

Bovine Papilloma Virus Plasmids Replicate Randomly in Mouse Fibroblasts throughout S Phase of the Cell Cycle

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Summary

Bovine papilloma virus (BPV) replicates as a multicopy nuclear plasmid in mouse fibroblasts. Using fluorescence activated cell sorting and mitotic selection procedures, we show that the replication of BPV occurs throughout S phase of the cell cycle and that replication is confined to S phase. After one round of chromosomal DNA replication, almost one quarter of BPV plasmids have replicated more than once, while a similar number of plasmids have not replicated at all. While multiple forms of BPV exist in the cell, all forms show the same pattern of replication. These results are consistent with a model in which BPV plasmids are chosen at random for replication throughout, and only during, S phase and support the view that the completion of S phase is a specifically activated event in the cell cycle rather than simply the end of one round of chromosomal DNA replication.

Introduction

Replication of chromosomal DNA in eukaryotic cells proceeds in a specific temporal order, with each chromosomal DNA segment replicating once per cell cycle at a characteristic time during S phase (Balzas et al., 1974; Hand, 1978; Laskey and Harland, 1981). We are interested in understanding the regulatory mechanisms that determine the temporal order of eukaryotic DNA replication and the relationship of ordered replication to gene expression. In both prokaryotic and eukaryotic cells, extrachromosomal replicons have been useful tools for investigating DNA replication, and in some instances have been shown to be subject to the same replication control processes as the cell's chromosomes. For example, it has been reported that each copy of the 2 μ plasmid of *Saccharomyces cerevisiae* replicates once during S phase of the cell cycle (Zakian et al., 1979). Similarly, the ARS units of yeast, when incorporated into extrachromosomal elements, replicate at the same time in the cell cycle as their counterparts on the chromosome (Fangman et al., 1983).

Bovine papilloma virus (BPV) exists as a multicopy nuclear plasmid in most rodent cell lines (Law et al., 1981). Once the BPV plasmid is established within a cell line, its copy number is maintained at a constant level (Law et al., 1981; Turek et al., 1982). Thus, regulatory mechanisms must ensure that there is an exact, or almost exact, doubling of BPV molecules during each cell cycle. It has been reported that the control of BPV copy number in mouse fibroblasts occurs by replication of each plasmid molecule once per cell cycle (Botchan et al., 1986), and that the

mode of replication for BPV is thus similar to the mode seen for individual segments of chromosomal DNA and for the 2 μ plasmid of yeast. Potentially, once per cell cycle replication of each BPV molecule could occur either by synchronous duplication of all of the plasmid copies present in the cell, or, alternatively, by a nonsynchronous mechanism that differentiates replicated plasmids from unreplicated ones.

To distinguish between these possibilities, we investigated the timing and mode of BPV replication in mouse C127 fibroblasts stably transformed with viral DNA. We observed that BPV replicates asynchronously throughout S phase of the cell cycle. We also found that the replication of BPV plasmids in synchronized cultures of mouse fibroblasts is not restricted to once per cell cycle, as is the replication of eukaryotic chromosomal DNA. Our findings are consistent with a model in which BPV molecules within the nuclear pool replicate randomly in a mode similar to that observed for bacterial plasmids.

Results

Replication of BPV Occurs throughout S Phase

To determine the timing of replication of DNA sequences in eukaryotic cells, we have developed a retroactive synchrony procedure that is rapid and simple. This procedure (Gilbert, 1986) avoids the use of cumbersome prospective synchrony techniques that employ replication-inhibiting drugs or deprive cells of nutrients for extended periods of time, altering cellular metabolism or the kinetics of DNA replication (Laughlin and Taylor, 1979; Taylor, 1977; Johnston et al., 1986). The procedure is applicable to any cell line that can be stained for DNA content and is limited only by the number of cells that can be harvested in each portion of the cell cycle. Since BPV exists as a multicopy plasmid (Law et al., 1981), BPV replication can be analyzed using relatively few cells.

ID13 is a transformed clone of the C127 mouse cell line carrying approximately 150 copies per cell of wild-type BPV (Turek et al., 1982). Logarithmically growing ID13 cells were pulsed with BUdR for 2 hr and stained with the DNA-specific dye chromomycin. Populations of cells in different stages of the cell cycle were obtained using the Fluorescence Activated Cell Sorter (FACS) by choosing the windows shown in the histogram displayed in Figure 1A; because each sorted fraction exhibits a similar fluorescence intensity, it represents a (synchronous) population of cells possessing a similar DNA content. To confirm the purity of fractions, 10⁴ cells from each fraction were reanalyzed (Figure 1B). Similar results were obtained in all analogous experiments carried out with ID13 and other cell lines stably transformed with BPV.

To quantitate the extent of BPV replication in various stages of the cell cycle, DNA isolated from FACS-synchronized cell populations was analyzed by centrifugation in cesium sulfate gradients to separate BUdR-substituted (heavy-light, or HL) DNA from the unsubstituted (light-

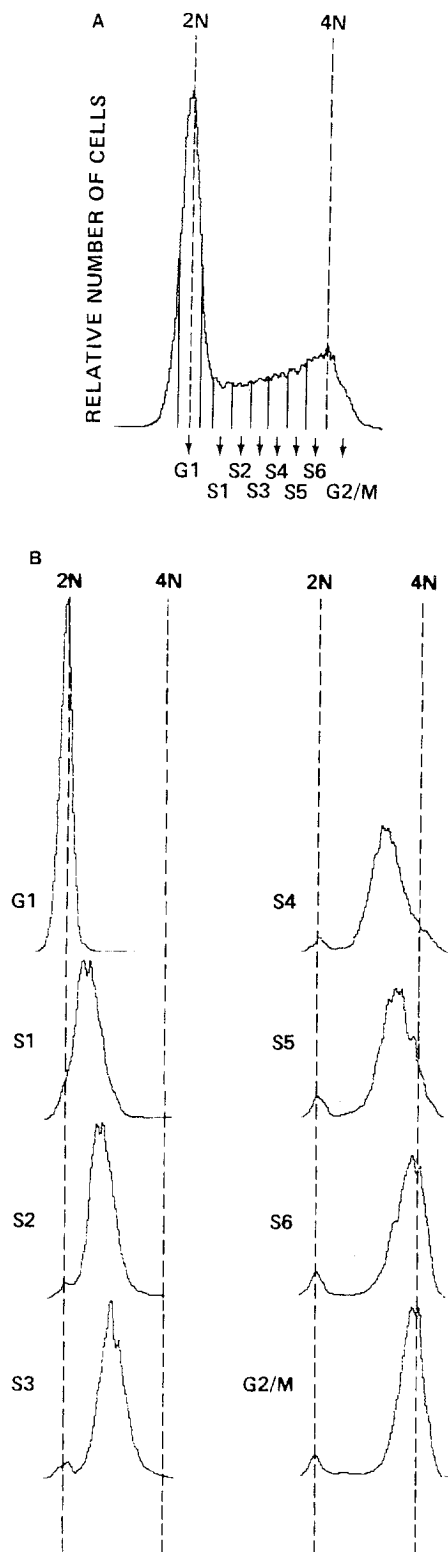


Figure 1. Analysis and Sorting of Chromomycin-Stained Cells
(A) Fluorescence histogram of logarithmically growing ID13 cells labeled for 2 hr with BudR and stained for DNA content with chromomycin A3. The relative number of cells is plotted on the ordinate and DNA content on the abscissa; N is the diploid DNA content of the cells, which are tetraploid. The six windows chosen for sorting synchronized populations of cells in different stages of the cell cycle are shown.

light, or LL) DNA. DNA contained in the fractions of each gradient was immobilized on nitrocellulose filters and hybridized with [32 P]-labeled BPV DNA. The radioactively labeled probe used in all of these experiments was a BPV/pML chimeric plasmid, pdBPV-1, which hybridizes only to the BPV DNA in the transformed C127 cells (Sarver et al., 1982). Hybridization to each filter was quantitated by densitometric scanning.

The results of a typical experiment (Figure 2A) show that BPV hybridizing material is abundant in the HL DNA in all of the fractions taken from S phase of the cell cycle. To monitor chromosomal DNA replication, we used as a hybridization probe a plasmid (pCHOR32; Crouse et al., 1982) containing a hamster Alu-like sequence (homologous to the mouse equivalent) that is highly repetitive and randomly dispersed (Haynes et al., 1981) (Figure 2B). The replication of a sequence known to be synchronously replicating (i.e., a mouse satellite DNA experimental control) was also analyzed in the same experiment (Figure 2C); replication of the satellite DNA was seen to occur primarily during S4–S6, consistent with what has been reported for this satellite DNA (Tobia et al., 1970, 1971). Some hybridization to BPV DNA, but not to the Alu-like probe, was seen at the bottom of the gradient. This may represent BPV molecules that have replicated more than once within the 2 hr labeling. However, the density of the gradients in these experiments was not adjusted to be optimal for the detection of DNA molecules labeled with BUdR in both strands (see Figure 6).

To specifically compare the timing of BPV replication relative to the replication of chromosomal DNA, ID13 cells were incubated with 14 C-TdR to radioactively label all chromosomal DNA prior to a 2 hr BUdR pulse and cell cycle fractionation. Fractions containing the HL DNA or the LL DNA from each gradient were separately pooled, and the DNA immobilized on nitrocellulose filters; these were exposed to X-ray film and the amount of 14 C-labeled DNA in each pool was quantitated by densitometry. The amount of BPV in the pooled gradient fractions was then determined by hybridizing the same filters to the [32 P]-labeled BPV or Alu-like sequence probes. The results (Table 1) indicate that in four separate experiments (Experiments I–IV) in which either four or six sections of S phase were sorted and analyzed, DNA that hybridized with BPV or the Alu-like sequence was present in the HL fractions collected throughout S phase of the cell cycle, and the distribution of the hybridizing BPV DNA correlated closely with the distribution of the chromosomal DNA. Table 1 also indicates that two additional clonal isolates of BPV-transformed mouse C127 cells (clone B and clone D) which have been isolated more recently than ID13 and which contain approximately 30 and 60 copies of BPV per cell, respectively (P. F. Lambert and P. Howley, personal com-

S1–S6 are sections 1–6 of S phase. The cell sorting profile seen here is for Experiment III in Table 1.

(B) Analysis of the fractions sorted from the windows shown in (A), demonstrating the degree of enrichment for cells with increasing model DNA content.

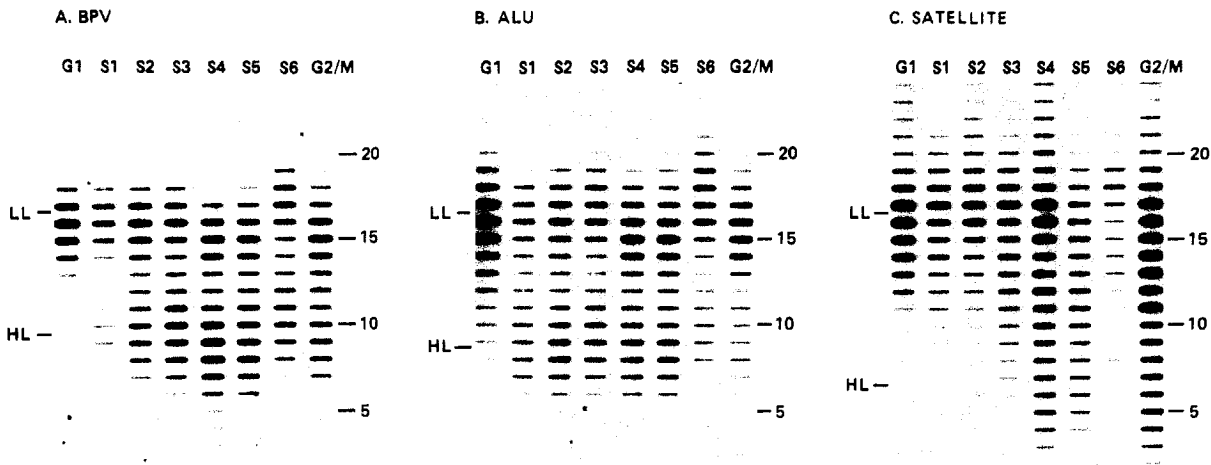


Figure 2. Analysis of Replicated (HL) and Unreplicated (LL) DNA

DNA samples, isolated from cell populations fractionated as in Figure 1, were centrifuged on CsSO₄ gradients and 24 fractions were collected from each gradient. DNA from equal volume aliquots of each fraction was immobilized on nitrocellulose filters using a slot-blot apparatus and hybridized to (A), BPV; (B), hamster Alu-like sequence; or (C), mouse satellite sequence probes. Each lane represents a single gradient prepared so that the center of the gradient is between HL and LL chromosomal DNA. The fraction numbers are presented on the right of each blot, and the locations of the HL and LL DNA are indicated on the left. The cell-cycle stage from which the DNA was isolated is indicated at the top of each lane. The results shown are for Experiment III of Table 1.

munication), yielded results identical to those seen with ID13.

Forms of BPV viral DNA that migrate in agarose gels more slowly than the monomeric form (Law et al., 1981) and which were shown to consist of a complex of extrachromosomal circular viral DNA molecules have been identified in BPV transformed mouse C127 cells. Potentially, the asynchronous replication we observed for DNA able to be hybridized with BPV could have resulted from

the aggregate effects of synchronous replication of each of the different molecular forms of the virus at different times in S phase, or from replication of integrated viral DNA concomitantly with chromosomal DNA sequences at the site(s) of integration. However, as seen in Figure 3, monomeric and nonmonomeric forms of BPV were represented equivalently in the HL peaks throughout all of S phase, indicating that all of the extrachromosomal forms of BPV replicate throughout S phase in these cell lines. In

Table 1. Replication Time of BPV

Experiment	Cell Line	DNA	Relative Amount of Replication/Time in Cell Cycle							
			G1	S1	S2	S3	S4	S5	S6	G2/M
I	ID13	BPV	9	30	25	25	31	31	23	22
		Chromosomal (¹⁴ C-TdR)	6	18	20	26	32	23	18	18
II	ID13	BPV	9	11	30	41	51	37	27	16
		Chromosomal (¹⁴ C-TdR)	10	21	33	40	46	45	46	46
III	ID13	BPV	2	31	35	51	56	54	46	37
		ALU	4	44	46	47	43	40	26	20
		Mouse Satellite	0	4	5	17	28	46	38	33
IV	ID13	BPV	3	23	26	28	27	—	—	22
		ALU	6	29	26	19	13	—	—	8
		Chromosomal (¹⁴ C-TdR)	8	37	37	48	40	—	—	33
V	ID13	BPV	1	32	41	38	32	—	—	28
VI	Clone B	BPV	1	26	41	35	32	—	—	28
VII	Clone D	BPV	7	45	51	43	26	—	—	26

Quantitation of the relative amounts of replication of total ¹⁴C-labeled chromosomal DNA, BPV DNA, and mouse ALU-like DNA in three separate experiments in which four (Experiments IV–VII) or six (Experiments I–III) sections of S phase were FACS sorted from ID13 cells. Numbers represent densitometric quantitation and are expressed as HL/HL + LL. The presence of HL DNA in G1 is due to incomplete purity in the synchronized cell populations and reflects the efficiency of cell sorting in each experiment. In Experiments I–IV, DNA isolated from the synchronized populations was cut with the restriction enzyme EcoRI, which linearizes BPV plasmids, prior to fractionation on CsSO₄ gradients, while in Experiments V, VI, and VII, DNA was cut with SacI, which does not cut BPV DNA, and the resulting HL and LL was analyzed by Southern blotting as in Figure 3.

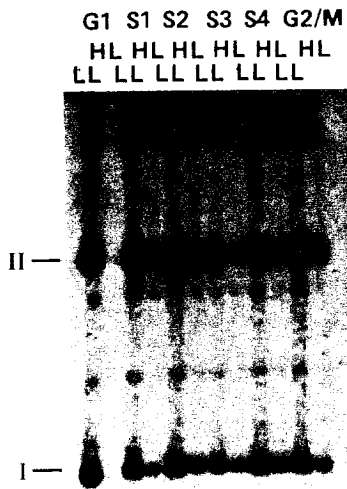


Figure 3. All Forms of BPV Replicate throughout S Phase
Southern blot of HL and LL DNA isolated from clone D cells taken at different times in the cell cycle. Similar results were obtained with DNA isolated from ID13 cells or from clone B.

addition, Southern blot analysis of DNA from ID13 cells and clones B and D indicated that only a small fraction of the BPV hybridizing material is integrated in the genome

of ID13 cells, and BPV sequences were not detected in the genomes of clones B and D. Identical bands were observed when DNA from the three cell lines was cut with three different enzymes that do not cut within the plasmids or was physically sheared by passage through a 22-gauge needle (data not shown). Moreover, nearly all BPV hybridizing material occurred as monomeric form III molecules when DNA was cut with enzymes that cleave each plasmid once.

Replication of BPV Is Confined to S Phase

The results described above indicate that BPV plasmids replicate throughout S phase. To determine whether replication of BPV also occurs in other phases of the cell cycle, we took two approaches: In the first, a pool of ID13 cells was synchronized by mitotic selection (see Experimental Procedures), and cellular DNA synthesis through the cell cycle was monitored by assaying the incorporation of ³H-TdR into high molecular weight DNA of cells taken at various times after replating. Separate aliquots of cells from this synchronized population were pulsed with BUdR, and the DNA was hybridized with probes for BPV or the Alu-like sequence, as seen in Figure 4. The results indicate that BPV replication proceeds throughout S phase and that no significant replication occurs during the period of the cell cycle corresponding to G1 (hours 0-4).

In the second approach, cells labeled for two generations with ¹⁴C-TdR were pulse-labeled with BUdR for 1.5

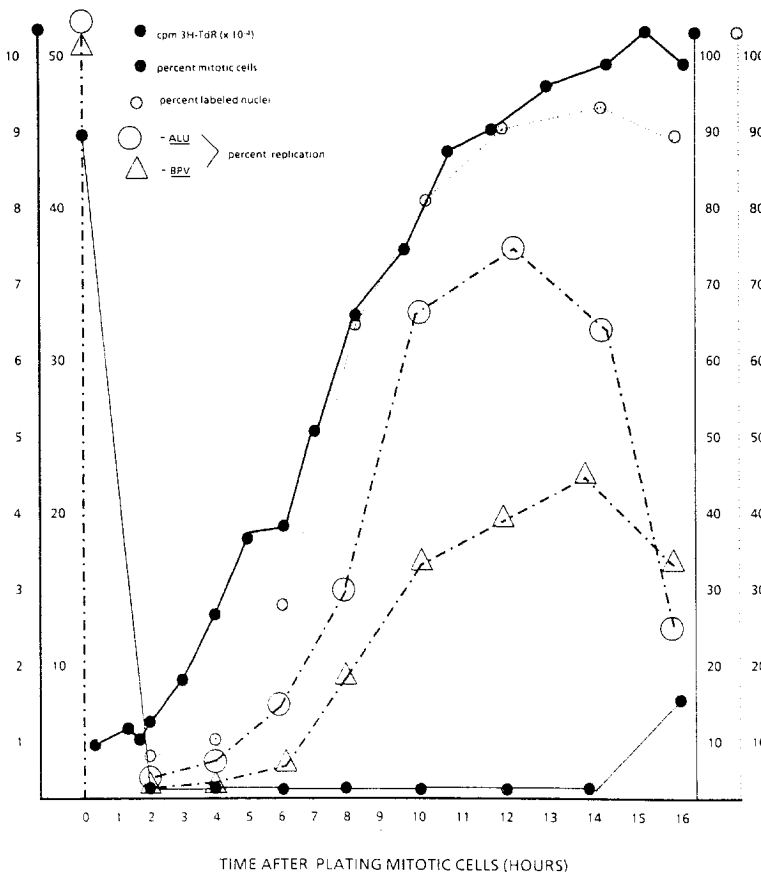


Figure 4. Replication in Cells Synchronized by Mitotic Selection

ID13 cells were synchronized by selective mitotic detachment and the same pool of mitotic cells was analyzed for the incorporation of ³H-TdR into high molecular weight DNA (●—●); the percentage of mitotic cells from count of at least 500 (●—●); the percentage of cells which were labeled with BUdR in a two-hour pulse (⊙—⊙); and the relative amounts of replication of BPV (Δ—Δ) and mouse-like Alu sequence (○—○) at the indicated times after replating the cells.

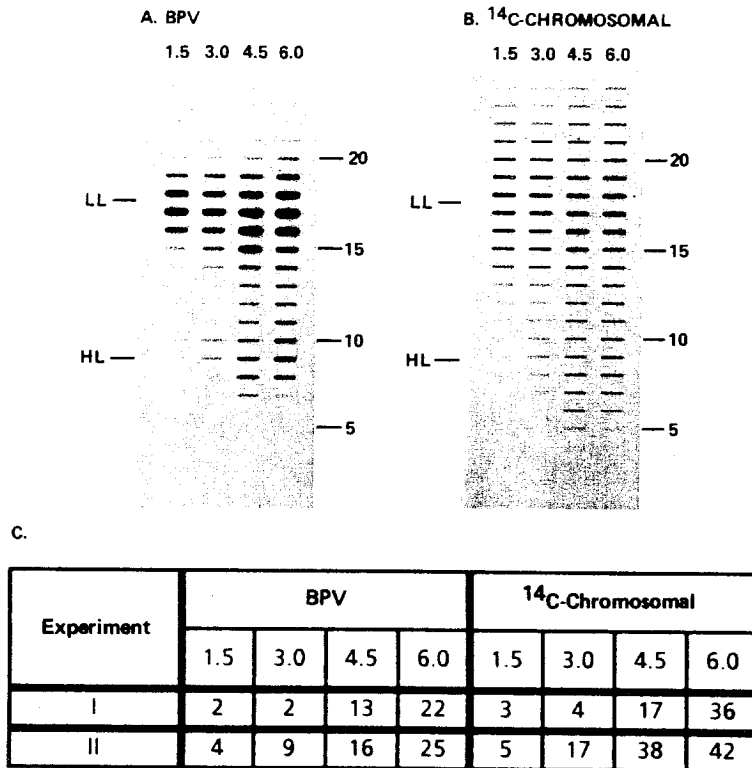


Figure 5. Replication in Late S Phase and G2
Logarithmically growing ID13 cells grown in ¹⁴C-TdR medium for two generations were labeled for 1.5 hr in BudR. The drug was then replaced with thymidine, and mitotic cells were collected at 1.5, 3.0, 4.5, and 6.0 hr after the start of the BudR pulse. HL and LL DNA from each of these samples was then resolved and analyzed as in Figure 2 except that before hybridizing to a [³²P]-labeled BPV probe (A), the filter was exposed directly to film to reveal the ¹⁴C labeled total chromosomal DNA (B). (C) The data from two such experiments is presented in a tabulated form after quantitating the relative replication (presented as HL/HL+LL) of BPV and chromosomal DNA by densitometry.

hr and then switched to media containing TdR for various times before being retroactively synchronized by mitotic selection. This procedure enabled analysis of replication during the last six hours of the cell cycle. Densitometric tracings of autoradiograms indicate that for BPV, as well as for chromosomal DNA, most of the detectable replication occurs before the last three hours of the cell cycle and that no significant replication of BPV occurs during G2 (Figure 5). We conclude that BPV replication is restricted to the period during which chromosomal DNA synthesis occurs, i.e., S phase of the cell cycle.

BPV Plasmids Replicate Randomly

Our finding that BPV plasmids replicate asynchronously throughout S phase precludes the possibility that maintenance of BPV copy number occurs by the simultaneous replication of all copies of the plasmid once per cell cycle. Maintenance of constant copy number by an asynchronous mode of replication potentially could occur by a mechanism that enables postreplicative plasmids to be distinguished from prereplicative molecules, or, alternatively, by a random replication mechanism similar to that observed for the control of copy number in bacterial plasmids (Rownd, 1969). These two possibilities can be distinguished experimentally by determining whether BPV plasmids can replicate more than once per cell cycle. To address this question, ID13 cells were pulsed for 10 hr with BUdR, a period of time longer than one S phase (6–8 hr) but less than one cell cycle (14 hr in log phase), and mitotic cells were collected at the end of this period of time. This population should contain cells that have completed only one round of replication in the presence of BUdR. DNA

isolated from such cells was then centrifuged to equilibrium in CsSO₄ gradients and the amounts of BPV and Alu-like sequences present in each fraction were quantitated as before (Figure 6A). Twenty-one percent of the BPV-hybridizing material banded as doubly substituted (heavy, heavy or HH) DNA, which necessarily must have proceeded through at least two rounds of replication in the presence of BUdR (see Table 2, first row). In addition, a significant proportion of the BPV molecules had not replicated at all during this period and were found in the LL fraction. In contrast, no mouse Alu-like sequences were found in the HH fraction, even after long autoradiography exposures.

Since BPV sequences replicate throughout S phase, the HH BPV DNA we observed in the above experiment potentially could have resulted from the replication of some molecules during the BUdR pulse at the end of one S phase and the re-replication of the same molecules at the beginning of the next S phase (still during the same pulse) in some of the contaminating cells in the mitotic preparation. Alternatively, a few cells may have been unusually rapid in their progression through the cell cycle and actually may have proceeded through more than one cell generation. To test these possibilities, we pulsed cells for increasing lengths of time. If either of the above mentioned artifacts are responsible for our observed results, then HH molecules should accumulate if one allows the cells to incorporate BUdR for longer periods of time. If, however, the experimental results reflect only BPV molecules that replicate within the first cell cycle, then the amount of HH DNA should remain essentially the same. The results of this experiment (Figures 6A–6D) show no

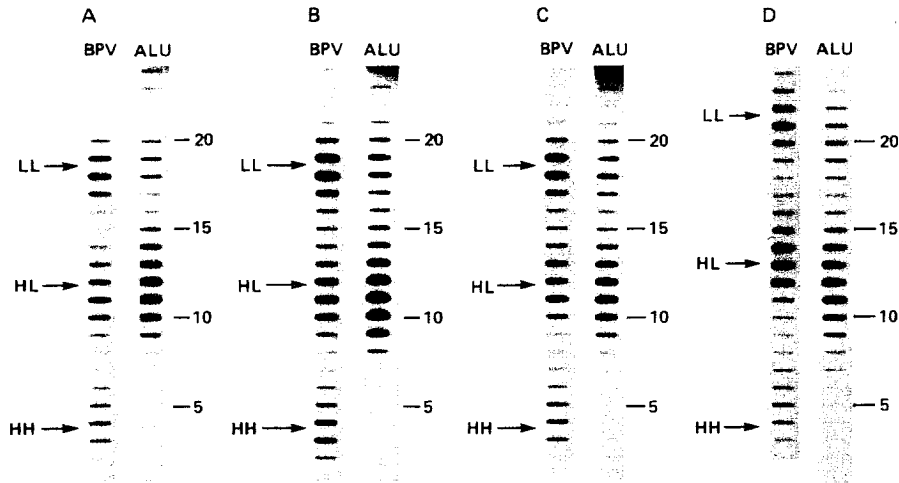


Figure 6. Density Analysis after One Cell Cycle

Density gradients of DNA from ID13 cells labeled for one cell cycle with BudR and prepared from mitotic cells as described in Experimental Procedures. Each fraction of the gradients was hybridized to [³²P]-labeled BPV or mouse Alu-specific probes. The positions of LL, HL, and HH DNA are indicated, and fractions are lined up for comparison of the results for each probe. A-D, ID13 cells labeled with BudR for 10, 12, 14, and 18 hr, respectively (see Experiments I-IV, Table 2). The amount of LL chromosomal DNA for BPV and Alu and the extent to which more LL than HH BPV DNA was observed was found to be variable from experiment to experiment (see Table 2). Overexposed autoradiograms are shown for Alu-like hybridization to emphasize the lack of HH chromosomal DNA.

relationship between the length of time of incubation of the cells in BudR and the amount of HH BPV molecules, indicating that BPV replication within one cell cycle is in fact being measured. We also examined BPV replication in clone B and clone D in the same manner and obtained similar results, indicating that the replication of BPV plas-

mids more than once per cell cycle is not a unique property of ID13 cells.

Table 2 summarizes the results of a number of experiments involving mitotically synchronized cells. In all cases, significant amounts of BPV DNA were found in the HH fraction; in no case were the Alu-like sequences ever

Table 2. Density Analysis after One Cell Cycle

Cell Line	Experiment	Length of Time in BUdR	BPV Probe			Alu Probe		
			HH	HL	LL	HH	HL	LL
ID13	I	10	21	42	37	0	78	22
ID13	II	12	17	40	43	0	82	18
ID13	III	14	20	42	38	0	89	11
ID13	IV	18	11	51	38	0	85	15
			*15	*56	*29			
Clone B	V	11	22	29	49	0	100	0
			*23	*32	*45			
Clone B	VI	14	18	39	43	0	100	0
			*24	*36	*40			
Clone D	VII	14	9	54	37	0	100	0
			*9	*53	*38			
ID13	VIII	10	*18	*56	*26	*0	*90	*10
ID13	IX	12	*21	*58	*21	*0	*86	*14
ID13	X	14	*20	*65	*15	*0	*95	*5
Clone D	XI	10	*28	*52	*20	*0	*94	*6
Clone D	XII	12	*21	*57	*22	*0	*93	*7
Clone D	XIII	14	*10	*53	*37	*0	*70	*30

Quantitation of HH, HL, and LL DNA from gradients analyzed as in Figure 6, presented as the percentage of the total hybridization on the gradient. Numbers denoted with an asterisk (*) indicate that DNA samples were cut with SacI prior to loading on CsSO₄ gradients, and the resulting HH, HL, and LL analyzed on Southern blots as in Figure 7. Other samples were cut with EcoRI prior to loading. In Experiments IV, V, VI, and VII aliquots of the same DNA samples were cut with EcoRI and SacI before gradient analysis; end results for both are shown. O = none detectable with exposures in the linear range for densitometry.

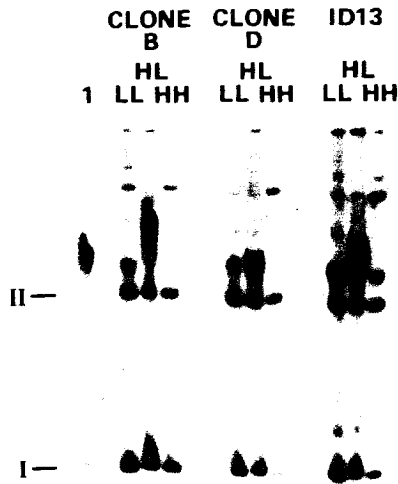


Figure 7. All Forms of BPV Replicate More Than Once per Cell Cycle Southern blots of LL, HL, and HH DNA from experiments with ID13, clone B, and clone D each labeled for 14 hr with BudR as in Figure 6C. Lane 1 is HH DNA prepared from clone B cells grown in BudR for 70 hr—a point when nearly all chromosomal DNA was also banding as HH DNA. HL DNA samples are smeared and overloaded on these gels because nearly all of the chromosomal DNA is HL. No HH chromosomal DNA is observed when gels for these experiments are stained with ethidium bromide. The band migrating just above the nicked circle (Form II) is variably present. Its presence does not correlate with any of the replication properties studied in this paper.

observed in the HH fraction, consistent with the expectation that chromosomal origins of DNA replication are used only once per cell cycle. Similar results were obtained for cells that were pulsed with BUdR for 10 hr and then sorted on the FACS to isolate the cells that had completed a round of replication (i.e., G2 and mitotic cells; data not shown).

Experiments were performed to determine the effect of varying the intracellular pool of BUdR (BUdR concentrations in the media ranged from 6 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$). We found that although the percent substitution of BudR for TdR increased to a plateau at 20 $\mu\text{g/ml}$ BudR, the relative amounts of HH, HL and LL remained essentially unchanged (data not shown). Finally, we wished to determine whether all, or only some, of the various forms of BPV DNA replicate more than once per cell cycle. For example, perhaps the monomeric forms of BPV replicate only once per cell cycle while the multimeric forms replicate more than once, with each BPV origin functioning only once in each cell cycle. Since similar proportions of HH DNA were obtained from three cell lines having three different ratios of monomers to nonmonomeric forms, different replication modes for the various forms of BPV seemed unlikely. However, to test this possibility directly, we separately pooled gradient fractions representing the HH, HL, and LL DNA and performed Southern analysis as described above. The results (Figure 7) show that all forms of BPV, including the form I monomers, are present

in the HH, HL, and LL DNA. Furthermore, densitometric tracings indicate that the relative proportions of form I monomer to total BPV DNA in the HH, HL, and LL are virtually the same. We conclude from this that all forms of BPV can replicate more than once per cell cycle.

Discussion

The experiments reported here indicate that bovine papilloma virus (BPV) is not subject to once per cell cycle replication control as are segments of chromosomal DNA. Instead, BPV molecules destined for replication are chosen randomly from the nuclear pool of BPV plasmids, replicated, and then reintroduced into the pool. BPV thus replicates according to a mode observed for many bacterial plasmids: some molecules replicate once during a particular cell cycle, some replicate more than once, and others do not replicate at all.

Our results also show that the replication of BPV occurs throughout S phase of the cell cycle and that BPV replication (like chromosomal DNA replication) is confined entirely to S phase. Replication of BPV plasmids throughout S phase is a general property of BPV replicons in mouse fibroblasts, and has been observed for three separate clonal isolates of BPV transformed cells that have been passaged for varying lengths of time and have different numbers of BPV copies. It is not known whether the duration of S phase (which classically is defined as the period of chromosomal DNA replication) is a specifically regulated event in the cell cycle, or whether it simply reflects the length of time required for the cell's chromosomes to complete one round of DNA synthesis. However, our finding that extrachromosomal BPV replicons also complete their DNA synthesis within the confines of S phase supports the view that the end of S phase is a regulated cell-cycle event.

The cell-cycle analysis of BPV replication reported here has employed a rapid and simple retroactive synchrony technique utilizing the Fluorescence Activated Cell Sorter (FACS) to isolate cells having a similar DNA content. The technique, which has been used previously to investigate the timing of replication of the 5S ribosomal RNA genes of *Xenopus laevis* (Gilbert, 1986), appears to be generally applicable for analysis of cell-cycle events. We have shown that the FACS method yields results similar to those obtained using prospective mitotic synchrony methods and to other methods employed to examine the timing of replication of mouse satellite DNA (Tobia et al., 1970, 1971).

Our finding that BPV molecules replicate randomly rather than once per cell cycle is in conflict with conclusions reached by others based on density labeling analyses of nonsynchronized cells (Botchan et al., 1986). Both our experiments and the earlier work have included the same clonal isolate of BPV transformed cells (ID13). As described above, our experiments were carried out also in other cell lines with identical results. Our observation that BPV plasmids are chosen randomly from the intracellular pool for replication may possibly explain the finding that rearrangements occurring in BPV plasmids can in-

crease their frequency rapidly in stably transformed clonal cell lines (Pintel et al., 1984; M. Calos, personal communication). Plasmids with deletions may complete their replication faster than larger plasmids that contain cloned DNA segments; these would be reintroduced into the nuclear pool following completion of replication and would be more likely to be replicated more than once in any cell cycle. Similar "taking over" of a population of replicons that contain spontaneously generated variants of cloned DNA segments has been observed for bacterial plasmids (Timmis et al., 1978; Brutlag et al., 1977).

Our experiments indicated that multimeric forms of BPV that migrate more slowly in agarose gels than the supercoiled monomers are present in different amounts in the three separate mouse fibroblast clones examined. While we do not know whether the nonmonomeric forms of BPV consist of catenanes, concatamers, or both types of molecules, we do know that the presence of multiple BPV replication units on a single molecule does not alter the timing of their replication; monomers and multimers appear to replicate in parallel throughout S phase. Moreover, evidence that the relative proportion of BPV monomers and multimers is similar in the LL, HL, and HH fractions we analyzed makes it unlikely that the BPV molecules that have replicated more than once per cell cycle result from initiation of replication at multiple loci on oligomeric BPV plasmids. While we have not directly investigated whether the replication of multimeric molecules more than once per cell cycle is due to their multiple replication origins and whether subsequent resolution of such multimers yields the HH monomers we have observed, the constancy of the monomer/multimer ratio in the pre- and post-replicated fractions of DNA we have analyzed is inconsistent with such a notion.

Random replication implies that the cell cannot distinguish plasmids that have replicated previously in a given cell cycle from those that have not replicated in that cycle. Mathematical models of plasmid replication have been studied in some detail in bacteria: in a purely random model, plasmids are chosen for replication one at a time, replicated, and returned to the intracellular pool *before* the next plasmid is chosen for replication. Such a replication mode gives a distribution of 25% HH, 50% HL, and 25% LL in density labeling experiments analogous to those reported here (Rownd, 1969; Zakian, 1979). The data we have obtained for BPV correspond closely to these theoretical ratios, but, in some instances, do not match them exactly. We believe that the amount of HH DNA detected in our studies is underestimated because the retroactive synchrony technique we employed to collect cells at the very end of the cell cycle is dependent on the percent of mitotic cells that can be harvested from a plate of growing cells. Since 80%–90% of the cells isolated are routinely mitotic, 10%–20% of the cells in the population are in other phases of the cell cycle and thus have not undergone a complete round of replication in the presence of BUdR by the time they are harvested. We would therefore expect that unreplicated molecules (LL DNA) and once-replicated molecules (HL DNA) will be overrepresented

relative to those molecules that have replicated more than once (HH DNA).

By analogy to the replication of bacterial plasmids, replication of BPV in stably transformed mouse fibroblasts until a characteristic copy number is achieved, at which point further replication will not occur until the next cell cycle. In contrast, initiation of DNA synthesis from chromosomal origins of replication occurs only once per cell cycle. Thus, while BPV replication, like chromosomal DNA replication, occurs during S phase of the cell cycle and is confined entirely to S phase, the replication mode for BPV plasmids appears to differ substantially from that seen for chromosomal DNA segments.

Experimental Procedures

Cell Culture and Labeling

ID13 cells and clones B and D are lines derived from mouse C127 cells transformed with wild-type bovine papilloma virus (Law et al., 1981). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cell lines were tested for mycoplasma contamination using the Gibco Mycotect assay subsequent to some of the experiments described in Schlosser et al. (1981). ID13 cells, but not clone B or clone D, were found to contain such contamination. ID13 cells were then cured of mycoplasma using Gibco BM cycline; experiments repeated using the cured lines gave results identical to those obtained prior to curing. ID13 cells were then retested to confirm that they had remained mycoplasma free throughout the duration of the repeated experiments.

For labeling with 5-bromo-2'-deoxyuridine (BudR), cells were incubated for the lengths of time indicated in the text with 30 µg/ml BudR unless otherwise stated.

Mitotic Selection Experiments

Mitotic selection of cells was performed by firmly tapping 175 cm² flasks containing 5 ml of media. Typically, about 2×10^5 cells are obtained from each subconfluent flask. To examine the percentage of mitotic cells for each experimental sample, an aliquot was added to 10 ml of media diluted with 4 vol of distilled water and incubated at 37°C for 10 min. A few drops of fixative (methanol:acetic acid; 3:1) were added and the cells were spun and resuspended in 2 ml of fixative and stored at 4°C. For analysis the swollen and fixed cells were spun and resuspended in 200 µl of fixative and dropped onto microscope slides. Slides were then stained for 30 min in aceto-orcein (Gibco), washed in ethanol for 5 min, and the percentage of mitotic cells scored. We found that 80%–90% of the cells in almost every sample were mitotic.

For prospective synchrony procedures, mitotic cells were pooled from several flasks and replated into two sets of plates containing warmed media. In one set of plates, the entry of cells into S phase was monitored by measuring the rates of incorporation of ³H-TdR as described in Schlosser et al. (1981). In the other set of plates, cells were pulse-labeled for 2 hr with BUdR at 2 hr intervals, detached from the plate, and analyzed for the percentage of cells that took up the BUdR label by the procedure of Dolbeare et al. (1983), the percentage of mitotic cells as described above, and the replication of BPV and Alu like sequences as described below.

Two types of experiments described in this paper utilized mitotic selection as a retroactive synchrony procedure. Experiments to determine whether sequences had undergone more than one round of replication are described below. The other type of experiment was designed to examine events occurring in G₂. Cells were grown for two generations in 175 cm² flasks containing media with 0.05 µCi/ml ¹⁴C-TdR, then 30 µg/ml BUdR was added for 1.5 hr. Mitotic cells were either collected at this time or the cells were washed with normal media and then transferred to media containing 100 µg/ml TdR and mitotic cells collected every 1.5 hr thereafter. Each flask was used only once for mitotic selection. One and a half hours was used as a labeling time instead of 2 hr in order to examine the G₂ period in greater detail. Mitotic cells collected at each time point were analyzed for the replication

of chromosomal DNA by exposing the nitrocellulose filters, which contained DNA obtained from analytical CsSO₄ gradients as described below, directly to pre-flashed Kodak XAR film. BPV replication was then analyzed and compared by probing these same filters with a BPV probe as described below.

Fluorescence-Activated Cell Sorter (FACS) Analysis and Sorting of Cells

To determine the timing of replication of sequences using the Fluorescence Activated Cell Sorter (FACS), logarithmically growing populations of cells were pulse-labeled with BUdR and then stained for DNA content with chromomycin A3 as described by Gray and Coffino (1979). Analysis and sorting were performed at the Stanford shared cell sorter facility using a FACS II (Becton-Dickinson) with a 495 nm long-pass filter. Cell suspensions (10⁷ cells per ml) were sorted at a flow rate of 4000 cells per sec and collected into siliconized glass tubes. To check fidelity of sorting, 50 µl of sorted cells were added to 150 µl of chromomycin staining solution and, after 30 min at room temperature, were reanalyzed on the FACS.

Separation of Replicated and Unreplicated DNA

Cells from each FACS collection tube were transferred to Eppendorf tubes, centrifuged, resuspended in 150 µl of DNA extraction buffer (1% NaDodSO₄/100 mM Tris Cl [pH 8.0]/200 mM EDTA with proteinase K at 100 µg/ml), and incubated at 56°C for at least 2 hr. Samples were then extracted once with phenol, once with phenol/chloroform (1:1 vol/vol), and once with chloroform, ethanol-precipitated, and resuspended in the appropriate restriction enzyme buffer with DNAase free RNAase A at 10 µg/ml. Either EcoRI or SacI enzymes were added and digestions checked by examining aliquots on mini-gels. When the RNA was completely digested, samples were loaded on CsSO₄ (Braunstein et al., 1982) gradients in 5.2 ml centrifuge tubes, the refractive index was adjusted to 1.3705, and gradients were centrifuged at 30,000 rpm in a VT180 (Bechman) rotor for at least 48 hr. Gradients were then collected from the bottom into 24 equal volume fractions, using a peristaltic pump.

Hybridization and Quantitation

Aliquots of the DNA in CsSO₄ solution from each of the gradient fractions or from pooled fractions were denatured and applied to nitrocellulose filters using a slot-blot apparatus according to the procedure of Brown et al. (1983). BPV hybridization was determined using the plasmid pdBPV-1 (a gift from M. Calos), which contains a complete BPV-1 genome. To measure chromosomal DNA replication by a hybridization technique, the plasmid pCHOR32 (Crouse et al., 1982) (a gift from D. Smouse) was used as a probe. This plasmid contains a hamster Alu-like sequence that is homologous to the mouse Alu-like sequence and is highly repetitive and randomly dispersed throughout the mouse genome (Haynes et al., 1981). Mouse satellite DNA was analyzed using satellite DNA prepared from L-929 cells in the laboratory of B. Hamkalo by the method of Manuelidis (1977) and given to us as a gift by B. Hamkalo. The probes used in these experiments were prepared by standard nick translation techniques which routinely yield a specific activity of 2–5 × 10⁸ cpm/µg. Quantitation of the amount of hybridization to each fraction of the gradients was performed using a Helena Laboratories Quick Scan R&D densitometer which has been modified to link up with Hoeffer gs350 software for rapid computer analysis of densitometric data.

Southern Blot Analysis

For Southern analysis of SacI-digested DNA fractionated on gradients as described above, the appropriate fractions (representing the LL, HL, or HH DNA) were pooled and dialyzed against distilled H₂O using a BRL mini-dialysis apparatus, lyophilized to 100 µl, and resuspended in 100 µl 10 mM Tris-Cl (pH 8.0)/1 mM EDTA (TE buffer). Sheared DNA samples were prepared by passing DNA in 0.5 ml TE buffer through a 22-gauge needle at least ten times. For all analyses, DNA was electrophoresed in 0.7% agarose gels until Xylene Cyanol dye had migrated 20 cm or more.

Analysis of Number of Replications per Cell Cycle

Logarithmically growing cells were labeled with BUdR for the times in-

dicated in the text and then mitotically synchronized as described above. DNA was prepared and analyzed on gradients as described except that the refractive index of the CsSO₄ was adjusted to 1.3715 prior to centrifugation.

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References

- Balzas, I., Brown, E. H., and Schildkraut, C. L. (1974). The temporal order of replication of some DNA cistrons. *Cold Spring Harbor Symp. Quant. Biol.* 38, 239–245.
- Botchan, M. R., Berg, L. J., Reynolds, J., and Lusky, M. (1986). The bovine papilloma virus replicon. In *Papillomaviruses*, D. Evered and S. Clark, eds. (New York: John Wiley & Sons), pp. 53–67.
- Braunstein, J. D., Schulze, D., DelGiudice, T., Furst, A., and Schildkraut, C. L. (1982). The temporal order of replication of murine immunoglobulin heavy chain constant region sequences corresponds to their linear order in the genome. *Nucl. Acids Res.* 10, 6887–6902.
- Brown, P. C., Tlsty, T. D., and Schimke, R. T. (1983). Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. *Mol. Cell. Biol.* 3, 1097–1107.
- Bruttig, D., Fry, K., Nelson, T., and Hung, P. (1977). Synthesis of hybrid bacterial plasmids containing highly repeated satellite DNA. *Cell* 10, 509–519.
- Crouse, G. F., Simonson, C. C., McEwan, R. N., and Schimke, R. T. (1982). Structure of amplified normal and variant dihydrofolate reductase genes in mouse sarcoma S180 cells. *J. Biol. Chem.* 257, 7887–7897.
- Dolbeare, F., Gratzner, H. G., Pallauicini, M. G., and Gray, J. W. (1983). Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA* 80, 5573–5577.
- Fangman, W. L., Hice, R. H., and Chiebowicz-Siedziwska, E. (1983). ARS replication during the yeast S-phase. *Cell* 32, 831–838.
- 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.
- Gray, J. W., and Coffino, P. (1979). Cell cycle analysis by flow cytometry. *Meth. Enzymol.* 58, 233–248.
- Hand, R. (1978). Eucaryotic DNA: organization of the genome for replication. *Cell* 15, 317–325.
- Haynes, S. R., Toomey, T. P., Leinwand, L., and Jelinek, W. R. (1981). The Chinese hamster Alu-equivalent sequence: a conserved, highly repetitive, interspersed deoxyribonucleic acid sequence in mammals has a structure suggestive of a transposable element. *Mol. Cell. Biol.* 1, 573–583.
- Johnston, R. N., Feder, J., Hill, A. B., Sherwood, S. W., and Schimke, R. T. (1986). Transient inhibition of DNA synthesis results in increased dihydrofolate reductase synthesis and subsequent increased DNA content per cell. *Mol. Cell. Biol.* 6, 3373–3381.
- Laskey, R. A., and Harland, R. M. (1981). Replication origins in the eucaryotic chromosome. *Cell* 24, 283–284.
- Laughlin, T. J., and Taylor, J. H. (1979). Initiation of DNA replication in

- chromosomes of Chinese hamster ovary cells. *Chromosoma* 75, 19-35.
- Law, M. F., Lowy, D. R., Dvoretzky, I., and Howley, P. M. (1981). Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proc. Natl. Acad. Sci. USA* 78, 2727-2731.
- Manuelidis, L. (1977). A simplified method for preparation of mouse satellite DNA. *Anal. Biochem.* 78, 561-568.
- Pintel, D., Merchlinsky, M. J., and Ward, D. C. (1984). Expression of minute virus of mice structural proteins in murine cells lines transformed by bovine papilloma virus-minute virus of mice plasmid chimera. *J. Virol.* 52, 320-327.
- Rownd, R. (1969). Replication of a bacterial episome under relaxed control. *J. Mol. Biol.* 44, 387-402.
- Sarver, N., Byrne, J. C., and Howley, P. M. (1982). Transformation and replication in mouse cells of a bovine papilloma virus-pML2 plasmid vector that can be rescued in bacteria. *Proc. Natl. Acad. Sci. USA* 79, 7147-7151.
- Schlosser, C. A., Steglich, C., deWet, J. R., and Scheffler, I. E. (1981). Cell cycle-dependent regulation of thymidine kinase activity introduced into mouse LMTK⁻ cells by DNA and chromatin-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* 78, 1119-1123.
- Taylor, J. H. (1977). Increase in DNA replication sites in cells held at the beginning of S phase. *Chromosoma* 62, 291-300.
- Timmis, K. N., Cabello, F., Andres, I., Nordheim, A., Burkhardt, H. J., and Cohen, S. N. (1978). Instability of plasmid DNA sequences: macro and micro evolution of the antibiotic resistance plasmid R6-5. *Mol. Gen. Genet.* 167, 11-19.
- Tobia, A. M., Schildraut, C. L., and Maio, J. J. (1970). Deoxyribonucleic acid replication in synchronized cultured mammalian cells. I. Time of synthesis of molecules of different average guanine + cytosine content. *J. Mol. Biol.* 54: 499-515.
- Tobia, A. M., Schildraut, C. L., and Maio, J. J. (1971). DNA replication in synchronized cultured mammalian cells. III. Relative times of synthesis of mouse satellite and main band DNA. *Biochim. Biophys. Acta* 246, 258-262.
- Turek, L. P., Byrne, J. C., Lowy, D. R., Dvoretzky, I., Friedman, R. M., and Howley, P. M. (1982). Interferon induces morphologic reversion with elimination of extrachromosomal viral genomes in bovine papilloma virus-transformed mouse cells. *Proc. Natl. Acad. Sci. USA* 79, 7914-7918.
- Zakian, V. A., Brewer, B. J., and Fangman, W. L. (1979). Replication of each copy of the yeast 2 micron DNA plasmid occurs during the S phase. *Cell* 17, 923-934.