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Use of Simian Virus 40 Replication To Amplify Epstein-Barr Virus Shuttle Vectors in Human Cells

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We have increased the copy number of Epstein-Barr virus vectors that also carry the origin of replication of simian virus 40 (SV40) by providing a transient dose of SV40 T antigen. T antigen was supplied in *trans* by transfection of a nonreplicating plasmid which expresses T antigen into cells carrying Epstein-Barr virus-SV40 vectors. A significant increase in vector copy number occurred over the next few days. We also observed a high frequency of intramolecular recombination when the vector carried a repeat segment in direct orientation, but not when the repeat was in inverted orientation or absent. Furthermore, by following the mutation frequency for a marker on the vector after induction of SV40 replication, it was determined that SV40 replication generates a detectable increase in the deletion frequency but no measurable increase in the frequency of point mutations.

Shuttle vectors based on Epstein-Barr virus (EBV) replicate autonomously in the nuclei of human cells. These vectors maintain copy numbers of approximately 1 to 50 per cell (27, 35, 38, 39) and are useful for a variety of studies. In particular, EBV vectors have proven valuable in studies of mutation in human cells (12, 14). A bacterial target gene carried on an EBV vector in human cells has a very low background mutation frequency. The frequency of mutations can be increased by exposure of the cells to mutagens. Resulting mutations can be quickly scored and analyzed at the DNA sequence level by transfer of the vector DNA to *Escherichia coli*. The sensitivity of this shuttle system would be increased if the copy number of the vectors per human cell were higher. More vector molecules could be purified from a given number of human cells. Also, there would be an increased chance for inducing mutation in the target gene before the lethal dose for the cell was reached. In addition to their value for mutation studies, human cell lines carrying high copy numbers of EBV vectors may also be valuable for studies of gene expression, protein overproduction, and replication (23).

Our strategy for increasing the copy number of EBV vectors involves exploiting the vigorous replication properties of simian virus 40 (SV40). SV40 vectors require the SV40 origin of replication and SV40 T antigen to replicate in human cells (1). The vectors undergo multiple rounds of replication and reach high copy numbers (10, 31, 36). Chimeric vectors containing the EBV replicon and the SV40 origin of replication might be expected to replicate in the SV40 mode during a period when T antigen is present. Transient provision of T antigen could produce a copy number boost, after which the vectors may resume an EBV replication mode.

The EBV replicon is composed of a *cis*-acting origin of replication, *oriP*, and the *EBNA-1* gene, which encodes a *trans*-acting protein that allows the initiation of replication from *oriP* (27, 35, 38, 39). Vectors containing this replicon appear to replicate in synchrony with the host cell chromosomes (2, 20) and are stably maintained at low copy numbers in human cells as long as selection for the vector is maintained (38). In contrast, SV40-based vectors have been shown to replicate continuously in several human and monkey cell lines, and vector replication is generally incompatible with long-term survival of the host cell (36). SV40 vectors can reach copy numbers ranging from 10,000 to 100,000 copies per cell (10).

We have previously shown that an SV40 origin of replication present on bovine papillomavirus (BPV)-SV40 chimeric vectors can be used to elevate the copy number of BPV in mouse cells (13). In those experiments, mouse cell lines carrying BPV-SV40 vectors at a copy number of approximately 100 per cell were fused to SV40 T-antigen-producing COS cells (17). This fusion allowed the activation of the SV40 replicon and caused a 50- to 100-fold amplification of vector copy number in these cells. Later experiments, reported by Roberts and Weintraub, demonstrated that BPV sequences on a different BPV-SV40 chimera limited runaway SV40 replication (29, 30).

The experiments reported here demonstrate that an SV40 replicon can be used to increase the copy number of EBV vectors, potentially allowing the creation of cell lines which stably maintain EBV vectors at high copy numbers. We and others previously observed a high mutation frequency associated with transfection of SV40 shuttle vectors into mammalian cells (8, 9, 24, 26). These mutations could have been due to SV40 replication or to the transfection process alone. The experiments in this study have allowed us to monitor the fidelity of replication initiated from the SV40 origin in the absence of any transfection-associated mutation. Also, because of the presence of repeat sequences on our vectors, we have made observations concerning intramolecular recombination.

MATERIALS AND METHODS

Plasmids. pMCi1, pMCi3, and pMCi5 are derivatives of the plasmid p220.2 (14). p220.2 is an 8.9-kilobase (kb) plasmid that contains the EBV *oriP* and *EBNA-1* sequences, the pBR322 ampicillin resistance gene and origin of replication, and the hygromycin resistance gene under the control of promoter and termination sequences from the herpes simplex virus type 1 thymidine kinase gene. *EBNA-1* is expressed from a fortuitous promoter in pBR322 (14; B.

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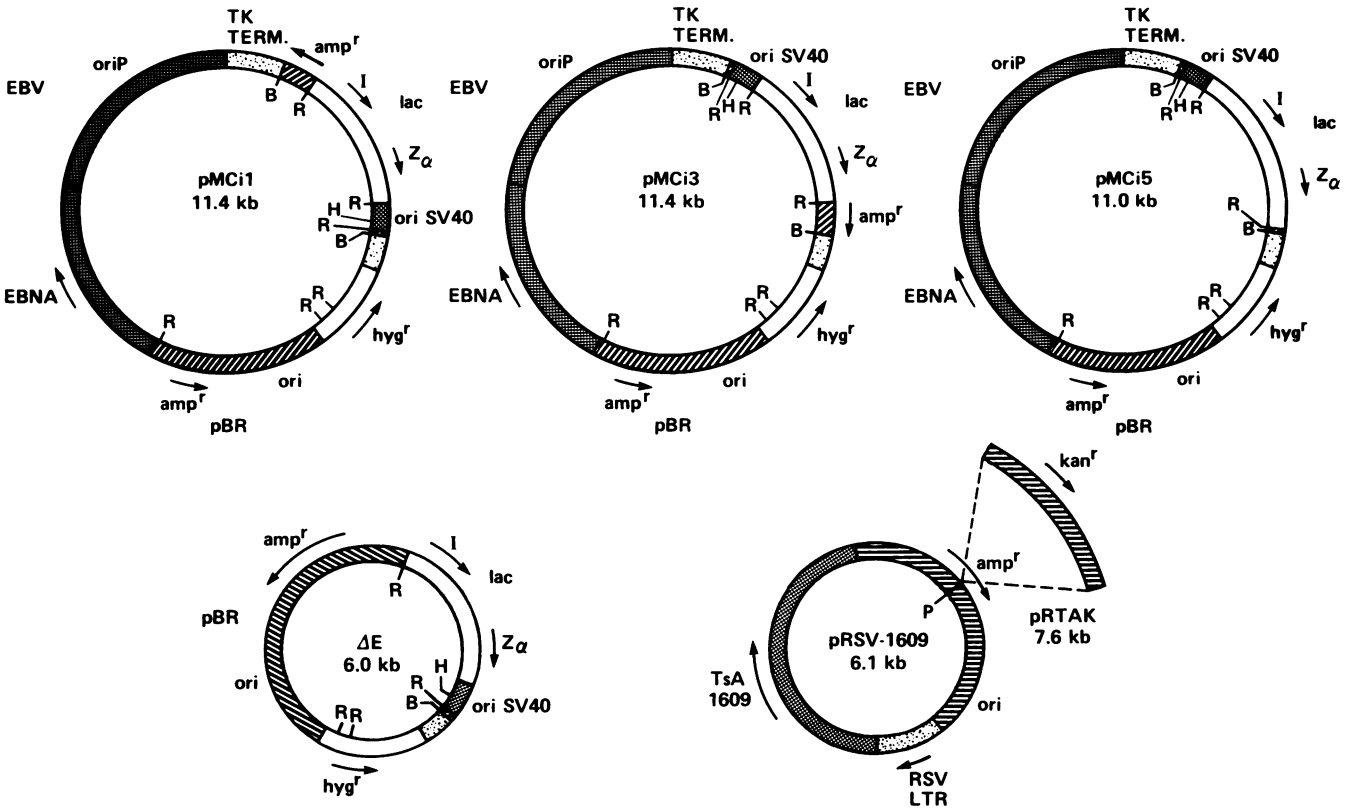

FIG. 1. Plasmids. Plasmid constructions are described in the text. Symbols: □, EBV or SV40 sequences; ▨, pBR322 or kanamycin resistance sequences; □, bacterial genes (*lacI*, hyg^r); □, herpes simplex virus thymidine kinase (TK) or Rous sarcoma virus (RSV) control regions. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; LTR, long terminal repeat.

Sugden, personal communication). p220.2 also contains the polylinker from pUC12 (*Sma*I-*Hae*III fragment) inserted into the *Nar*I site in the herpes simplex virus thymidine kinase termination sequences. pMCi5 was constructed by the insertion of a 2.0-kb fragment containing the sequences for *lacI*, *lacZ*α, and the SV40 origin of replication into the polylinker of p220.2. This 2.0-kb fragment was isolated from the plasmid pSVi2 (9) by partial digestion with *Eco*RI. To convert the 2.0-kb *Eco*RI fragment to *Bam*HI ends, it was ligated to the vector pUCR (a derivative of pUC19 containing two polylinkers in inverted orientation about their *Eco*RI sites, separated by a spacer fragment [D. J. Van Den Berg, R. B. DuBridge, and M. P. Calos, unpublished results]), which had been completely digested with *Eco*RI to produce an intermediate containing the 2.0-kb fragment, pUCR-*lacI*/SV40. pUCR-*lacI*/SV40 was subsequently digested with *Bam*HI to produce the desired 2.0-kb *lacI*/SV40 fragment with *Bam*HI ends. This fragment was then inserted into the unique *Bam*HI site in the polylinker of p220.2 to create pMCi5. The orientation of this *lacI*/SV40 fragment in pMCi5 is shown in Fig. 1.

pRTAK is a derivative of the plasmid pRSV-1609 (28). pRSV-1609 is a 6.05-kb plasmid which contains the gene for a temperature-sensitive SV40 T antigen under the transcriptional control of the long terminal repeat of the Rous sarcoma virus, as well as the pBR322 ampicillin resistance gene and origin of replication. pRTAK was created by the insertion of the aminoglycoside 3'-phosphotransferase gene, which confers resistance to kanamycin (Kanamycin Resistance GenBlock; Stratagene Corp., La Jolla, Calif.), into the ampicillin resistance gene of pRSV-1609. The gene was isolated on a 1.5-kb *Pst*I fragment and ligated to the *Pst*I partial digestion products of pRSV-1609 to produce pRTAK.

Tissue culture. The cell line 1.11 and a pMCi3 population are derivatives of 293 human embryonic kidney cells (18). These lines were described previously (14). The cell line 5.4 and the pMCi3 population used in this study were created from 293S (34), a suspension-adapted derivative of 293, by transfection with 5 μg of DNA per 100-mm-diameter dish by the calcium phosphate coprecipitation technique (37). Selection was applied 4 days after transfection by addition of medium containing 200 μg of hygromycin B (Calbiochem-Behring, La Jolla, Calif.) per ml. This level of selection was continuously maintained for all cell lines carrying EBV vectors. All of the cell lines described above were grown in Dulbecco modified Eagle medium with 10% fetal calf serum, penicillin, and streptomycin in a 10% CO_2 incubator.

Copy number amplification. pRTAK was introduced into the cell lines 1.11 and 5.4 and the pMCi3 population, by both calcium phosphate coprecipitation and electroporation. For calcium phosphate coprecipitation, cell lines were transfected with 2.5 μg of pRTAK DNA per 60-mm-diameter dish 1 day after being split 1:20 into medium without hygromycin. One day after transfection, growth medium was replaced with fresh nonselective medium. Four days after transfection with pRTAK, confluent 60-mm-diameter dishes were harvested and plasmid DNA was recovered by the method of Hirt (21). When electroporation was used to introduce pRTAK, cells were transfected in 1 ml of electroporation buffer with 20 μg of pRTAK DNA and 400 μg of carrier DNA (denatured salmon sperm DNA; Sigma Chemical Co., St. Louis, Mo.) per 7×10^6 cells (11). Electroporated cells

were plated into 60-mm-diameter dishes containing nonselective medium so that confluent dishes could be harvested 4 days after transfection. One-fourth of the total plasmid DNA recovered from a confluent 60-mm-diameter dish was digested with *Hin*dIII or with *Dpn*I and *Hin*dIII, electrophoresed on a 0.7% agarose gel, and transferred to GeneScreen Plus (New England Nuclear Corp., Boston, Mass.) by using 0.4 M NaOH as the transfer buffer. Filters were probed with plasmid DNA labeled with ^{32}P by random primer extension (16). Hybridization was performed at 65°C in a buffer consisting of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate, 5× Denhardt solution, 100 μg of denatured salmon sperm DNA per ml, and ~10^7 cpm of probe (100 ng; specific activity ≥ 1×10^8). Washing was done under high-stringency conditions according to the New England Nuclear protocol at 65°C. Hybridized filters were wrapped in Saran Wrap and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Mutation analysis. Vector DNA was transformed into the competent *E. coli* MC1061 F'150 Kan *recA*. Cells were plated, and LacI$^-$ colonies were detected as described previously (9, 14, 24). A Biotran II automatic colony counter (New Brunswick Scientific Co., Inc., Edison, N.J.) was used to count the colonies. Plasmid DNA was purified from each LacI$^-$ colony by the small-scale alkaline lysis procedure (7), digested with *Eco*RI, and examined on a 0.7% agarose gel to determine the mutational type.

RESULTS

Plasmids and cell lines. The construction of plasmids pMCi1 and pMCi3 has been described (14; Fig. 1). They are shuttle vectors containing two sequences from EBV, *oriP* and *EBNA-1*, that allow autonomous replication in human cells. In addition, these vectors contain the SV40 origin of replication and the *lacI* gene as a target for scoring mutation. To construct pMCi5, a fragment containing the sequences for *lacI*, *lacZ*α, and the SV40 origin of replication was isolated from a previously constructed SV40-based shuttle vector, pSVi2 (9). This fragment was inserted into the EBV-based shuttle vector p220.2 (14) to create pMCi5 (Fig. 1). The vectors pMCi1, pMCi3, and pMCi5 have similar compositions, but differences in the arrangement of the components lead to differences in mutation and recombination frequencies, as described below.

The source of SV40 T antigen was pRSV-1609 or pRTAK. pRSV-1609 contains all the sequences necessary for the expression of a temperature-sensitive SV40 T antigen in human cells (28). pRTAK was constructed by inserting a fragment conferring resistance to kanamycin into the ampicillin resistance gene of pRSV-1609. The elimination of ampicillin resistance in this vector abolishes the possibility of its recovery in bacteria when vector DNA is extracted from human cells, transformed into *E. coli*, and plated on medium containing ampicillin.

293, a human embryonic kidney cell line immortalized by the early region of adenovirus type 5 (18), was used to create cell lines carrying the EBV vectors extrachromosomally. A clonal cell line, 1.11, carrying the vector pMCi1, and a nonclonal population of cells containing pMCi3 have both been described (14). A cell line containing pMCi5 was created as follows. 293S cells were split 1:20 onto 100-mm-diameter dishes and transfected with pMCi5 the following day by calcium phosphate coprecipitation. Selection with hygromycin was applied 4 days after transfection, resulting in a population of cells containing pMCi5. To obtain a cell line free of transfection-induced mutation in *lacI*, subcloning of the population was undertaken. Several of the resulting clones were propagated in culture, and their background mutation frequencies were characterized by plasmid rescue and transformation into *E. coli*. Mutations in the *lacI* gene were scored as blue colonies on plates containing X-Gal (data not shown). Plasmids with no mutation in *lacI* appeared as white colonies. In this manner, most of the clones were found to be free of mutation, and one of these clones, 5.4, was selected for further study.

Another cell line derived from 293, *tsA*201, has been used previously (formerly called 293/*tsA1609*neo; 14). *tsA*201 carries the gene for SV40 T antigen integrated into its genome and produces T antigen constitutively. A simian cell line, COS-7, contains a replication-defective copy of SV40 and also produces T antigen constitutively (17). Consequently, any plasmid with an SV40 origin of replication will replicate in either of these cell lines. *tsA*201 and COS-7 were used for the transient replication assays described below.

Copy number amplification. All the cell lines carrying chimeric EBV-SV40 vectors maintained a stable vector copy number of approximately 10 to 50 per cell (14). Our strategy to raise this copy number by switching to an SV40 replicative mode involved bringing the SV40 T antigen into the cell in *trans*, via transfection with pRTAK. We will use the term boost to refer to this procedure. Accordingly, the cell lines 1.11 and 5.4 and the pMCi3 population were electroporated with 15 μg of pRTAK. Fractions from each electroporation were plated onto 60-mm-diameter plates and grown to confluence in 4 days. Plasmid DNA was isolated by the method of Hirt (21), and one-fourth of the total plasmid DNA recovered from each 60-mm-diameter dish was cut with the restriction enzyme *Hin*dIII, run on a 0.7% agarose gel, and Southern blotted. The results of this experiment are shown in Fig. 2. Longer time courses indicated that copy number of the vector reached a maximum by approximately 4 days after transfection with pRTAK.

Densitometry scannings indicated an average amplification in plasmid DNA for each cell line of approximately 10-fold. This result was confirmed by comparing the number of bacterial colonies recovered when equal portions of the recovered DNA from the boosted cell lines and unboosted controls were transformed into *E. coli*. For example, plasmid DNA extracted from one-third of a 60-mm-diameter dish of 5.4 cells yielded 2,344 bacterial colonies, whereas an equivalent amount of extract from boosted cells gave 32,897. Similar results were also produced when calcium phosphate coprecipitation was used as the method of transfection of pRTAK. It appears that the maximum copy achieved by boosting is similar (at least 1,000 copies per cell) regardless of initial copy number. For example, the degree of amplification was observed to be at least an order of magnitude higher when cell lines carrying lower initial copy numbers of EBV vectors were boosted in the same way (data not shown).

To determine whether the EBV sequences on our vectors inhibit SV40 replication, a transient replication assay was done. This assay measured the levels of replicated DNA for three plasmids, pMCi5, pBOP, and pSV2-neo, 3 days after introduction into COS-7 cells. Plasmid DNA was isolated and digested with the restriction enzyme *Dpn*I. This restriction enzyme cuts only DNA with an adenine methylation pattern characteristic of bacteria and removes any input DNA that failed to replicate in the COS-7 cells over this period. The positive control for SV40 replication was the vector pSV2-neo (33), which contains the SV40 origin and

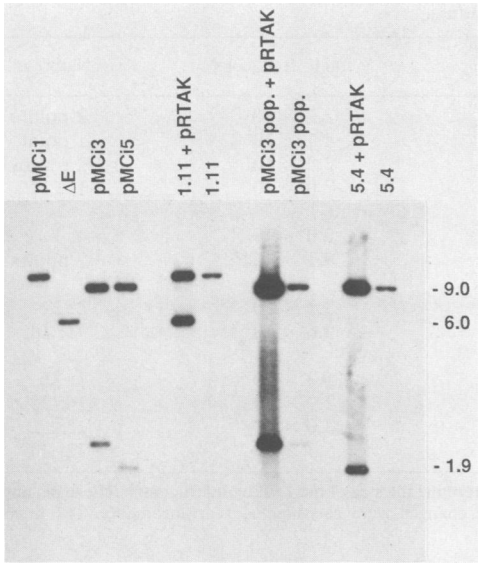

FIG. 2. Copy number amplification of EBV-SV40 vectors. pMCi1, ΔE, pMCi3, and pMCi5 marker lanes each contain 1 ng of vector DNA digested with *Hin*dIII. The lanes 1.11 + pRTAK, pMCi3 pop. + pRTAK, and 5.4 + pRTAK represent one-fourth of the total plasmid DNA recovered from a confluent 60-mm-diameter dish (6×10^6 cells), digested with *Hin*dIII, for each respective cell line 4 days after transfection with pRTAK. Lanes 1.11, pMCi3 pop., and 5.4 correspond to the plasmid DNA recovered from an equivalent number of cells that did not receive the pRTAK vector. The filter was probed with ^{32}P-labeled pMCi5 DNA. The 6.0-kb band in the 1.11 + pRTAK lane is not an expected product of digestion of pMCi1 with *Hin*dIII but rather a digestion product of ΔE, a derivative of pMCi1 formed by intramolecular recombination.

the gene encoding neomycin resistance but no EBV or BPV sequences. The plasmid pBOP contains the entire BPV genome as well as the SV40 origin (13), while pMCi5 contains EBV sequences and the SV40 origin. The results of this experiment are shown in Fig. 3. All three plasmids replicated to similar levels, indicating that the *oriP* and *EBNA-1* sequences do not inhibit the SV40 replication of pMCi5 and that SV40 replication is dominant over EBV replication. Likewise, inhibition of SV40 replication was not found for BPV sequences in the context of the pBOP vector. Identical results were obtained when the human cell line *tsA*201 was used as the SV40 replication-permissive cell line rather than COS-7 (data not shown).

Recombination. We noted that boosts of the cell line 1.11 with pRTAK yielded an unexpected 6-kb band on Southern blots (Fig. 2). Moreover, when plasmid DNA recovered from such a boost was transformed into *E. coli*, >90% of the colonies screened were found to contain a 6.1-kb plasmid, rather than the original 11-kb pMCi1 vector. Restriction mapping of this smaller plasmid showed it to be a derivative of pMCi1, deleted for all EBV sequences. This derivative form of pMCi1 was referred to as ΔE. It was postulated from the map of pMCi1 that the formation of ΔE was due to intramolecular recombination between a region in the full-length ampicillin gene present on the vector and a 300-base-pair (bp) direct repeat of the 5′ end of this gene. This repeat was a by-product of the cloning strategy and is located adjacent to the sequences for *lacI* (Fig. 1).

The related vector, pMCi3, also contains this 300-bp repeat, but in an inverted orientation. Another plasmid, pMCi5, was constructed to contain no repeated sequences.

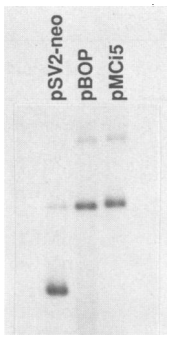

FIG. 3. Transient replication in COS-7. COS-7 cells were transfected via calcium phosphate coprecipitation with 5 μg of either pSV2-neo (5.6 kb), a control plasmid containing the SV40 origin of replication and neomycin resistance gene but no EBV or BPV sequences; pBOP (10.9 kb), a plasmid containing the entire 8-kb BPV genome as well as the SV40 origin of replication; or pMCi5 (11.0), the EBV-SV40 plasmid described in the text. Each lane contains one-fourth of the uncut replicated DNA (forms I and II) isolated from a 60-mm-diameter dish 72 h after transfection. Each sample was digested with the restriction enzyme *Dpn*I to remove unreplicated input DNA (see text), run on a 0.7% agarose gel, and blotted, and the filter was probed with ^{32}P-labeled pBR322 DNA. All three vectors contain equivalent amounts of pBR322 sequences, so all three have equivalent homology to this probe.

Boosting of cell lines carrying these three related vectors was done to determine the effects of various arrangements of the repeat sequence on recombination efficiency. Upon the induction of SV40 replication (by the introduction of T antigen via transfection with pRTAK), neither pMCi3 nor pMCi5 was found to produce prominent derivative plasmids of any type. Southern blots of boosts of these two vectors showed only the expected bands upon digestion with *Hin*dIII (Fig. 2), though minor amounts of heterogeneous species cannot be ruled out. Deletion or inversion of the segment between the inverted repeats of pMCi3 would produce *Hin*dIII fragments of 7.3 kb or 5.0 and 6.0 kb, respectively. These fragments were not detected. We conclude that there is little or no recombination of EBV vectors lacking directly repeated sequences.

To rule out any effects of the pRTAK plasmid itself on the recombination observed with pMCi1, transient SV40 replication experiments were carried out in *tsA*201. pMCi1, pMCi3, ΔE, and pMCi5 DNA (2.5 μg each) were transfected via calcium phosphate coprecipitation into *tsA*201 cells. The plasmid pHyg (35), which lacks an SV40 origin of replication and should not replicate, was also added as a negative control. These plasmids were allowed to replicate for 4 days in the SV40-permissive environment of *tsA*201. Plasmid DNA was then collected and digested with *Dpn*I. Subsequent digestion with *Hin*dIII and blotting gave results identical to those obtained from boosting in *trans* with pRTAK; pMCi1 recombined to form ΔE, and both pMCi3 and pMCi5 gave only the bands expected from digestion with *Hin*dIII. These findings confirm that the observed instability of pMCi1 during the SV40 replication of a boost is due to the presence of two 300-bp directly repeated sequences rather than any inherent incompatability of these SV40 and EBV sequences to reside on the same vector.

A time course of a 1.11 boost was performed to examine vector copy number and the effect of recombination over a longer time. 1.11 was electroporated with pRSV-1609, and fractions of this electroporation were plated onto 60-mm-diameter dishes so that confluent dishes could be harvested

TABLE 1. *lacI* mutation frequencies

Source	Plasmid	No. of colonies screened	No. of LacI mutants	LacI$^-$ frequency	Nature of mutations[a]
Bacterial cells	pMCi1	95,257	2	2.1×10^{-5}	2 points
	ΔE	241,183	1	4.1×10^{-6}	1 point
	pMCi3	91,356	2	2.2×10^{-5}	2 rearrangements
	pMCi5	288,799	20	7.0×10^{-5}	19; 1[b]
Human cells	pMCi1	249,455	5	2.0×10^{-5}	4; 1
	pMCi5	76,556	7	9.1×10^{-5}	7 points
SV40 boost	pMCi1	365,200	116	3.2×10^{-4}	2; 22
	pMCi5	1,225,094	118	9.6×10^{-5}	71; 18
Transient SV40 replication	pMCi1	18,652	236	1.3×10^{-2}	1; 35
	ΔE	25,265	74	2.9×10^{-3}	16; 20
	pMCi3	19,313	39	2.0×10^{-3}	15; 11
	pMCi5	234,522	124	5.3×10^{-4}	22; 20

[a] A number of the *lacI* mutant plasmids were examined by *Eco*RI restriction digestion to determine the size of the 1.7-kb *lacI* fragment. Those plasmids showing no change in size were classified as harboring putative point mutations. Those with size changes were classified as rearrangements. The majority of the rearrangements were simple deletions.

[b] Number of points; number of rearrangements.

at 2, 3, 4, and 5 days. These samples were digested with *Hin*dIII and examined by Southern blotting as before. The results of this experiment clearly demonstrate the emergency of ΔE by 2 days and its accumulation over time (Fig. 4). Once a ΔE molecule has been formed, it would be expected to have a replicative advantage over pMCi1 because of its smaller size (9, 32).

To directly quantitate the replicative advantage of ΔE, it was compared to pMCi5 during transient replication in *tsA*201. pMCi5 was used because it is approximately the same size as pMCi1 but does not undergo intramolecular recombination. Plasmid DNA from ΔE carrying a point mutation in *lacI* was isolated. Transformation of this DNA into *E. coli* gives only blue colonies on plates containing X-Gal. Wild-type pMCi5, which gives only white colonies upon transformation, was used. pMCi5 and ΔE DNA were mixed so that samples of this mixture transformed into *E. coli* gave a 1:1 ratio of white and blue colonies. This mixture of ΔE and pMCi5 was transfected into *tsA*201 via calcium phosphate coprecipitation and allowed to replicate for 4 days. Plasmid DNA was recovered, digested with *Dpn*I, and transformed into *E. coli*. The ratio of blue colonies (ΔE) to white colonies (pMCi5) was found to be 90% ΔE to 10% pMCi5 (4,924 blue colonies of 5,509 total colonies screened). Therefore, the smaller plasmid increased its proportion from 50 to 90% during the 4-day replication period.

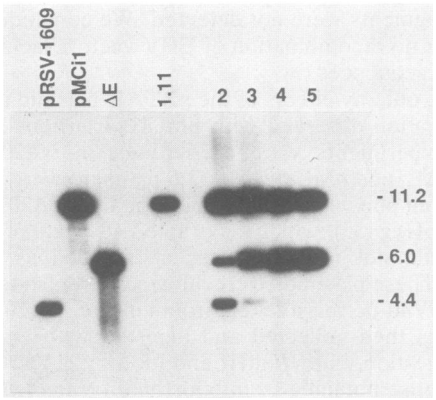

FIG. 4. Time course of copy number amplification in 1.11. The marker lanes pRSV-1609, pMCi1, and ΔE each contain 2 ng of plasmid DNA digested with *Hin*dIII. Lanes 2, 3, 4, and 5 correspond to one-fourth of the total plasmid DNA recovered from confluent 60-mm-diameter dishes of 1.11 cells harvested at 2, 3, 4, and 5 days posttransfection with pRSV-1609 and digested with *Hin*dIII. The filter was probed with ^{32}P-labeled pMCi1 DNA. Lane 1.11 represents one-fourth of the amount of pMCi1 (11.2 kb) recovered from a confluent 60-mm-diameter dish of 1.11 cells that did not receive pRSV-1609. pRSV-1609 (4.4 kb) does not replicate in human cells and is lost by day 5. However, ΔE (6.0 kb), the smaller derivative of pMCi1, appears at day 2 and increases over the course of the experiment.

Mutation frequency. One of our primary goals in amplifying the copy number of EBV vectors was to increase the efficiency of the shuttle vector system for the study of mutation in human cells. Therefore, we wanted to investigate whether there was any mutational effect associated with copy number amplification via SV40 replication that would contribute to the background mutation observed in our shuttle vector system. The boost protocol provides a direct way to measure the level of mutation associated with SV40 replication, free from any effects of transfection. For comparison, we first characterized the mutation frequencies and types of mutations obtained with our vectors when replicated using the EBV origin in human cells, the plasmid origin in bacterial cells, and the SV40 origin when transfected into *tsA*201 and replicated transiently.

To determine the background mutation frequency for *lacI* in bacteria, pMCi1, pMCi3, and pMCi5 were transformed into *E. coli* and blue colonies were scored. pMCi1 and pMCi3 had mutation frequencies of approximately 2×10^{-5} (14; Table 1), while that of pMCi5 was 7×10^{-5} (Table 1). Most of the mutants had a *lacI* restriction fragment of wild-type size and were putative point mutations. The reason for the higher mutation frequency for pMCi5 is not clear. It seems to be a property of the plasmid, since several independent DNA preparations gave the same value. The background mutation frequency for the plasmids in *E. coli* should define the lower limit of background mutation attainable for plasmid rescued into bacteria from a human cell line carrying that plasmid.

The background mutation frequencies for pMCi1 and pMCi5 when carried in human cells as autonomous EBV

replicons were determined (Table 1). The background mutation frequency for 1.11, a clonal 293 derivative carrying pMCi1, has been reported as 6.4×10^{-6} (14). The mutation frequency of a pMCi1-carrying clonal derivative of a xeroderma pigmentosum complementation group D lymphoblastoid cell line was approximately 2×10^{-5} (L. H. Lam and M. P. Calos, unpublished results). Neither of these figures is statistically different from the background mutation frequency of pMCi1 in *E. coli*, which is 2.1×10^{-5}. The background mutation frequency for 5.4, the cell line carrying the pMCi5 vector, was obtained by harvesting confluent 60-mm-diameter dishes of unboosted cells, purifying plasmid DNA by Hirt extraction, transforming the DNA into *E. coli*, and scoring blue colonies. Plasmid DNA from each blue colony was isolated by the mini-prep procedure, digested with *Eco*RI, and run on a 0.7% agarose gel to determine the type of mutation. In this way, the background frequency of mutation for 5.4 was found to be 9.1×10^{-5} and most of the mutations were found to be of the point mutation class. This value is similar to the bacterial background mutation frequency for pMCi5. Therefore, passage of the plasmids in human cells as EBV replicons does not significantly increase their mutation frequency.

To ascertain whether boosting induced an elevated mutation frequency, plasmid DNA from boosts of 1.11 was transformed into *E. coli*. A mutation frequency of 3.2×10^{-4} was observed, indicating a 16-fold mutational effect associated with boosting via SV40 replication. Essentially all the increase in mutation frequency is accounted for by deletions (Table 1). Since we have demonstrated that these smaller molecules have a replicative advantage, they will tend to become overrepresented. Thus, the 16-fold increase in mutation frequency is a maximum value. This result is further complicated by the fact that boosting of the pMCi1 vector leads to the formation of ΔE. Consequently, the great majority of mutations recovered from a boost of 1.11 are deleted for EBV sequences in addition to containing deletions into *lacI*. It is not clear whether the observed increase in mutation frequency is due to the recombination associated with the formation of the ΔE molecule, the boost process, or both. It could also represent the normal levels of deletion-formation associated with any DNA molecule, with the deleted molecules receiving a selective advantage during SV40 replication (see Discussion).

On the other hand, boosts of 5.4 (pMCi5) were found to give a mutation frequency of 9.6×10^{-5}, a number similar to the pMCi5 background frequencies of mutation in both human cells and bacteria. Most of the mutations recovered from a boost of this vector were found to be point mutations. Thus, copy number amplification of an EBV-SV40 chimeric vector by temporarily inducing SV40 replication does not produce a significant increase in the frequency of point mutations.

The mutation frequencies were determined for the same plasmids after transfection and replication for several days in *tsA*201. Comparison of these frequencies with those associated with boosting would give a direct measure of the contribution of the transfection process to the mutation frequencies observed in the transient replication assay. To determine the amount and types of mutation incurred by transient SV40 replication, pMCi1, pMCi3, pMCi5, and ΔE were introduced into *tsA*201 by calcium phosphate coprecipitation, and plasmid DNA was recovered after 4 days. This DNA was then transformed into *E. coli*, blue colonies resulting from mutation in *lacI* were counted, and mutations were analyzed as described above. The results of these experiments, summarized in Table 1, demonstrate the high (≈1%) level of mutation induced by transfection, as has been previously reported (8, 9, 24, 26).

As expected, ΔE, pMCi3, and pMCi5 were found to have much lower mutation frequencies than pMCi1 in these transient replication experiments. Plasmids pMCi1, ΔE, pMCi3, and pMCi5 all carry *lacI* and the SV40 origin. However, in pMCi3 and pMCi5, the SV40 origin of replication is located directly adjacent to the target gene for mutation, *lacI*, and in the case of ΔE, the ampicillin gene flanks *lacI*. Therefore, in these vectors, the *lacI* gene is flanked on both sides by selected sequences, i.e., by the SV40 origin or the ampicillin gene on its 5′ end and by the α fragment of *lacZ* (required for the formation of a blue colony) on its 3′ end. The consequence of this flanking is that plasmids carrying deletions extending out of *lacI* cannot be recovered as blue colonies. Only point mutations and small deletions within the *lacI* gene will be detected as mutants. In pMCi1, on the other hand, the SV40 origin is located adjacent to the *lacZ*α sequences, leaving *lacI* unflanked on its 5′ end and thus sensitive to these deletion events (Fig. 1). As predicted, the types of mutations recovered from transient replication in *tsA*201 were different for pMCi1 compared with pMCi3 and pMCi5. The mutations found for pMCi1 were predominately deletions, whereas in pMCi3 and pMCi5 point mutations predominated (Table 1). The higher mutation frequencies associated with transient replication, compared with those associated with boosts, indicated that most of the mutations were associated with transfection rather than SV40 replication per se.

DISCUSSION

We have shown that the copy number of EBV vectors carrying the SV40 origin of replication can be raised by providing transient exposure to SV40 T antigen. The mechanism by which EBV copy number is normally set is unknown. Both EBV and vectors derived from it have low copy numbers (22). EBV has been shown to replicate early in S phase (20) and appears to replicate only once per cell cycle (2). Our EBV shuttle vectors contain *oriP* and *EBNA-1* as their only viral sequences, representing approximately 2% of the viral genome (5). Vector copy number may be influenced by the amount of DNA entering the nucleus at transfection, the levels of EBNA-1, the magnitude of selection imposed, and/or some limiting cellular factor. EBNA-1 levels are not likely to be regulated in a meaningful way on our vectors, since the native EBNA promoter is not present and EBNA is apparently expressed from a fortuitous promoter in pBR322.

After introduction of SV40 T antigen by transfection of a nonreplicating T-antigen expression vector, the EBV-SV40 vector undergoes a copy number boost of at least an order of magnitude at the population level. At the level of the individual cell the copy number increase would be higher, since only a fraction of the cells actually receive T antigen. We have also observed that cell lines initially carrying very low EBV vector copy numbers boost to the same final magnitude as higher copy number lines, thus undergoing a greater relative boost in the process. The upper limit may be set by the amount of T antigen present. The subset of cells expressing the highest level of T antigen appear to have the highest copy numbers (S. B. Haase, S. S. Heinzel, and M. P. Calos, unpublished observations).

The EBV sequences on the vector do not appear to inhibit SV40 replication, as shown by the vigorous replication of

EBV-SV40 plasmids in permissive cells expressing T antigen. In contrast, Roberts and Weintraub (29, 30) reported that in the case of a BPV-SV40 chimera, BPV sequences impose a negative control on SV40 replication. Two comments are warranted. First, not all BPV-SV40 chimeras demonstrate inhibited replication. Our previous work (13) showed that a BPV-SV40 chimera, pBOP, resident in a mouse cell, underwent extensive amplification upon fusion to COS-7 cells. This observation suggested little or no inhibition of SV40 replication by BPV, a finding confirmed by transient replication of pBOP in COS-7 cells (Fig. 3). Therefore, although pBOP contains all of BPV, it does not show inhibitory effects on SV40 replication. It appears that the Roberts and Weintraub results are not generalizable for all BPV-SV40 vectors. The reason for the discrepancy may be that in their vector the BPV E1 gene is driven by the SV40 promoter, while in our vector it is driven by its native promoter. It may be necessary to overproduce BPV E1 in order to get an appreciable inhibition of SV40 replication.

Second, the question must be raised of whether the EBV sequences on our vector should be expected to have inhibitory effects on SV40 replication. For BPV it is known that the E1 gene product has both negative and positive effects on BPV replication (6). For the EBNA-1 protein of EBV, only positive effects on replication have been demonstrated (27). Indeed, we observe no inhibition of SV40 replication by the EBV sequences on our vectors.

An unexpected observation made in the course of this work was high-frequency recombination at 300-bp direct repeat sequences separated by several kilobases in pMCi1 during SV40 replication. This recombination was manifested by the appearance of a deleted form of the plasmid retaining the SV40 origin of replication. This deleted form of the vector became the predominant plasmid in the population within a few days. Because of the direct repeat at the borders of the deletion, we interpret it as a product of intramolecular recombination. The frequency of recombination is difficult to estimate because the deleted form has a replication advantage over the parental vector. This advantage increases the proportion of ΔE geometrically with the number of rounds of replication. Still, the initial frequency of recombination must be appreciable to account for the numbers of recombinant molecules observed. We do not know whether this type of intramolecular recombination also occurs during the replication of EBV, which contains many direct repeats. Our failure to observe ΔE in cell lines such as 1.11, which carry pMCi1, could reflect a low recombination efficiency under EBV replication and/or selective disadvantage of the individual recombination products in this situation.

In contrast to the high degree of recombination seen in pMCi1, no detectable recombination was observed for pMCi3. This plasmid contains the identical 300-bp repeats separated by a similar distance. However, they are in an inverted rather than direct orientation. No prominent deletion or inversion events mediated by the repeats or any other sequences on the plasmid were detected. We conclude that recombination in a circular molecule is much more frequent at direct rather than inverted repeats. This observation is in keeping with the prevalence of deletions at direct repeats in bacteria and other organisms (3), which may also be mediated by recombination. For example, recombination at tandem direct repeats has been observed in yeast (15), as has recombination between nearby *Alu* sequences in direct repeat orientation in human cells (25). The absence of rearrangement in pMCi5, which lacks repeat sequences, lends further support to the argument that the rearrangement in pMCi1 is due to a specific sequence arrangement and not to any inherent interference between EBV and SV40 replicons.

This experimental protocol has allowed us to determine the mutation frequency of a marker gene on an SV40 replicon independent of transfection. When SV40 vectors are transfected into human or monkey cells, a high mutation frequency in marker genes located on the vectors is observed, as reported here and previously (8, 9, 24, 26). This frequency can be as high as 1% or more on vectors in which large deletions can be tolerated. These mutations could be due to SV40 replication and/or to damage incurred during transfection. Arguments based on the kinetics of mutation formation and the appearance of mutations in nonreplicating DNA led us to conclude that most of the mutations were due to transfection (24). This conclusion is borne out by the data in the present study.

For pMCi5 the mutation frequency does not change after SV40 replication is induced. It remains at the same low level we observed when the vector replicated as EBV in human cells or as a plasmid in *E. coli*. For pMCi1 there is also no increase in the frequency of point mutations during SV40 replication. Because of the arrangement of sequences flanking *lacI*, large deletions cannot be scored in pMCi5, whereas they are tolerated in pMCi1. Indeed, a 16-fold increase in deletions was detected for pMCi1 during the replication period. However, large deletions will rapidly become overrepresented in the SV40 replication pool because of their selective advantage. As demonstrated above, a smaller molecule will increase its proportion in the population over time, presumably because it replicates more quickly and reenters the replication pool sooner. No such replication advantage for deletions can exist for replicons which initiate only once per cell cycle, such as EBV or chromosomal replicons. Therefore, the increase in deletions seen during SV40 replication may be due to a higher inherent frequency of deletion formation by the SV40 replicon, amplified by the selective advantage a deleted molecule will hold in a multiple-round replication situation. At this stage, we cannot rule out a role for the pMCi1 recombination in generating deletions producing *lacI* mutant molecules. This idea is being tested with appropriate constructions.

Further evidence suggesting an elevated frequency of deletions during replication of SV40 vectors was reported by Ashman and Davidson (4). They found a mutation frequency of approximately 0.7% for the *gpt* gene of pSV2-gpt integrated into the genome of Chinese hamster cells, upon excision after fusion with COS cells. All of the mutations were found to be deletions. Thus, these authors also observed deletion formation, though the frequency was 20 times higher than that of pMCi1 after an SV40 boost (0.03%). The greater mutation frequency in their experiment may be related to some aspect of the excision process or of the specific plasmid used. Also, we have previously demonstrated that rodent cells have a greater propensity to form deletions in vector DNA than human cells, and this species difference may play a role in the quantitative difference between our results and theirs. The generation of deleted derivatives upon serial passage of SV40 is also consistent with the generation of deletions during SV40 replication (19).

A comparison of the mutation frequencies from transient replication with those incurred during a boost makes it clear that most of the mutations observed in transient replication were associated with the transfection process alone. For example, the transient replication mutation frequency for pMCi1 is 50 times higher than that observed during an SV40 boost. Therefore, at least 98% of the mutations seen in the

transient replication must have been associated with transfection.

Induction of SV40 replication is an effective way to increase EBV vector copy number. It is also relatively mutation-free, particularly if the target gene is flanked by selected or screened sequences. However, we have found that the elevated copy number after the introduction of T antigen is gradually lost during the weeks after the boost. We would like to stabilize the high EBV copy number in the absence of any further SV40 replication, since SV40 replication is difficult to control and introduces an intense selective advantage for molecules with deletions or other alterations that give them a replication advantage (19). The loss of vectors over time may be due to one or more of a number of factors, including selective disadvantage of cells carrying a high vector copy number, insufficient levels of *EBNA-1* to maintain the elevated vector number, and the lack of selection for a high-copy-number plasmid. Current work in the laboratory is investigating these possibilities. The effort to create stable, high-copy-number cell lines should produce useful vector systems. The manner in which this goal is achieved should also yield new information about replication in mammalian cells.

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