased at increments of 1.5 ml/min, and 100-ml (200-ml for double-chamber elutriation) fractions were collected at each speed, up to speeds of 24, 26, 37.5, and 35 ml/min, respectively. The pump speeds at which the various populations emerged varied slightly. Cells were immediately fixed in 70% ethanol after collection and stored until analyzed on the FACS as described previously (14). Appropriate fractions were pooled and reanalyzed on the FACS.

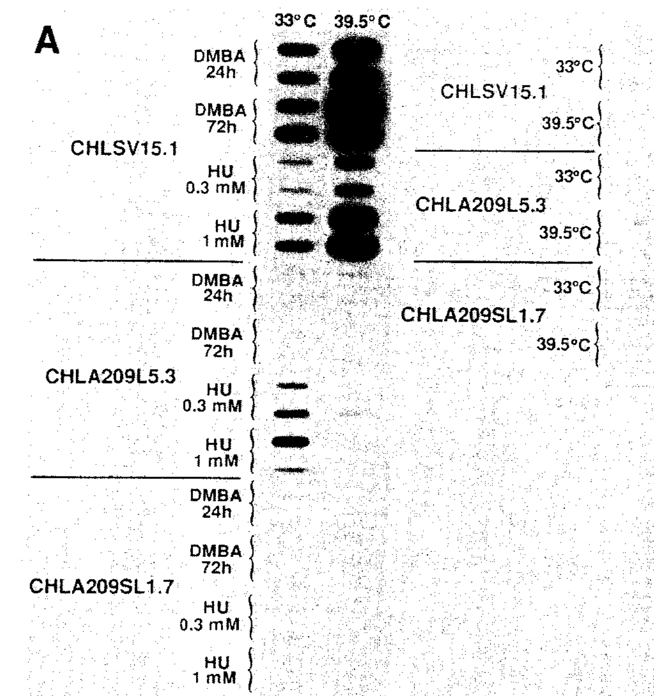
Separation of Heavy/Heavy (HL) and Light/Light (LL). DNA was extracted from the various cell populations as described by Gilbert and Cohen (14), except that cells were suspended in 4 ml of DNA extraction buffer instead of 100 μl. DNA was then digested with *Bgl*II in 200 μl of restriction enzyme buffer, and the entire volume was added to a Beckman Quick-Seal VTi80 tube. The tubes were then filled to the top with ultrapure Cs_2SO_4 (BRL) at a refractive index of 1.3705 and centrifuged in a Beckman VTi80 rotor at 30,000 rpm for 60 h. Gradients were fractionated into 22 to 24 fractions, and the optical density at 260 nm (OD_{260}) for each fraction was checked. Fractions representing the HL peak and six surrounding fractions were pooled, added to fresh VTi80 tubes containing 200 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]), and fractionated as before. The HL peak and six surrounding fractions were then dialyzed in a BRL mini-dialysis device, lyophilized, and quantitated by absorbance at 260 and 280 nm. The relative amounts were verified by comparing the UV fluorescence when 25 ng of DNA in 10 μl was mixed with 10 μl of 0.5-μg/ml ethidium bromide.

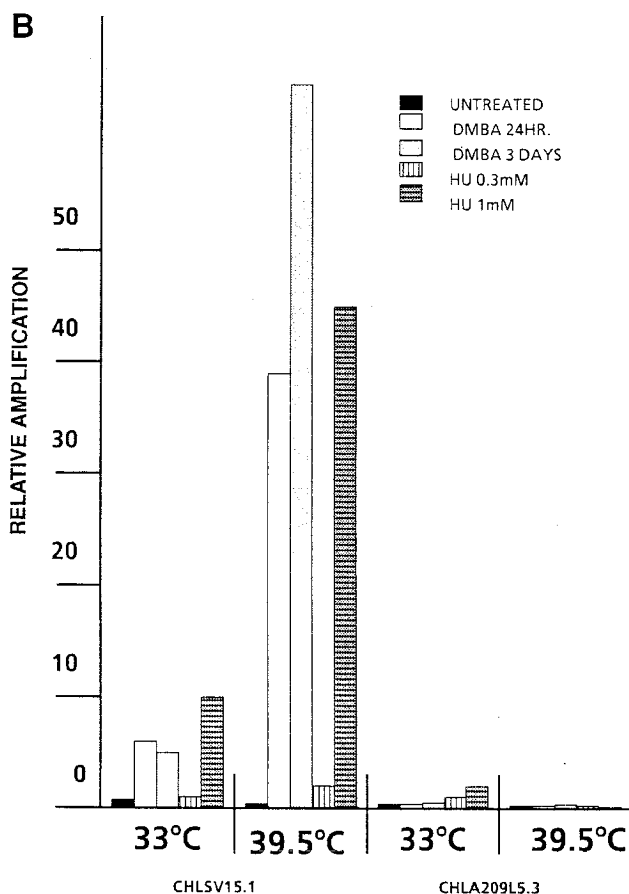
RESULTS

Properties of three SV40-transformed Chinese hamster cell lines. Two of the three cell lines we investigated (CHLA209SL1 and CHLA209L5) contain an SV40 DNA molecule that is integrated at a single chromosomal location and expresses a temperature-sensitive T antigen (*tsA209*) (27). The third cell line, CHLSV15, is transformed with wild-type SV40; these cells gave a single hybridizing band by Southern blot analysis upon digestion of genomic DNA with *Bgl*II and a single band upon in situ hybridization with an SV40 DNA probe (J.-B. Ravnan, D. M. Gilbert, and S. N. Cohen, unpublished observations).

Prior to their use in our studies, all three of the cell lines were subcloned in soft agar at 33°C, and it was verified that DNA rearrangement in the vicinity of the insert did not occur in the subclones selected for further study (such rearrangement was frequently observed in other subclones [D. M. Gilbert, Ph.D. thesis, Stanford University, 1989]). The subcloned cell lines were designated CHLA209SL1.7, CHLA209L5.3, and CHLSV15.1.

It has been shown that carcinogen treatment can cause the amplification of SV40 DNA in Chinese hamster cells and that this process is T-antigen dependent (34). To verify the activity of T antigen and its ability to interact with the SV40 origin of replication in these Chinese hamster cell lines, each of the subclones was treated at the permissive and nonpermissive temperatures with either dimethylbenzanthracene or hydroxyurea, and the DNA isolated from these cells was hybridized with an SV40 probe (Fig. 1). With SV15.1, harboring a wild-type T antigen, amplification occurred at 33°C but was 5- to 10-fold greater at 39.5°C. In contrast, CHLA209L5.3 amplified at 33°C, albeit to a lesser extent than SV15.1, and CHLA209SL1.7 showed little if any amplification of SV40 at 33°C; as expected, neither of these temperature-sensitive cell lines amplified SV40 at 39.5°C. The amount of amplification of SV40 observed in the three cell lines upon fusion is consistent with the level of T-antigen immunofluorescence observed microscopically and by FACS analysis (data not shown), indicating that the relative replication-promoting activity of T antigen in these cell lines is SV15.1 > CHLA209L5.3 > CHLA209SL1.7. The occurrence of amplification in these experiments also indicates





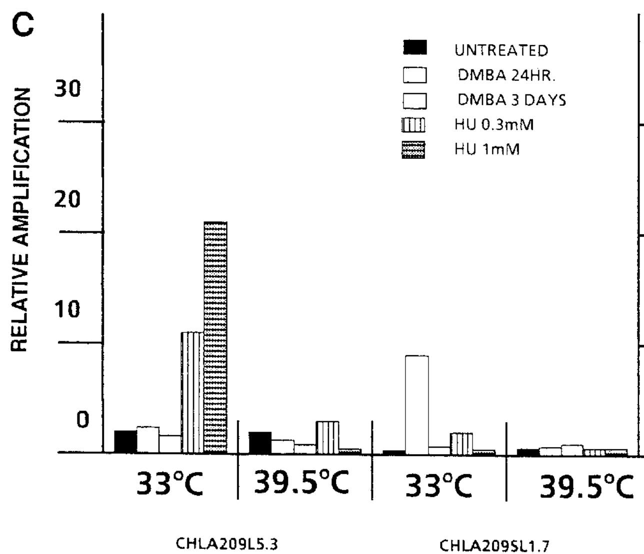


FIG. 1. Carcinogen-induced SV40 amplification. Cells were treated with different doses of hydroxyurea (HU) for 24 h or with dimethylbenzanthracene (DMBA) at 0.1 μg/ml for either 1 or 3 days. Some plates were shifted to 39.5°C at the time of drug addition. On the fifth day after the addition of drugs, DNA was isolated and hybridized to an SV40 probe (A). Portions of this DNA were also hybridized to a Chinese hamster *Alu*-like repeat sequence (7) to aid in quantitation of total cellular DNA loaded on each slot. The amount of SV40 hybridization relative to *Alu* hybridization was then plotted on bar graphs (B and C). Each bar represents the average of the results from two independent plates of cells, shown as duplicate slots for each condition in panel A. The scale in graph C is expanded 10-fold relative to the scale in graph B.

that the T-antigen proteins and SV40 *ori* sequences in these cell lines are capable of interacting functionally under the appropriate conditions.

Cloning the sites of integration of SV40. To determine whether the timing of replication of SV40 is independent of the timing of replication of the integration site, we wished to compare the timing of replication of SV40 and the sequences flanking SV40 to homologous, allelic sequences within the same cells that are not linked to SV40. This required the isolation of the sequences flanking SV40 in each of the three cell lines, including the two cell lines studied by Marchionni and Roufa (27). Lambda EMBL3 (Gigapack-plus; Stratagene) libraries were made from *Bgl*II-cut genomic DNA from each cell line, and phages containing SV40 inserts were purified and mapped. Purified restriction fragments from the flanking chromosomal DNA were tested for their ability to hybridize to a single *Bgl*II restriction band of the appropriate size in genomic Southern blots.

After testing 15 probes spanning the entire flanking sequence of CHLA209SL1, we concluded that SV40 has integrated into a nest of middle and highly repetitive DNAs in this cell line. In the case of CHLSV15, a 2.2-kilobase (kb) fragment that hybridized to a single band of the predicted size for allelic sequences in CHLA209L5 and CHLA209SL1 genomic DNA was found. However, we could not identify a similar band in CHLSV15 DNA, indicating that the allelic locus is either rearranged or not present. Therefore, the flanking sequences in these two cell lines were not experimentally useful as probes for the chromosomal integration sites.

In the case of CHLA209L5, a 0.7-kb fragment hybridized to bands of the predicted size. A restriction map of the phage insert from CHLA209L5 and the location of the unique 0.7-kb probe are shown in Fig. 2. Figure 3 shows DNA from CHLSV15, CHLA209L5, and CCL16 (a Chinese hamster

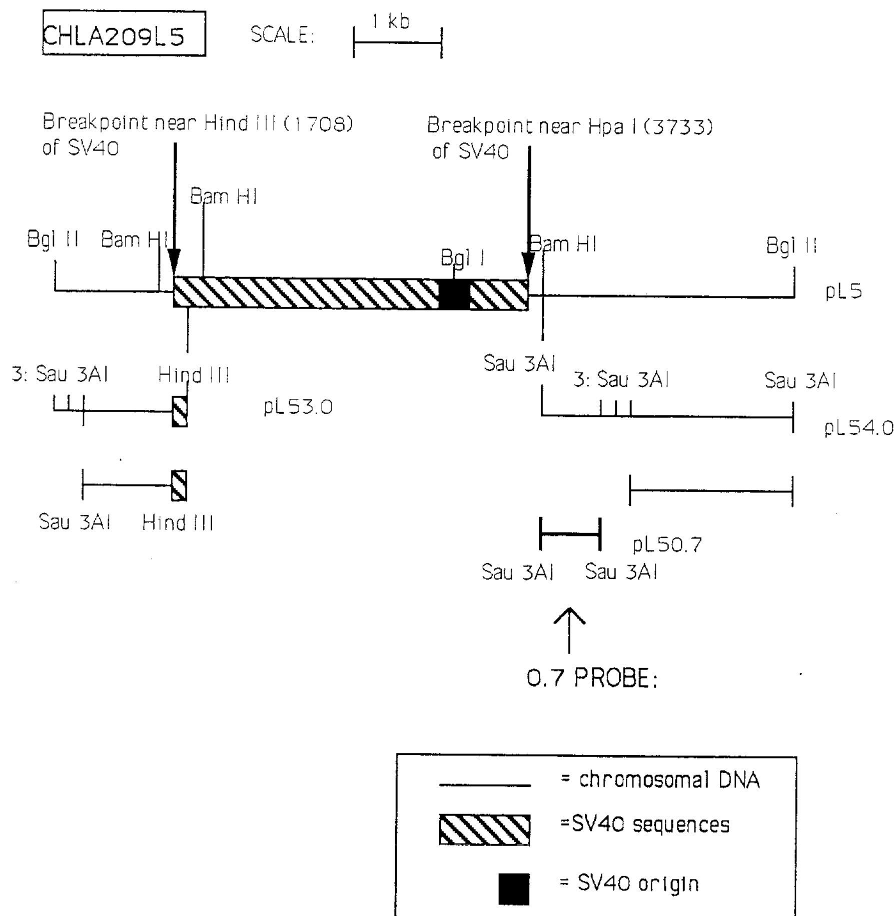


FIG. 2. Restriction maps of cloned genomic fragments containing SV40 inserts in CHLA209L5. The 0.7-kb fragment shown is the 0.7-kb unique-sequence probe used for the experiments described in this report. All other fragments shown contain repetitive DNA sequences. For probes that have been cloned into pBR322, the plasmid name is shown to the right of the fragment.

lung cell line not transformed with SV40), cut with *Bgl*II and hybridized with the 0.7-kb probe isolated from the CHLA209L5 genome. Each of the three DNA preparations showed a band of the size predicted for the *Bgl*II genomic fragment lacking an SV40 insert. CHLA209L5 DNA had an additional hybridizing band of the size of the *Bgl*II fragment containing the SV40 insert. Furthermore, when this filter was stripped and hybridized with an SV40 probe, only the larger band hybridized in CHLA209L5, while neither of these bands hybridized in CCL16 or CHLSV15 DNA.

Timing of replication of SV40-linked and -nonlinked fragments containing the 0.7-kb fragment in CHLA209L5.3. Marchionni and Roufa (27) reported that in CHLA209L5, SV40 sequences replicated earlier at the permissive temperature for T antigen than at the nonpermissive temperature and suggested that SV40 was programmed by T antigen to replicate early in S phase. Therefore, the timing of replication of integrated SV40 in this cell line should be early and independent of the normal timing of replication of the chromosomal region into which it integrates at the permissive temperature for T antigen, but dependent upon and at the same time as the chromosomal region into which it integrates at the nonpermissive temperature for T antigen.

CHLA209L5.3 cells were either grown at the permissive temperature continuously (experiments I, II, and III, Table 1) or shifted to the nonpermissive temperature and passaged for either 4 days (experiments V and VI, Table 2) or 11 days (experiment VII, Table 2). Cells grown under these conditions were then labeled for 2 h with BUdR and separated into the populations shown in Fig. 4 by centrifugal elutriation as described in Materials and Methods. Replicated DNA from each of these populations (designated S1 to S4 for the different times in S phase) was isolated on density gradients as described in Materials and Methods and subjected to Southern blot analysis probing with the 0.7-kb chromosomal DNA fragment flanking the SV40 insert. Since SV40 and one of the alleles hybridizing with the 0.7-kb probe are located on the same *Bgl*II restriction fragment, the timing of replication of SV40 could be represented by examining the timing of replication of the 0.7-kb allele linked to SV40. Using the 0.7-kb probe and genomic Southern blot analysis, we could therefore directly compare the timing of replication of SV40 with the natural timing of replication of the same chromosomal location lacking SV40 on the homologous chromosome. The results (Fig. 5A) indicate that the allele of 0.7 kb that is linked to SV40 replicated earlier than the unlinked allele; the SV40-linked allele hybridized more intensely than the unlinked allele in HL DNA prepared from early-S-phase cells, while the reverse was true with HL DNA prepared from the late-S-phase fractions.

Because the exact interval of S phase at which the sequences showed the majority of their replication differed

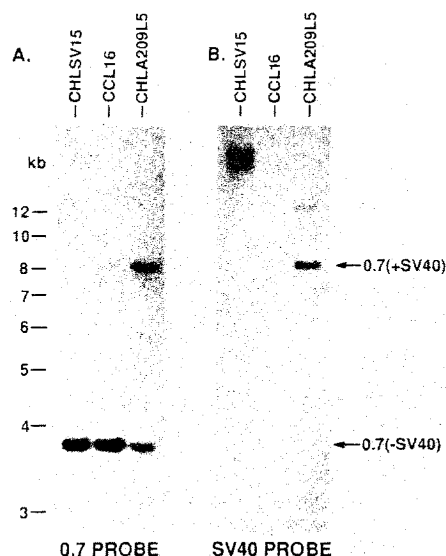


FIG. 3. Southern blots of hamster DNA probed with the 0.7-kb probe containing chromosomal DNA sequences that flank the SV40 insert. CHLSV15, CCL16, and CHLA209L5 DNAs were cut with *Bgl*II (which does not cut within SV40), and Southern hybridization was performed. (A) Hybridyzed with the 0.7-kb probe. (B) The same blot stripped and hybridized with labeled SV40 DNA. The band seen in the CHLSV15 lane is the location of the SV40 insert in CHLSV15. The extra band was sometimes seen and is consistent with a partially digested restriction fragment. The positions of the SV40-linked chromosomal DNA fragments detected by the probe and its unlinked homolog are indicated as 0.7(+SV40) and 0.7(−SV40), respectively.

in different experiments, we compared their timing of replication with that of two control sequences, v-*fes* and v-Ki-*ras*, which previously were shown to replicate early and late, respectively, in Chinese hamster cells (20). The timing of replication of SV40 and the 0.7-kb fragment relative to these controls was similar in each experiment (Tables 1 and 2), verifying the efficacy of each elutriation. In every experiment, both alleles of the flanking sequence replicated in middle S phase, and the linked allele replicated prior to the unlinked allele (Tables 1 and 2 and Fig. 6). When the same DNAs were hybridized with an SV40 probe, the timing of replication was found to be the same as with the SV40-linked 0.7-kb allele (Fig. 6 and Tables 1 and 2), verifying that the flanking sequences adjacent to SV40 and within the same gradient-purified *Bgl*II fragment replicated coordinately with SV40 and that the timing of replication of the SV40-linked 0.7-kb allele is representative of the SV40 molecule. This observed allelic difference in the timing of replication occurred whether cells were passaged at the permissive temperature or the nonpermissive temperature (Tables 1 and 2 and Fig. 6), indicating that T antigen is not the factor that was causing SV40 and its linked chromosomal sequences to replicate earlier than the natural timing of replication of the chromosomal location unlinked to SV40.

Replication of SV40. It can be seen from the results presented in Tables 1 and 2 that SV40 replicated in the early-middle fraction of S phase in CHLA209L5.3 and certainly later than the early-replicating cellular homolog of v-*fes*, indicating that even in the presence of active T antigen, integrated SV40 DNA molecules are not programmed to replicate in the first quarter of S phase. In addition, the differences observed in the timing of replication

TABLE 1. Replication of SV40, SV40-linked and unlinked 0.7-kb fragments, v-*fes*-homologous, and v-Ki-*ras*-homologous sequences in CHLA209L5.3 cells at 33°C[a]

Expt no.	Probe	Relative concn (% of total specific hybridization)[b]			
		S1	S2	S3	S4
I	v-*fes*	48	30	12	10
	v-Ki-*ras*	5	25	39	31
	SV40	ND	ND	ND	ND
	0.7(+SV40)[c]	20	47	28	5
	0.7(−SV40)[c]	16	43	31	10
II	v-*fes*	38	30	15	18
	v-Ki-*ras*	9	27	31	33
	SV40	21	38	25	16
	0.7(+SV40)[c]	14	40	28	18
	0.7(−SV40)[c]	6	34	34	26
III	v-*fes*	32	25	27	16
	v-Ki-*ras*	7	10	36	47
	SV40	11	28	33	28
	0.7(+SV40)[c]	10	28	32	30
	0.7(−SV40)[c]	7	8	40	45
IV	v-*fes*	ND	44	29	27
	v-Ki-*ras*	ND	8	38	54
	SV40	ND	ND	ND	ND
	0.7(+SV40)[c]	8	36	33	23
	0.7(−SV40)[c]	3	14	39	43

[a] HL DNA, purified from elutriated populations of CHLA209L5.3 in the indicated portions of S phase (S1 to S4) after growth at 33°C, was probed with the indicated specific probes.

[b] Relative concentrations of specific sequences in the HL DNA are expressed as a percentage of the total specific hybridization in all four DNA samples.

[c] The probe was, in both cases, the 0.7-kb DNA fragment shown in Fig. 2. The data shown are for the two chromosomal DNA loci detected by this probe.

TABLE 2. Replication of SV40, SV40-linked and unlinked 0.7-kb fragments, v-*fes*-homologous, and v-Ki-*ras*-homologous sequences in CHLA209L5.3 cells at 39.5°C[a]

Expt no.	Time at 39.5°C (days)	Probe	Relative concn (% of total specific hybridization)			
			S1	S2	S3	S4
V	4	v-*fes*	37	23	23	17
		v-Ki-*ras*	12	16	31	41
		SV40	ND	ND	ND	ND
		0.7(+SV40)	26	29	26	19
		0.7(−SV40)	12	19	35	34
VI	4	v-*fes*	19	33	25	23
		v-Ki-*ras*	6	21	28	45
		SV40	ND	ND	ND	ND
		0.7(+SV40)	14	30	28	28
		0.7(−SV40)	6	25	26	43
VII	11	v-*fes*	30	33	20	17
		v-Ki-*ras*	10	19	34	37
		SV40	ND	ND	ND	ND
		0.7(+SV40)	22	26	28	24
		0.7(−SV40)	11	18	33	38

[a] HL DNA, purified from elutriated populations of CHLA209L5.3 cells in the indicated portions of S phase after growth initially at 33°C and then at 39.5°C for the indicated lengths of time, was probed with the indicated specific probes. See also Table 1, footnotes *b* and *c*.

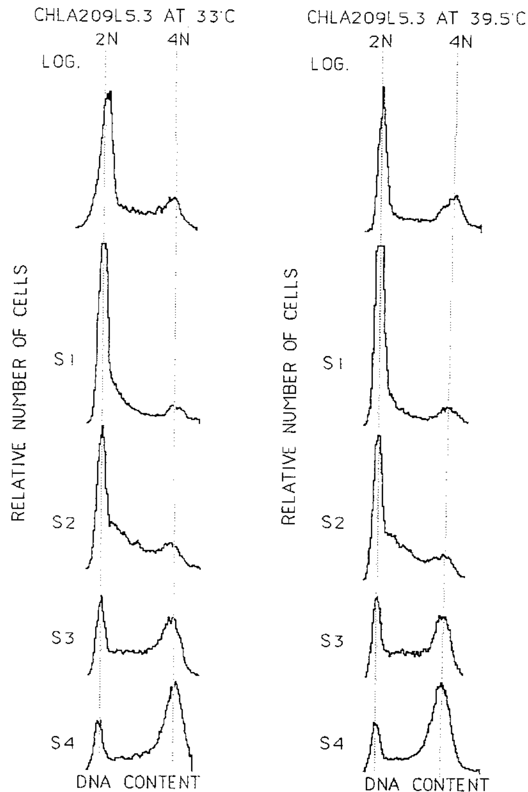


FIG. 4. DNA histograms of CHLA209L5.3 elutriated fractions. Logarithmically growing CHLA209L5.3 cells, grown at 33°C continuously or shifted to 39.5°C, were labeled for 2 h with BUdR and stained for DNA content with chromomycin A3 (LOG). Cells were fractionated by centrifugal elutriation, analyzed for DNA content, and then pooled into the four populations shown representing different times in S phase (S1 to S4). Shown are histograms from experiments III and VI of Tables 1 and 2. Pump speeds (in milliliters per minute) for the elutriated fractions in these experiment are as follows. Cells grown at 33°C: S1, 17.0 to 18.5; S2, 18.5 to 20.0; S3, 20.0 to 22.5; S4, 22.5 to 24.0. Cells grown at 39.5°C: S1, 18.0 to 19.0; S2, 19.0 to 21.0; S3, 21.0 to 22.5; S4, 22.5 to 26.5.

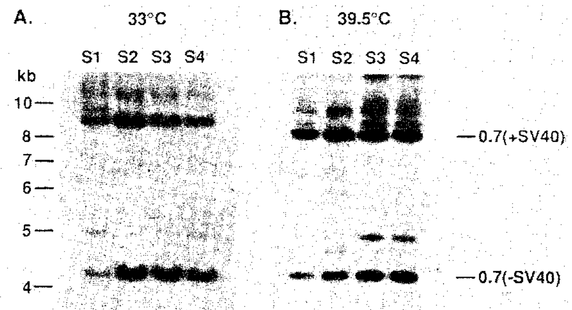


FIG. 5. Timing of replication of 0.7-kb alleles in CHLA209L5.3. DNA isolated from the cell populations in Fig. 4 was digested with *Bgl*II and replicated (HL) DNA was isolated on Cs_2SO_4 gradients. Equal quantities of purified HL DNA were then used for Southern blotting and hybridization with the 0.7-kb probe, which resides within the same *Bgl*II restriction fragment as SV40 in CHLA209L5.3. (A) Cells maintained at 33°C (experiment II). (B) Cells shifted to 39.5° (experiment VII). Panels A and B are from separate gels, electrophoresed for similar distances. BRL 1-kb ladder molecular size standards are shown for panel A only but are very similar for panel B.

of the inserts versus the chromosomal DNA sequences at the SV40 integration site were independent of the presence of T antigen activity.

To investigate whether T antigen programs SV40 to replicate in early-middle S phase (albeit not in very early S phase) independent of its integration site, we determined the timing of replication of SV40 in CHLA209SL1.7 at the permissive temperature and in CHLSV15.1, which shows high levels of T-antigen biological activity (Fig. 1). These cell lines were labeled with BUdR and fractionated by centrifugal elutriation (Fig. 7). Replicated DNA was isolated as described above and hybridized with either SV40 or the various control probes for sequences that replicate at particular times in S phase. Table 3 summarizes the results, comparing the timing of SV40 replication with that of early- and late-replicating controls and with the timing of replication of the middle-S-phase-replicating 0.7-kb sequence (not linked to SV40 in these cell lines).

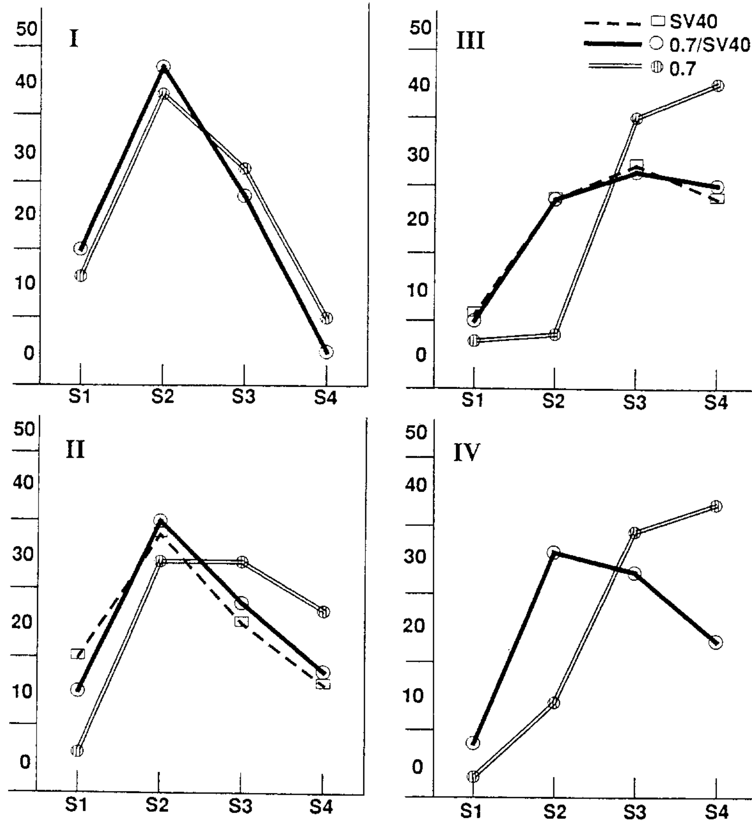
Figure 8 graphically compares the timing of replication of SV40 with that of the middle-S-phase-replicating 0.7-kb sequence shown in Table 3. Comparison of Fig. 8 with Fig. 6 and of Fig. 3 with Table 1 shows that the SV40 insert in CHLSV15.1 consistently replicated later than the SV40 insert in CHLA209L5.3 and very close to the same time as the late-middle-S-phase-replicating 0.7-kb locus. The results for the SL1.7 cell line suggest that its replication time is intermediate between those of SV15.1 and L5.3. While the relatively poor elutriation obtainable with this cell line (Fig. 7) yields less repeatable results, our data for SL1.7 are included because of the importance of this cell line to the interpretations of Marchionni and Roufa (27). Despite the observed variability, the results obtained for SL1.7 also demonstrate the lack of early replication of SV40 in this cell line.

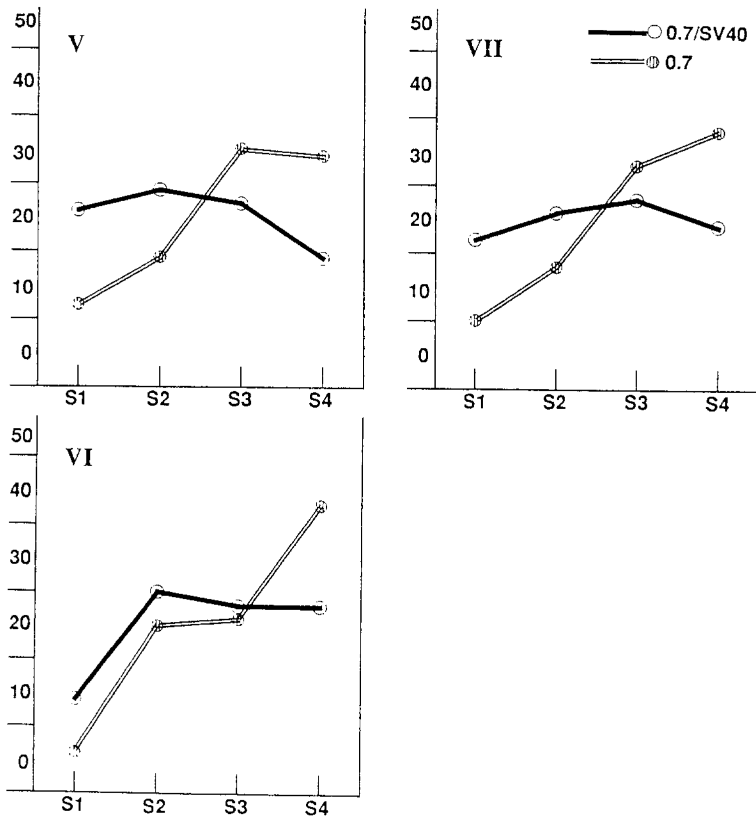
DISCUSSION

Our results show that the timing of replication of SV40 in Chinese hamster cells is influenced by the chromosomal position into which it integrates and that the timing of replication of SV40 is not determined expressly by the level of activity of T antigen. Therefore, while T antigen may have some effect on the timing of replication of SV40, T antigen does not appear to program integrated SV40 to replicate during a specific interval of S phase. Our results also indicate that SV40 may replicate slightly later at the nonpermissive temperature in the CHLA209L5.3 cell line (Fig. 6 and Tables 1 and 2), consistent with the slight shift observed by Marchionni and Roufa (27) with this cell line. The cell line in which Marchionni and Roufa observed the greatest difference in timing of replication upon a temperature shift, CHLA209SL1, grows very poorly at the nonpermissive temperature, and we did not attempt to elutriate this cell line at 39.5°C (see Materials and Methods).

Marchionni and Roufa (27) have reported that in some of

FIG. 6. Replication timing of the SV40-linked 0.7-kb allele and its unlinked homolog. Graphic representation of the replication times in CHLA209L5.3 of the linked (0.7+SV40) and unlinked (0.7−SV40) alleles of the 0.7-kb DNA fragment from Tables 1 and 2; the experiment numbers are indicated in the upper left of each panel.


L5.3 AT 33℃
I
II
III
IV
SV40
0.7/SV40
0.7
0
10
20
30
40
50
S1
S2
S3
S4


L5.3 AT 39.5℃
V
VI
VII
0.7/SV40
0.7
0
10
20
30
40
50
S1
S2
S3
S4

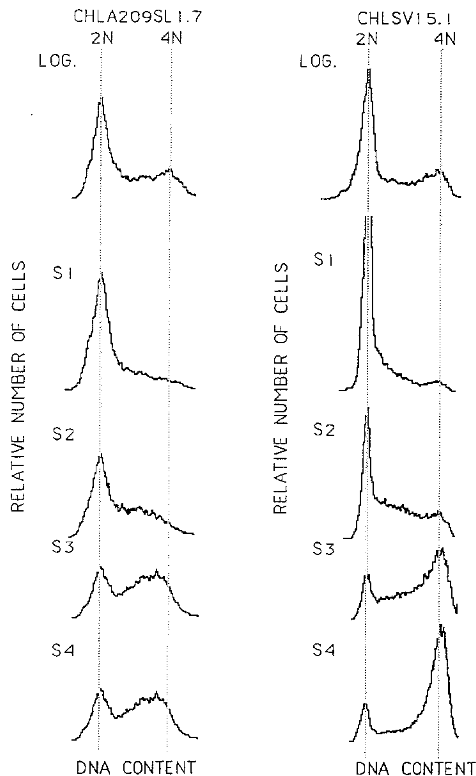


FIG. 7. DNA histograms of CHLSV15.1 and CHLA209SL1.7 elutriated fractions. Logarithmically growing CHLSV15.1 and CHLA209SL1.7 cells were labeled for 2 h with BUdR and stained for DNA content with chromomycin A3 (LOG). Cells were fractionated by centrifugal elutriation, analyzed for DNA content, and then pooled into the four populations shown representing different times in S phase (S1 to S4). Shown are histograms from experiments XII and IX of Table 3. Pump speeds (in milliliters per minute) for these fractions are as follows. CHLA209SL1.7: S1, 18.5 to 20.0; S2, 20.0 to 23.5; S3, 23.5 to 27.0; S4, 27.0 to 30.5. CHLSV15.1: S1, 16.0 to 17.5; S2, 17.5 to 20.0; S3, 20.0 to 23.5; S4, 23.5 to 30.0.

the cell lines (CHLA209L5 and CHLA209SL1) used in our study, the timing of replication of SV40 occurs early in S phase at the permissive temperature. We have found that SV40 is replicated in early-middle S phase in these cell lines. It is possible that this small difference results from the different methods used to determine timing of replication and to present the data. Marchionni and Roufa (27) synchronized their cells by mitotic selection, analyzed the timing of replication of SV40 by liquid hybridization, and presented their results as an absolute amount of replicated SV40 per cell cycle interval, comparing the timing of replication of SV40 with the time of apparent onset of chromosomal DNA replication. We were interested in determining the timing of replication of DNA sequences at the specific chromosomal location at which SV40 was integrated, which required analysis by Southern blotting, and we were not able to obtain enough replicated DNA for such analysis by either the FACS retroactive synchrony method (13, 14) or mitotic selection (27). We therefore synchronized cells by centrifugal elutriation and have presented the results in terms of the relative amount of hybridization of SV40 to equal amounts of replicated chromosomal DNA, comparing the timing of replication of SV40 with that of other specific chromosomal sequences.

TABLE 3. Replication of SV40, 0.7-kb fragment, v-*fes*-homologous, and v-Ki-*ras*-homologous sequences in CHLA209SL1.7 and CHLSV15.1[a]

Cell line (growth temp)	Expt no.	Probe[b]	Relative concn (% of total specific hybridization)[c]			
			S1	S2	S3	S4
CHLA209SL1.7 (33°C)	VIII	v-*fes*	33	29	18	20
		v-Ki-*ras*	8	13	34	45
		SV40	10	23	37	30
		0.7	22	34	23	21
	IX	v-*fes*	36	35	15	14
		v-Ki-*ras*	12	22	31	35
		SV40	19	35	23	23
		0.7	16	36	30	18
CHLSV15.1 (37°C)	X	v-*fes*	50	24	13	13
		v-Ki-*ras*	17	23	43	17
		SV40	15	31	31	23
		0.7	16	32	30	22
	XI	v-*fes*	31	27	24	18
		v-Ki-*ras*	8	17	40	35
		SV40	13	27	41	19
		0.7	20	23	36	21
	XII	v-*fes*	42	26	18	14
		v-Ki-*ras*	7	11	32	50
		SV40	19	24	31	26
		0.7	18	20	37	25

[a] HL DNA, purified from elutriated populations of CHLA209SL1.7 cells grown at 33°C (permissive temperature) and CHLSV15.1 cells (high levels of T-antigen activity) grown at 37°C in the indicated portions of S phase, was probed with the indicated specific probes.
[b] 0.7, Probe specific for the 0.7-kb fragment.
[c] See Table 1, footnote *b*.

Furthermore, in neither of the two cell lines investigated by us and by Marchionni and Roufa (27) did Marchionni and Roufa find replication at the very beginning of S phase. In CHLA209SL1, they found that SV40 replicated biomodally, both early and late in S phase. The cell cycle separation afforded by centrifugal elutriation might not resolve such a bimodality in our experiments. In CHLA209L5, Marchionni and Roufa found that SV40 replicated shortly after the onset of cellular DNA synthesis and later than the time of replication of SV40 in a third cell line (CHLA30L1). In any case, it is clear from our results that SV40 is not among the earliest-replicating sequences in these cell lines; we have used an internal control (v-*fes*) that replicated earlier than SV40 in every cell line and in every experiment.

Although Marchionni and Roufa (27) found that SV40 replicated early in all three cell lines at the permissive temperature, they did observe differences in the timing of replication of SV40 between the different cell lines, including the bimodal replication of SV40 in CHLA209SL1 mentioned above. Based on T-antigen levels reported previously in these cell lines, Marchionni and Roufa suggested that SV40 might be programmed to replicate earlier in cell lines that express higher levels of T antigen. Our results do not support this interpretation; CHLSV15.1, which contains a much higher level of T-antigen activity than either CHLA209L5.3 or CHLA209SL1.7 (Fig. 1), replicated the SV40 insert in late-middle S phase. Clearly, the number of individual clones examined is not sufficient to determine whether transformed cells can replicate SV40 at all times in S phase.

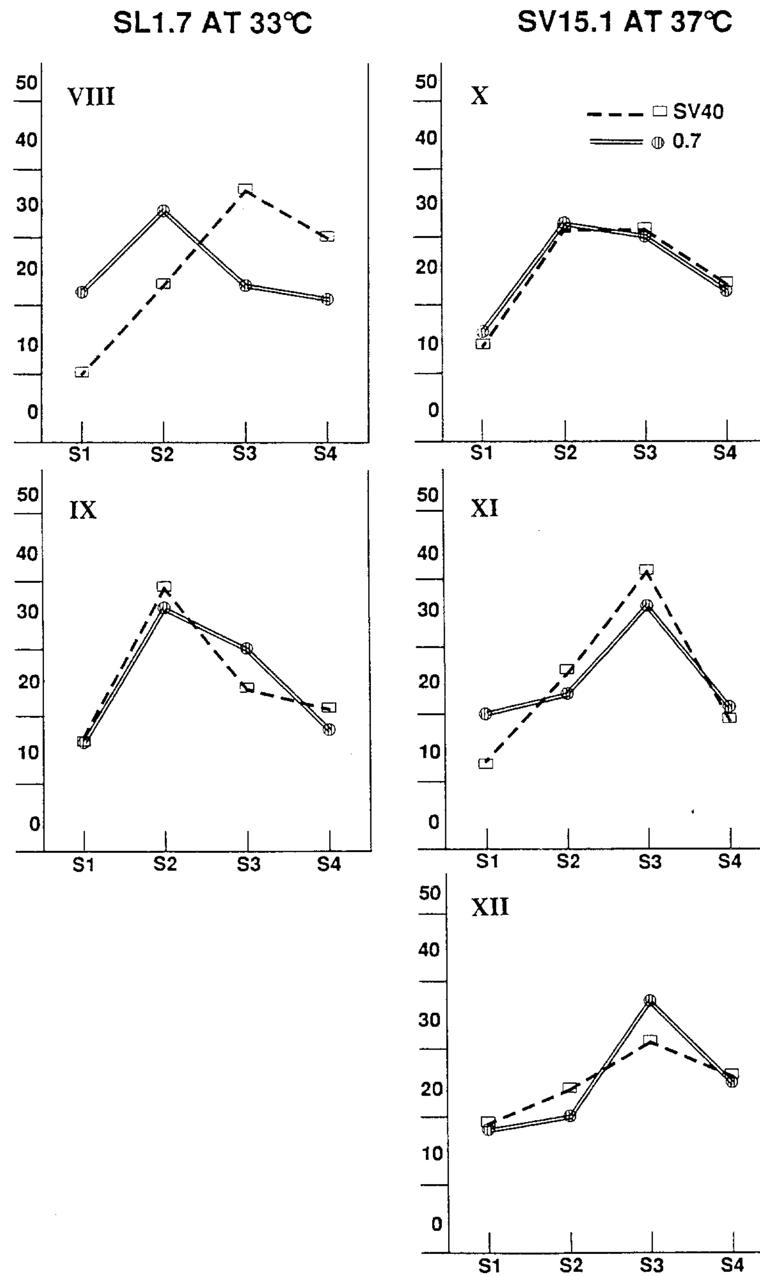


FIG. 8. Replication time of integrated SV40 in the different cell lines. Graphic comparison of the replication times, presented in Table 3, of SV40 and the 0.7-kb fragment in CHLA209SL1.7 and CHLSV15.1. Experiment numbers are indicated in the upper left of each graph. These graphs should be compared with those in Fig. 6.

Since SV40 replicates in late-middle S phase in one of these cell lines (CHLSV15.1), our results also demonstrate that the transcription of mRNA from the SV40 early promotor does not require early replication, in contrast to what has been proposed for many other genes (15). Our results do not, however, exclude the possibility that replication of SV40 in the very late stages of S phase precludes SV40 early-promoter activity.

Our data show that an SV40-linked chromosomal sequence replicates earlier in S phase than its homologous, allelic counterpart not linked to SV40 in a *tsA* SV40-transformed Chinese hamster lung fibroblast cell line. Furthermore, we found that this difference in the timing of replication of the two alleles is not T-antigen dependent. Assuming that no undetected structural rearrangements have occurred in either of the alleles assayed, the observed difference in timing of replication of homologous alleles could be due to an influence of T-antigen-independent elements of SV40 on the timing of replication of sequences adjacent to the site of integration. Potentially, alterations of chromatin structure in the vicinity of the SV40 insertion by the arrangement of nucleotides on the enhancer-promotor sequence and the presence of the resulting DNA-binding proteins could alter timing of replication; alternatively, the act of transcription from the SV40 early promotor could disrupt timing of replication (5, 8, 32). Actively transcribed

genes generally are early replicating (15, 18); however, it is not known whether early replication is a cause or an effect of gene expression.

A second possible explanation for these results is that inherent differences exist in the timing of replication of different alleles of the same chromosomal sequence and that the presence of SV40 in one of these alleles has simply allowed the detection of those differences by providing a restriction fragment length difference. Distinguishing between these two possibilities would require the identification of a restriction fragment length polymorphism in the vicinity of the SV40 integration site in order to distinguish the timing of replication of the different 0.7-kb fragment alleles in cell lines in which they are both unlinked to SV40. The timing of replication of different alleles of the same gene sequence at other heterozygous loci where restriction fragment length polymorphisms have been identified has not been investigated. In the case of X chromosome inactivation, however, it is evident from cytogenetic studies showing disparate replication of the active and inactive X chromosomes (40) that identical gene sequences on separate chromosomes are capable of replicating at widely different times in S phase. Furthermore, functional hemizygosity of autosomal gene sequences has been inferred for several genes based on the frequency of mutagenic inactivation of the phenotype and its reversion upon 5-azacytidine treatment (1, 34). It is possible, when only one of two alleles within a cell is active, that a difference in the timing of replication of the two alleles is associated with this difference in transcriptional activity. However, while the allelic differences in timing of replication reported in this article are highly reproducible, they are not large and thus may not affect transcription.

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