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Regulated Replication of an Episomal Simian Virus 40 Origin Plasmid in COS7 Cells

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The replication of a simian virus 40 (SV40) origin-containing plasmid, pSLneo, in stably transfected COS7 cells has been studied. pSLneo contains the SV40 origin of replication and encodes the positive selectable marker for G418 resistance. In transient replication assays, pSLneo replicates to a high copy number in COS7 cells. Uncontrolled SV40 plasmid replication has been reported to be lethal to such transfected cells. Thus, it was anticipated that extensive plasmid replication would preclude isolation of permanent cell lines containing pSLneo. However, significant numbers of G418-resistant colonies arose after transfection of COS7 cells with pSLneo. Cell lines established from these drug-resistant colonies contained between 100 and 1,000 extrachromosomal pSLneo copies per cell. Episomal plasmid DNA in pSLneo/COS7 lines was stably maintained after 2 months of continuous culture in selective medium. Bromodeoxyuridine labeling and density shift experiments demonstrated that replication of pSLneo closely paralleled that of cellular DNA. On average, plasmid DNA did not replicate more than once during a single cell generation period. Regulation of pSLneo replication appeared to be negatively controlled by a *cis*-acting mechanism. Endogenous copies of episomal pSLneo remained at a stable low copy number during the simultaneous, high-level replication of a newly transfected plasmid encoding SV40 large T antigen in the same cells. These results indicate that regulated replication of an SV40 origin plasmid can be acquired in a cell and does not require the presence of additional genetic elements. The molecular mechanism by which cells enforce this regulation on extrachromosomal SV40 plasmids remains to be defined.

Mammalian chromosomal DNA replication involves the coordinate function of a large number of independent origins of DNA synthesis (8, 15, 16). Faithful duplication of the cell genome requires a cellular control mechanism to ensure that each replication origin initiates replication only once during a single cell cycle. In the absence of such control, repeated initiations by a single replication origin would result in amplification of adjacent DNA sequences. The problem of regulating multiple replication origins is compounded by the observation that chromosome replication generally proceeds by a distinct temporal and spatial program (3, 11, 26; reviewed in reference 15). A replication origin that functions early in the S phase must be prevented from reinitiating replication during the remainder of the S phase, despite the continued presence of all factors necessary for initiation. It has been suggested that reinitiation is prevented by a *cis*-acting negative control mechanism that distinguishes newly replicated DNA from unreplicated DNA. However, at present there is only limited experimental evidence to support the existence of such a mechanism (21, 23).

A major obstacle to the study of replication control has been the difficulty in isolating chromosomal origins of DNA replication from mammalian cells. The bovine papillomavirus (BPV) and the Epstein-Barr virus (EBV) both replicate in a fashion that closely parallels duplication of cell chromosomes. In most cases, BPV and EBV persist as multicopy extrachromosomal plasmids in infected cells, with each viral episome replicating only once per cell generation period (1, 2). BPV and EBV rely upon the cellular DNA replication apparatus for their duplication. It is likely that these viruses also exploit the cellular mechanisms that regulate DNA replication to ensure long-term, stable maintenance of their own genomes in infected cells. These features of BPV and EBV genome replication make these viruses attractive model systems for the study of replication control.

In contrast to BPV and EBV, simian virus 40 (SV40) bypasses cellular controls on replication and exhibits uncontrolled, high-level replication in permissive cells (27). Plasmids containing the SV40 origin of replication are rapidly amplified when transfected into COS7 cells (5). These cells constitutively express SV40 large T antigen (T-Ag), the only viral protein required for SV40 replication. SV40 plasmids undergo multiple rounds of duplication within one cell generation, usually accumulating to over 10,000 plasmid copies per cell just 48 h after transfection. High-level, runaway replication by SV40 plasmids is thought to be lethal to cells (22, 24). Thus, very few COS7 colonies are reportedly obtained if one selects for the presence of an SV40 origin plasmid in a stable transfection assay (22, 28).

In a model system described by Roberts and Weintraub, BPV genetic elements imposed a regulated mode of replication upon SV40 in a *cis*-dominant fashion (22, 23). Chimeric plasmids containing both the SV40 origin of replication and the BPV genome replicated only to low levels in COS7 cells when tested in transient replication assays. In these experiments, suppression of SV40 replication was dependent upon the presence of specific *cis*- and *trans*-acting BPV genetic elements. Furthermore, it was possible to establish stable COS7 lines containing relatively low copy numbers of extrachromosomal BPV-SV40 hybrid plasmids. The BPV-SV40 plasmids in the stable COS7 lines replicated in a controlled fashion, with each plasmid undergoing only one duplication per cell cycle period (23). Introduction of a second plasmid, which contained an SV40 origin and expressed additional T-Ag, resulted in runaway replication of the newly transfected plasmid, while in the same cells the endogenous

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BPV-SV40 hybrid plasmids remained at a constant, low copy number. This result indicated that all *trans*-acting factors necessary for runaway SV40 replication were present in these cells; thus, replication of BPV-SV40 composite plasmids must be negatively controlled by a *cis*-acting mechanism. Replication control was suggested to be mediated through BPV genetic elements because SV40 plasmids lacking BPV sequences could not establish permanent COS lines.

The experiments presented here were originally undertaken to determine whether EBV sequences could similarly control SV40 replication in EBV-SV40 hybrid plasmids transfected into COS cells. In these experiments, substantial numbers of COS colonies were obtained after stable transfection of a control plasmid containing no EBV DNA sequences, pSLneo. This plasmid harbors only an SV40 origin and a selectable marker gene (for G418 resistance). Transfection by pSLneo was predicted to be lethal, since it replicates in a runaway fashion in short-term assays. Surprisingly, G418-resistant COS colonies arising after transfection with pSLneo could be readily cloned to derive permanent cell lines. The pSLneo/COS lines stably maintained between 100 and 1,000 extrachromosomal pSLneo copies per cell, and the replication of pSLneo DNA in these cells occurred in a regulated fashion (on average, once per cell generation period). Furthermore, pSLneo replication appeared to be negatively regulated by a *cis*-acting mechanism. These results indicate that controlled episomal replication of SV40-based plasmids can be imposed by a cellular mechanism that does not require the presence of exogenous viral DNA sequences. It remains possible that processes controlling pSLneo replication in this system are related to cellular mechanisms that regulate chromosomal DNA replication.

MATERIALS AND METHODS

Plasmid constructions. The construction of pSLneo was described previously (18). pSLneo, a 4.5-kb derivative of pSV2neo (25), contains the *Hin*dIII (nucleotide [nt] 5172)-to-*Pvu*II (nt 272) SV40 region encompassing the origin of replication (nucleotide numbering according to the system of Tooze [27]). The SV40 early promoter in this plasmid directs the expression of the bacterial neomycin phosphotransferase gene (*neo*), which provides G418 resistance in mammalian cells (25). A control plasmid that is defective for SV40 replication, pSLori−, was constructed from pSLneo by introducing a small deletion within the SV40 origin. pSLneo was digested with *Sfi*I, which cuts within the origin palindrome (nt 5234). The resulting 3-bp overhang was removed by subsequent treatment with T4 DNA polymerase, and the plasmid was recircularized to create pSLori−. pSLori− does not detectably replicate in COS7 cells when tested in a transient assay. pSVL was constructed by cloning a 7.4-kb *Eco*RI fragment of bacteriophage lambda DNA into the unique *Eco*RI site within pSLneo. The plasmid pSVTAg (gift from T. Shenk's laboratory) contains the *Hpa*II (nt 346)-to-*Bam*HI (nt 2533) SV40 early region, including the origin of replication and T-Ag gene, cloned into pML2 (a derivative of pBR322) (19).

Transfection assays. COS7 cells (5) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Confluent 100-mm-diameter plates of cells were trypsinized, and the cells were replated (1:10 dilution) 1 day before transfection. Typically, 1 μg of plasmid DNA, with 10 μg of salmon sperm carrier DNA, was transfected by the calcium phosphate coprecipitation method (6). The cells were subjected to a glycerol shock 3 to 4 h after transfection and refed with fresh medium. For some experiments, plasmid DNA (without carrier DNA) was transfected by the DEAE-dextran method (20). For transient replication assays, cells were harvested for preparation of DNA from 16 to 68 h after transfection. For isolation of permanent cell lines, cells were split (1:10) 3 days after calcium phosphate transfection into medium containing 500 μg of G418 per ml. Drug-resistant colonies generally arose after 10 to 12 days in selective media. Individual G418-resistant COS7 colonies were isolated approximately 3 weeks after transfection by trypsinization in glass cloning rings and then replated into 30-mm-diameter dishes. Cell cultures were expanded into 100-mm-diameter plates and split 1:5 or 1:10 during subsequent passaging. Throughout expansion and growth of G418-resistant cell lines, selection was maintained at 500 μg of G418 per ml. Total DNA was prepared from transfected cells by the method of Roberts and Weintraub (22). In some cases, the low-molecular-weight DNA fraction was isolated by the method of Hirt (12). For Southern blot analysis, DNA was fractionated on agarose gels, denatured, and electroblotted onto nylon membranes. ^{32}P-labeled probes were prepared by nick translation of plasmid DNA, resulting in specific activities of about 10^8 dpm/μg or greater. Hybridizations and washes were carried out as described by Church and Gilbert (4).

Density transfer analysis. Subconfluent 100-mm-diameter plates of cells were incubated in medium containing 50 μM bromodeoxyuridine (BUdR) for 24 or 88 h. Total cellular DNA was harvested and fractionated by CsCl density gradient centrifugation as described by Roberts and Weintraub (23). Fractions (0.4 ml) were collected by dripping from the tube bottom. The refractive index of every other fraction was determined to monitor fraction densities. Fractions were desalted by passage over G-50 spin columns and transferred to a nylon membrane with a Minifold II slot blot apparatus (Schleicher & Schuell). The blot was probed first with ^{32}P-labeled pBR322 to detect plasmid DNA. A control sample of COS7 DNA was included on the same blot to ensure that the probe was not binding nonspecifically to chromosomal DNA sequences. Following autoradiography, the pBR322 probe was stripped from the blot with boiling water. Complete removal of the first probe was confirmed by autoradiography. Cellular DNA in the fractions was subsequently detected by rehybridizing the blot with ^{32}P-nick-translated DNA from CV-1 cells (which do not contain SV40 DNA sequences). The relative amounts of plasmid DNA and cellular DNA within each fraction were measured by analysis of appropriate autoradiographs with a Bio-Rad model 620 densitometer.

Flow cytofluorimetry. Cells were prepared for flow cytometry essentially as described by Khochbin et al. (13). Fixed, permeabilized cells were incubated with a T-Ag-specific monoclonal antibody, pAb423 (10) (undiluted culture supernatants). For each cell line analyzed, a blank in which fixed cells were incubated in medium lacking the primary antibody was also prepared. Samples were then incubated in a 1:1,000 dilution of goat anti-immunoglobulin conjugated with fluorescein isothiocyanate (Cappel Laboratory). Fluorescein isothiocyanate fluorescence of treated cells was quantitated by analysis with a fluorescence-activated cell sorter. The mean fluorescence (log mean channel number) of 10,000 cells was measured for each sample. Specific fluorescence (SF) was defined as mean fluorescence of the cell line − mean fluorescence of the corresponding blank. The fluorescence of

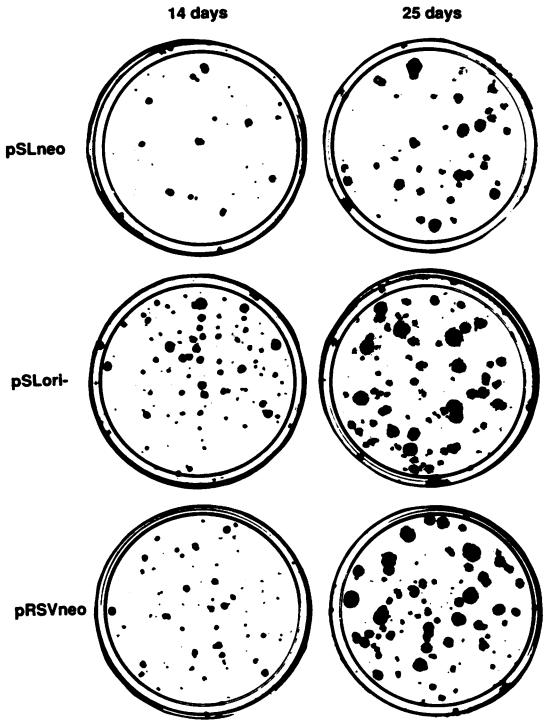

FIG. 1. Production of G418-resistant COS7 cell colonies by transfection with SV40-based plasmids. COS7 cells were transfected with 1 μg of the indicated plasmid DNA and grown in medium containing 500 μg of G418 per ml. Plates were stained with Giemsa stain after 14 days (left) or 25 days (right) in selective medium.

a cell line relative to COS7 cells was calculated as follows: relative fluorescence = $10^{(\text{SF of cell line/SF of COS7 cells}) - 1}$

RESULTS

Establishment of stable COS7 lines containing extrachromosomal pSLneo DNA. The original goal of these experiments was to determine whether DNA sequences from the EBV origin of replication could act to negatively control SV40 replication in EBV-SV40 chimeric plasmids. By analogy to BPV, the presence of certain EBV sequences on an SV40-neo plasmid might permit isolation of permanently G418-resistant COS7 colonies due to the suppression of runaway SV40 replication. As a control for these experiments, an SV40-based plasmid lacking any EBV sequences, pSLneo (18), was also tested. pSLneo contains a portion of the SV40 early region encompassing the origin of replication, a gene encoding G418 resistance (*neo*) expressed from the SV40 early promoter, and bacterial plasmid sequences derived from pML2 (19). It was anticipated that pSLneo would yield few, if any, G418-resistant COS7 colonies, because its overreplication would be lethal to cells. However, pSLneo consistently produced unexpectedly high numbers of G418-resistant COS7 colonies (Fig. 1), with an estimated transfection efficiency of 0.001%. These pSLneo colonies were not abortive in the sense that they continued to grow after 3 weeks of G418 selection (Fig. 1) and could be established into cell lines with high efficiency (>50%). Furthermore, the numbers of G418-resistant colonies obtained with pSLneo were only slightly reduced (two- to threefold lower) relative to those obtained with plasmids that lacked a functional SV40 origin (pSLori− and pRSVneo [Fig. 1]) and require integration of their DNA for long-term maintenance. pSLori− is identical in structure to pSLneo, except that the functional SV40 origin has been eliminated by a 3-bp deletion within the origin palindrome. pRSVneo completely lacks SV40 origin sequences and expresses G418 resistance from the Rous sarcoma virus long terminal repeat. The capacity of pSLneo to produce colony numbers comparable to those produced by pSLori− suggested that the replication of pSLneo was not substantially toxic to the COS7 cells that produced these colonies.

Multiple independent G418-resistant colonies were cloned and expanded in culture to establish cell lines. To examine the state of the transfected pSLneo DNA in these cell lines, low-molecular-weight DNA was prepared by the method of Hirt (12) and analyzed by Southern blotting (Fig. 2). Independent pSLneo transfected COS7 lines (termed SL1, SL3, and SL10, etc.) contained between 100 and 1,000 extrachromosomal copies of pSLneo per cell. Extrachromosomal DNA was not detected in four G418-resistant lines established after transfection with a plasmid lacking a functional SV40 origin (i.e., pRSVneo [Fig. 2]). Much of the pSLneo DNA persisted as form I closed circular DNA. Higher-molecular-weight species were also present in most clones and appeared to be multimers or concatemers of pSLneo. Digestion of the Hirt extracts with *Bam*HI, which cuts once within pSLneo, reduced the higher-molecular-weight material to a single species of unit length (Fig. 2). Southern blot analysis of high-molecular-weight DNA confirmed that the pSLneo DNA was predominantly episomal in these cell lines (data not shown), although the possibility of a small number of integrated pSLneo copies could not be excluded. Similar copy numbers (500 to 1,000 per cell) and higher-molecular-weight species were observed in cell lines containing BPV-SV40 chimeric plasmids (23).

To assess the stability of pSLneo episomes, one pSLneo/COS7 cell line, SL1 (containing approximately 400 pSLneo copies per cell), was passaged extensively either in the presence or in the absence of G418. The copy number of the pSLneo plasmid DNA in the cultures was monitored by Southern blot analysis of Hirt extracts (Fig. 3). In the presence of G418, very little of the pSLneo DNA was lost from the culture. After 2 months of continuous culture in G418 medium, approximately 200 copies of pSLneo per cell remained (P17 [Fig. 3]). These results indicate that pSLneo was capable of stable extrachromosomal maintenance in COS7 cells. When the cells were passaged in the absence of G418 selection, the plasmid DNA was eventually (4 to 6 weeks) lost from the culture (Fig. 3). The instability of pSLneo in the absence of selection is similar to the observed rate of loss of BPV-SV40 plasmids under equivalent conditions (23).

Regulated replication of pSLneo. It was possible that pSLneo/COS7 cell lines were heterogeneous cultures wherein runaway plasmid replication within a fraction of the cells provided resistance to the remaining population (which lacks the plasmid). It has been reported that this situation accounts for cell lines that maintain extrachromosomal SV40 plasmids harboring a GPT-based selectable marker (23, 28). Alternatively, pSLneo replication might proceed in a regulated fashion analogous to BPV-SV40 plasmids in stable COS7 lines, wherein the timing of plasmid replication closely paralleled cellular DNA replication. Density transfer experiments were performed to examine the replication of pSLneo DNA in stably transfected COS7 cells. G418-resistant COS7 colonies arising after transfection with pSLneo were pooled and expanded in culture. Subconfluent plates of cells were

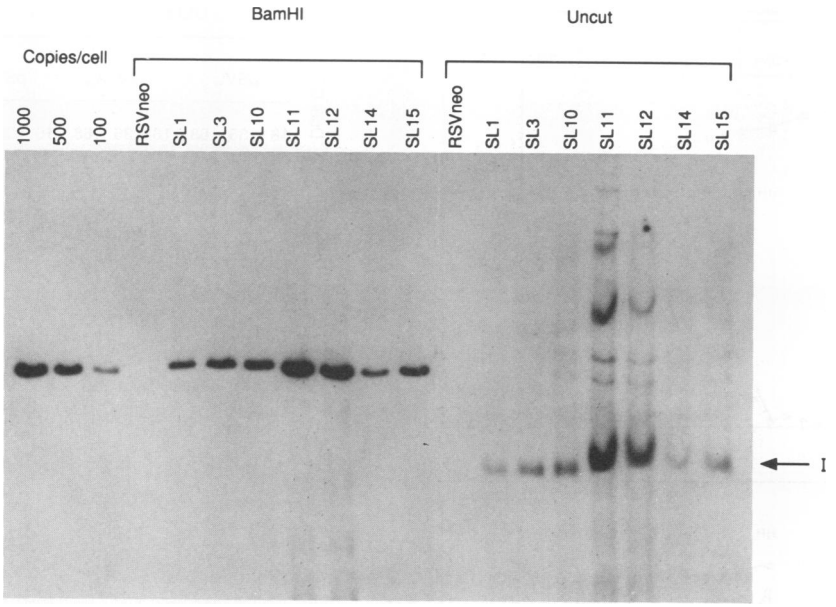

FIG. 2. Southern blot analysis of low-molecular-weight DNA from G418-resistant pSLneo/COS7 cell lines. DNA was prepared from seven independent G418-resistant clones (SL1, SL3, SL10, SL11, SL12, SL14, and SL15) isolated after stable transfection of COS7 cells with pSLneo. One pRSVneo/COS7 clone (RSVneo) was also analyzed. DNA prepared from 10^6 cells was fractionated on an agarose gel either uncut (right) or after digestion with *Bam*HI (middle). DNA was then blotted and probed with ^{32}P-labeled pBR322 to detect plasmid sequences. Copy number controls (left) were made by mixing appropriate quantities of *Bam*HI-digested pSLneo with the Hirt extract from 10^6 COS7 cells. The arrow indicates the position of form I circular plasmid DNA.

then incubated in media containing BUdR for either 24 h (less than the estimated cell generation time of 36 h) or 88 h. Total DNA was prepared from the cells and fractionated on CsCl density gradients, and the amount of pSLneo or cellular DNA in each fraction was determined by slot blot analysis (Fig. 4). This analysis revealed that the replication of pSLneo DNA closely paralleled the replication of chromosomal DNA sequences. Twenty-four hours after the addition of BUdR, significant amounts of pSLneo DNA were present only in the heavy-light hybrid DNA fractions and were not detected in the heavy-heavy (both DNA strands substituted with BUdR) portion of the gradient (Fig. 4, top), indicating that pSLneo plasmids did not on average replicate more than once during this period. Only after cells were incubated in BUdR for times greater than one cell division (88 h) did we detect substantial amounts of pSLneo DNA in the heavy-heavy density fractions (Fig. 4, bottom). Similar results were obtained after analysis of a cloned pSLneo/COS7 cell line (SL1) (data not shown). While the possibility of low levels of uncontrolled pSLneo replication cannot be totally excluded, these results indicate that the majority of pSLneo plasmids in stable COS7 lines replicated in a regulated fashion and closely followed cellular DNA duplication. Furthermore, these results suggest that there are very few "jackpot" cells within the culture that spontaneously undergo uncontrolled pSLneo replication.

***cis*-acting negative control of pSLneo replication.** pSLneo replicates to high levels (10,000 copies per cell) in a transient replication assay of COS7 cells (see Fig. 6). However, in stable pSLneo/COS7 cell lines, pSLneo is maintained at a lower copy number (100 to 1,000 per cell) and replicates in a controlled fashion. To examine the nature of this apparent suppression of pSLneo replication in stable pSLneo/COS7 lines, we performed transfection assays with two different test plasmids. The first plasmid, pSVL, is identical to

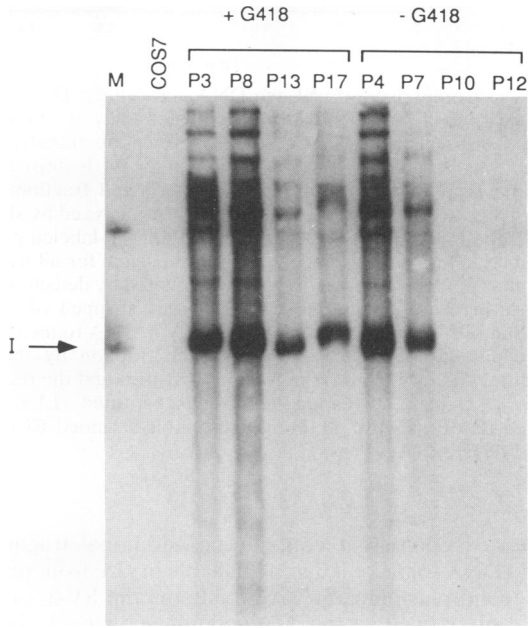

FIG. 3. Stability of extrachromosomal pSLneo DNA in COS7 cells. A pSLneo/COS7 cell line, SL1, was passaged in the presence (+ G418) or absence (− G418) of drug selection. Typically, the cells were passaged at confluency (trypsinized and split 1:10 or 1:5) every 4 days. Seventeen passages (P17) corresponds to 2 months in continuous culture. Cells were harvested at the indicated passage number for isolation of low-molecular-weight DNA. DNA corresponding to 10^6 cells was analyzed by Southern blotting. Plasmid DNA was detected by hybridization with ^{32}P-labeled pBR322. Lane M contains 50 pg of uncut pSLneo DNA to serve as a marker. The arrow indicates the position of form I pSLneo DNA.

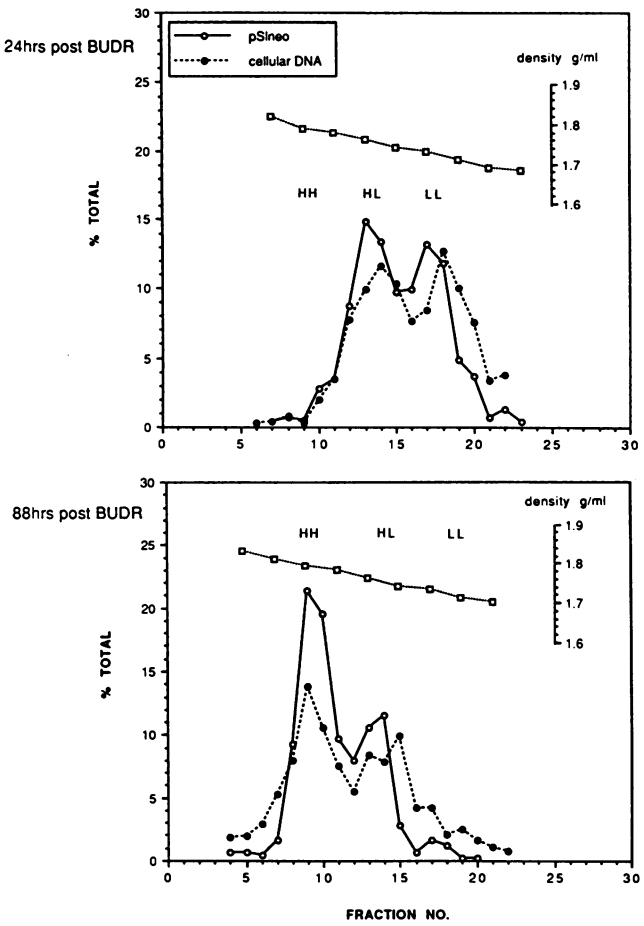

FIG. 4. Comparison of pSLneo DNA and cellular DNA replication in pSLneo/COS7 cells by density transfer analysis. A culture of pooled G418-resistant COS7 colonies derived by transfection of pSLneo was incubated in BUdR medium for 24 h (top) or 88 h (bottom). Total cellular DNA was harvested and fractionated on CsCl gradients. A portion of each fraction was analyzed by slot filter hybridization. The filter was first probed with ^{32}P-labeled pBR322, and the relative amount (percentage of total signal for all fractions) of pSLneo DNA in each fraction was quantitated by densitometry of the resulting autoradiograph. The filter was stripped of the first probe and rehybridized with ^{32}P-labeled CV-1 DNA to measure the relative amount of cellular DNA in each fraction by the same procedure. Also shown are the fraction densities and the regions of the CsCl gradient corresponding to unsubstituted (LL), single-stranded BUdR-substituted (HL), and double-stranded BUdR-substituted (HH) DNA.

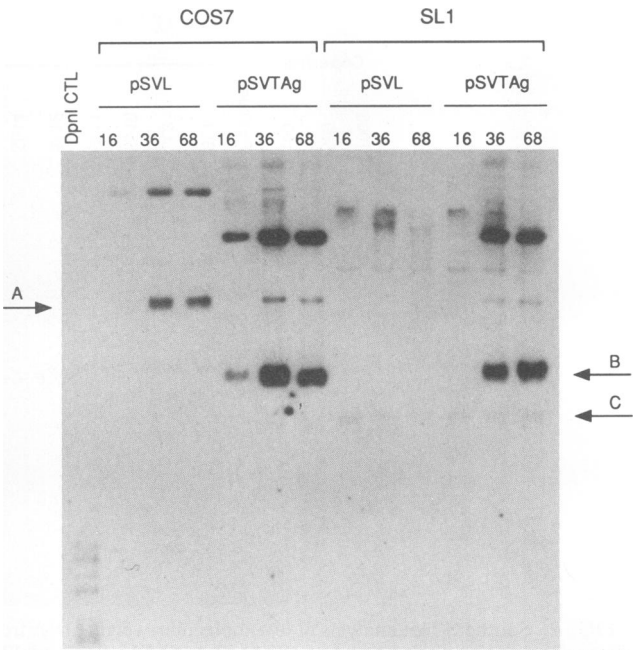

FIG. 5. Replication of pSVL and pSVTAg in pSLneo/COS7 cells. pSVL and pSVTAg were transfected into either COS7 cells (left) or SL1 cells (right), a pSLneo/COS7 cell line. Both plasmids contain the SV40 origin of replication; pSVTAg also encodes the T-Ag gene. Total DNA was harvested 16, 36, or 68 h after transfection. In each case, 2 μg of recovered DNA was digested with *Dpn*I to eliminate unreplicated DNA, Southern blotted, and hybridized to ^{32}P-labeled pBR322. Arrows indicate the positions of form I plasmid DNA for pSVL (A), pSVTAg (B), and the endogenous copies (about 400 per cell) of pSLneo within SL1 cells (C). To ensure that *Dpn*I eliminated all unreplicated plasmid DNA, 1 ng of pSLneo was mixed with 2 μg of COS7 DNA (DpnI CTL) and treated in parallel with the other samples.

pSLneo except that it contains an additional fragment of lambda DNA to make it distinguishable in size from pSLneo. The second plasmid, pSVTAg, contains the SV40 origin of replication as well as the T-Ag-coding sequences. Replication of these plasmids in COS7 cells and SL1 cells, which contain approximately 400 copies of episomal pSLneo, was tested. Total DNA was isolated from the cells 16, 36, or 68 h after transfection of plasmid DNA, digested with *Dpn*I to eliminate unreplicated DNA, and analyzed by Southern blotting (Fig. 5). pSVL replicated to high levels in COS7 cells (Fig. 5, arrow A); however, very little pSVL replication was detected in SL1 cells. This suggested that a *trans*-acting factor necessary for SV40 replication was limiting in SL1 cells. The limiting factor was T-Ag because pSVTAg, which encodes additional T-Ag, replicated to high levels in SL1 cells (Fig. 5, arrow B). Significantly, the copy number of the endogenous pSLneo plasmids in SL1 cells (arrow C) was unaffected by the transfection and overreplication of the T-Ag-encoding pSVTAg plasmid. This result suggests that insufficient T-Ag levels were not solely responsible for limiting the replication of pSLneo in SL1 cells. Since pSVTAg overreplicated in SL1 cells, all *trans*-acting factors necessary for runaway replication of SV40 origin plasmids must be present. Therefore, maintenance of the resident pSLneo plasmids at a low copy number under these conditions indicates that pSLneo replication in SL1 cells is negatively controlled by a *cis*-acting mechanism. Similar results were reported for COS7 lines containing BPV-SV40 composite plasmids (23). Newly transfected plasmids that expressed additional T-Ag overreplicated, while the copy number of the endogenous BPV-SV40 plasmids remained unaffected. The results presented here indicate that *cis*-acting negative control of SV40 replication can also occur in the absence of BPV or EBV genetic elements.

This suppression of pSLneo replication could be explained by mutation of the pSLneo DNA sequences (for example, within the SV40 origin of replication) to make up a less active substrate for the replication machinery. This explanation predicts that plasmid DNA isolated from pSLneo/COS7 cell lines will be defective for high-level replication when retransfected into COS7 cells. To examine this possibility, a

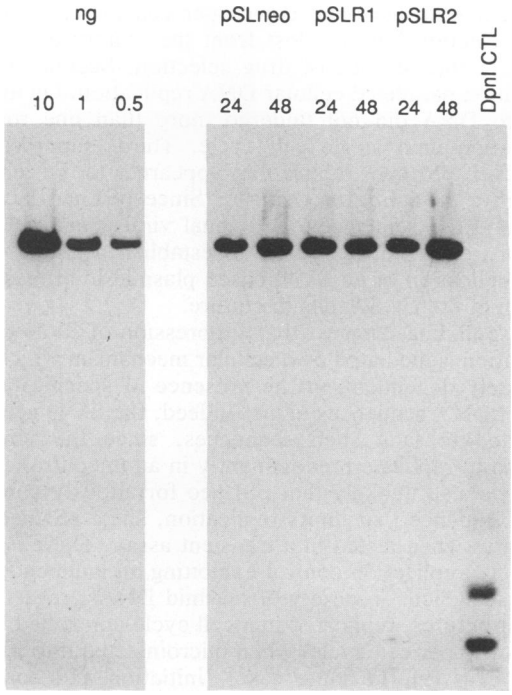

FIG. 6. Transient replication analysis of pSLneo plasmids rescued from pSLneo/COS7 cells. Two plasmids, pSLR1 and pSLR2, were rescued in *E. coli* from the Hirt extract of a pSLneo/COS7 cell line (SL1) and subsequently transfected into COS7 cells. For comparison, the parental plasmid, pSLneo, was tested in parallel. One microgram of plasmid DNA was transfected in each case. Total DNA was harvested 24 or 48 h after transfection, digested with *Dpn*I and *Bam*HI, Southern blotted, and probed with ^{32}P-labeled pBR322 to detect plasmid sequences. Copy number controls (left) contained the indicated amounts of *Bam*HI-digested pSLneo DNA. One nanogram corresponds to approximately 5,000 copies per cell, assuming a 10% efficiency of transfection. One nanogram of pSLneo, mixed with 2 μg of COS7 DNA, was treated in parallel with the other samples to ensure that *Dpn*I completely digested unreplicated DNA (DpnI CTL).

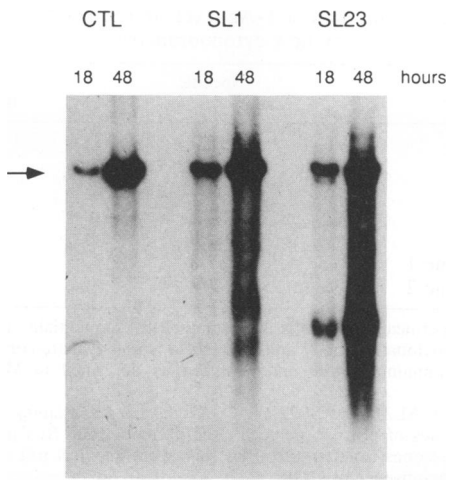

FIG. 7. Transient replication analysis of pSLneo DNA purified from pSLneo/COS7 cell lines. COS7 cells were transfected by the DEAE-dextran method with low-molecular-weight DNA preparations from two pSLneo/COS7 cell lines (SL1 and SL23) or with pSLneo DNA purified from *E. coli* (CTL). DNA corresponding to approximately 5 ng of pSLneo was transfected in each case. The low-molecular-weight DNA extract from about 5×10^6 COS7 cells was included as carrier DNA in the CTL transfection. Low-molecular-weight DNA was then isolated 18 or 48 h after transfection, digested with *Bam*HI (single site within pSLneo), and Southern blotted. Plasmid sequences were detected by hybridization with ^{32}P-labeled pBR322.

number of plasmids present in SL1 cells were isolated by transforming competent *Escherichia coli* (7) with Hirt DNA preparations. Plasmids rescued from SL1 cells were identical to pSLneo by a number of criteria. First, the plasmids produced the same pattern of restriction enzyme-generated fragments as pSLneo (data not shown). Second, the SV40 origin DNA sequences from two rescued plasmids were determined and found to be identical to pSLneo. In addition, all sequences were identical to the published sequence of the wild-type *Pvu*II (nt 272)-to-*Hin*dIII (nt 5172) SV40 origin region. Third, the rescued plasmids (pSLR1 and pSLR2) had the same capacity for overreplication in COS7 cells as pSLneo when tested in a transient replication assay (Fig. 6). From these results, it may be concluded that the *cis*-acting negative control of pSLneo replication does not involve genetic changes in the plasmid structure.

Modifications such as DNA methylation could be important for suppression of pSLneo replication. However, such modifications of plasmid DNA would not be maintained in the experiment described above, in which pSLneo plasmids were first rescued and then replicated in *E. coli*. This possibility has been tested by monitoring the replication capacity of extrachromosomal plasmid DNA purified directly from pSLneo/COS7 cells (without prior isolation in *E. coli*). Low-molecular-weight DNA was prepared from two independent pSLneo/COS7 cell lines (SL1 and SL23). An initial Southern blot was performed to quantitate the amount of episomal pSLneo present in each Hirt DNA preparation (data not shown). Portions of the low-molecular-weight DNA samples corresponding to approximately 5 ng of pSLneo DNA were then transfected into COS7 cells by the DEAE-dextran method (this transfection procedure appeared to be more efficient than the calcium phosphate method with small amounts of DNA). For comparison, 5 ng of pSLneo purified from *E. coli* was also transfected. Low-molecular-weight DNA was isolated 18 and 48 h after transfection, and replication of pSLneo was monitored by Southern blot analysis (Fig. 7). pSLneo DNA from two pSLneo/COS7 cell lines (Fig. 7, SL1 and SL23) showed the same capacity for replication as pSLneo DNA purified from *E. coli* (Fig. 7, CTL). This result suggested that suppression of pSLneo replication in stable pSLneo/COS7 lines did not involve DNA modifications (such as methylation) that are maintained upon purification of low-molecular-weight DNA.

Analysis of T-Ag in pSLneo/COS7 cell lines. The transfection data described above strongly suggest that suppression of runaway pSLneo replication occurs by a *cis*-acting mechanism. If negative control of pSLneo replication is mediated in *cis*, then all *trans*-acting factors required for high-level SV40 replication should remain intact in pSLneo/COS7 cells. The inability of pSVL to replicate in SL1 cells (see above) suggested that T-Ag in these cells was not available for replication of newly transfected plasmids. In order to test the capacity of T-Ag produced by SL1 cells to support SV40 replication, SL1 cells were passaged for several weeks in the absence of G418. During this time, pSLneo plasmids were lost from the culture (Fig. 3), permitting isolation of SL1-derived cell lines that had been cured of plasmid DNA. The

TABLE 1. Quantitation of T-Ag levels in pSLneo/COS7 cell lines by flow cytofluorimetry[a]

Cell line[b]	Specific fluorescence[c]	Relative fluorescence[d]
COS7	92.0	1.00
SL1	65.1	0.51
SL3	67.7	0.54
SL21	68.1	0.55
SL22	69.0	0.56
SL1-p, clone 1	87.6	0.89
SL1-p, clone 2	92.8	1.02

[a] Fixed, permeabilized cells were incubated sequentially in anti-T-Ag specific monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and analyzed as described in Materials and Methods.

[b] SL1, SL3, SL21, and SL22 are COS7 cell lines containing 100 to 1,000 episomal copies of pSLneo per cell. SL1-p clone 1 and SL1-p clone 2 are independent clones isolated after SL1 cells were cured of pSLneo DNA by passage in the absence of G418.

[c] Specific fluorescence represents the mean fluorescence (log mean channel number) of the sample after adjustment for nonspecific fluorescence.

[d] Relative fluorescence refers to the specific fluorescence compared with that of COS7 cells (defined as 1.00) (see Materials and Methods for method of calculation).

cured SL1 cells (SL1-p) were equivalent to COS7 cells in their capacity to support high-level replication (5,000 to 10,000 copies per cell) of transfected SV40 plasmids in a transient assay (data not shown). Since SL1-p cells were derived from SL1 cells, this result indicates that T-Ag in the parental SL1 cell line was not genetically defective for high-level SV40 plasmid replication. Had there been a heritable defect in T-Ag or other *trans*-acting components of the replication machinery in SL1 cells, SL1-p cells would not have supported overreplication of SV40 plasmids.

T-Ag in pSLneo/COS7 cell lines was also examined by biochemical methods. Immunofluorescence experiments using the T-Ag monoclonal antibody pAb423 demonstrated nuclear localization of T-Ag in pSLneo/COS7 cell lines (not shown). T-Ag levels in a number of cell lines were quantitated by flow cytometry (results summarized in Table 1). The four pSLneo/COS7 lines tested (SL1, SL3, SL21, and SL22) contained 40 to 50% less T-Ag than the parental COS7 cells. There is some heterogeneity of T-Ag levels within COS7 cells; thus, it is possible that COS7 cells expressing less T-Ag were selected for during isolation of pSLneo/COS7 lines. However, when plasmid DNA was eliminated from SL1 cells by passaging in the absence of G418 (as in Fig. 3), the amount of T-Ag returned to COS7 levels (SL1-p [Table 1]). This finding suggests that SL1 cells do not have a heritable reduction in T-Ag expression. Instead, diminished T-Ag levels in SL1 cells might reflect altered T-Ag metabolism (i.e., reduced half-life) due to the involvement of T-Ag in replication of plasmid DNA within these cells. In either case, while lower T-Ag levels might be important for limiting the extent of pSLneo replication, such reductions do not by themselves provide a mechanism for the observed *cis*-acting suppression of pSLneo replication.

DISCUSSION

The experiments presented here demonstrated that an SV40-based plasmid, pSLneo, can replicate in a regulated fashion in stably transfected COS7 cells. The properties of pSLneo replication in these cells were similar to those reported for BPV-SV40 composite plasmids in several respects. First, pSLneo DNA was stably maintained at 100 to 1,000 extrachromosomal copies per cell under continuous G418 selection but was lost from the culture after 4 to 6 weeks in the absence of drug selection. Second, pSLneo replication paralleled cellular DNA replication. On average, pSLneo DNA did not undergo more than one round of duplication in a single cell cycle. Third, suppression of high-level pSLneo replication appeared to be enforced through a *cis*-acting mechanism. Since pSLneo lacks any BPV or EBV sequences, additional viral genetic elements are apparently not necessary to establish negative control over replication of an SV40-based plasmid in at least some portion of the COS7 cells in culture.

These findings suggest that suppression of SV40 plasmid replication is mediated by a cellular mechanism which is not absolutely dependent on the presence of specific negative control DNA sequences in *cis*. Indeed, the SV40 genome is predicted to lack such sequences, since the virus has evolved to replicate predominantly in an uncontrolled fashion. It is also unlikely that pSLneo fortuitously contains a DNA sequence that limits replication, since pSLneo overreplicates when tested in a transient assay. There is precedence for replication control exhibiting an independence of DNA sequence. A variety of plasmid DNAs, regardless of their structures, replicated in a cell cycle-controlled fashion (only once per cell cycle) when microinjected into *Xenopus laevis* eggs (9). In some cases, initiation and control of plasmid DNA replication in human cells occurs with little apparent requirement for specific DNA sequence elements (14).

Suppression of overreplication of pSLneo plasmids in stable COS7 lines appears to act in *cis*. Supertransfection of pSLneo/COS7 cells with a SV40 plasmid encoding additional T-Ag resulted in unchecked replication of the newly transfected plasmid, while the copy number of the resident pSLneo episomes remained constant. Experiments were performed to assess the nature of the suppression mechanism. *cis*-acting control did not involve mutation of pSLneo DNA. Plasmid DNA that persisted in COS7 cells was largely identical in structure to wild-type pSLneo and displayed equivalent potential for high-level replication when rescued in *E. coli* and reintroduced into COS7 cells. Plasmid DNA purified directly from Hirt extracts of pSLneo/COS7 cells exhibited the capacity for runaway replication upon retransfection into COS7 cells. These data suggest that suppression does not involve simple covalent modification of plasmid DNA, such as methylation. These experiments do not rule out possible alterations imparted by the cell which are not retained during the isolation of plasmid DNA, such as compartmentalization of plasmid DNA or association with cellular proteins. Conceivably, replication may be repressed by an origin-binding cellular protein or by an epigenetic mechanism involving the stable association of chromatin proteins with pSLneo DNA.

The mechanism that dictates regulated replication appears to be heritable to daughter plasmid molecules. Our results suggest that once replication control has been established, it is maintained through future cell generations. Density transfer experiments indicated that very few cells in a culture escape negative control and overreplicate pSLneo. Furthermore, relatively low pSLneo copy numbers were maintained over many cell divisions and were unaffected by introduction of a T-Ag-expressing plasmid. Heritability appears to reside in pSLneo episomes and not the cellular environment, since pSLneo/COS7 cells that had been cured of pSLneo DNA supported runaway SV40 plasmid replication.

An apparent self-contradiction posed by these findings is

that one plasmid, pSLneo, replicated in two different modes: runaway (in transient assays) and controlled (in stable cell lines). One explanation for this might be the operation of an inefficient (or slow) process for the imposition of controlled replication upon pSLneo. In a transient replication assay, a fraction of transfected pSLneo DNA may, in fact, replicate in a controlled fashion. This phenomenon would be masked in a short-term assay by high-level replication of other plasmid molecules that have escaped replication control. In contrast, in a stable transfection experiment, cells that have managed to control pSLneo replication may be selected for because their growth is not impaired by overreplication of plasmid DNA. The presence of BPV genetic elements in *cis*, while not required, would then function to substantially enhance the efficiency (or rapidity) with which SV40 plasmid replication is brought under negative control. This is consistent with the observations that BPV-SV40 plasmids exhibit detectable reductions in replication in a transient assay and give rise to G418-resistant COS7 colonies with increased frequency (22).

There are no clear reasons at this time why pSLneo is able to establish stable G418-resistant cell lines, while others have reported that SV40-*neo*-based plasmids produce only abortive drug-resistant COS7 colonies (22). In the studies reported here, about five times more plasmid DNA (1 μg versus 200 ng) was employed in stable transfection assays than in other studies (22), which may also have contributed to the ability to isolate stable pSLneo/COS7 lines. Conceivably, differences in the plasmid constructions used may be important. However, the capacity to produce stable G418-resistant COS7 cells is not unique to pSLneo, since transfection of pSV2neo also yielded stable COS7 lines containing episomal plasmid DNA (data not shown).

SV40 DNA replication provides a particularly attractive model system for study of eukaryotic DNA replication in that T-Ag is the only viral protein required for this process and cell-free replication systems are available (17). A substantial limitation of SV40 as a model for chromosome replication is its failure to exhibit analogous regulation of DNA replication. These studies may have removed this limitation, and further study of negative control of SV40 plasmid replication in stable COS7 lines may well extend the utility of SV40 as a model to include regulation of replication origins. It is possible that the mechanisms that control pSLneo replication in COS7 cells are directly related to those that govern chromosomal DNA replication.

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