Amplification of a Bovine Papillomavirus-Simian Virus 40 Chimera

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A chimeric plasmid, pBOP, containing bovine papillomavirus (BPV) and the origin of replication from simian virus 40 (SV40) was constructed. The plasmid was established in mouse cells, where it was maintained stably as an autonomous BPV replicon. Lines carrying pBOP were fused to cells of COS-7, a simian line producing SV40 T antigen. Replication dependent on the SV40 origin and having the kinetics and approximate amplitude of an SV40 infection ensued. SV40 replication is therefore dominant over BPV replication, and the SV40 origin can conveniently be used to amplify lower-copy-number plasmids in mammalian cells.

Bovine papillomavirus (BPV) and simian virus 40 (SV40) are both animal viruses whose genomes consist of a small double-stranded circle of DNA. However, the life cycle and mode of replication of the two viruses differ radically. The BPV genome replicates autonomously in infected nuclei (6). This replication requires gene products and *cis*-acting sequences encoded in the early region of the BPV genome (9, 10, 17). Only in terminally differentiated epidermal cells are virions produced (13, 18). It is only in this situation that the BPV genome is amplified to high levels. By contrast, in tissue culture cells BPV replicates as a plasmid to the extent of 50 to 100 copies per cell (6), its replication appearing to closely resemble that of the host chromosomes. The copy number of BPV is stably maintained without selection, implying that a mechanism exists to correctly apportion plasmid copies to the daughter cells during mitosis (9, 10).

SV40 replication represents a contrasting situation (1, 3). After infection of permissive cells, the SV40 early region is transcribed, allowing SV40 T-antigen production. This protein induces a set of host genes involved in DNA replication. It also catalyzes the initiation of DNA synthesis from the SV40 origin of replication. Bidirectional replication from the SV40 origin can reinitiate repeatedly on the same molecule during the DNA synthesis period (16), generating approximately 100,000 genome copies per cell. Virus production and cell lysis then ensue. In contrast with stable BPV replication, lytic-phase SV40 replication is not coordinated with that of the host chromosomes.

Both viral genomes replicate in tissue culture cells, and it was of interest to study the behavior of artificially constructed chimeras containing the origins of replication of both viruses. By placing the molecules in a cellular environment allowing activation of both origins, we could study the interaction of the two replication modes.

We constructed a plasmid, pBOP, which contains all of the BPV genome, 300 base pairs of SV40 including the origin of replication, and a derivative of the bacterial plasmid pBR322 (Fig. 1). The construct can replicate in *Escherichia coli* cells by virtue of its pBR322 sequences. It can replicate in simian cells if T antigen is provided to activate replication from the SV40 origin. For example, the plasmid replicates in COS-7 cells, but not in CV-1 cells (data not shown). In mouse cells, pBOP would be expected to replicate by using the origin and early gene product(s) of BPV. BPV also encodes transforming functions (12, 17). Therefore, focus

formation can be used to screen for cells harboring BPV. Accordingly, we transfected the contact-inhibited mouse cell line C127 with pBOP by the calcium phosphate coprecipitation technique and picked transformed foci 2 to 3 weeks later. Nine lines were characterized for the presence of pBOP DNA. Small-circular DNA was purified from the cells by Hirt extraction (5). A portion of the extracted DNA from each line was transformed into *E. coli*, and ampicillin-resistant bacterial colonies resulted in each case. For one of the lines, BOP-6, the DNA from 110 of the colonies was examined after *Eco*RI digestion, and no rearrangements were detected. Southern analysis confirmed the presence of supercoiled and nicked pBOP-sized plasmid DNA in BOP-6 cells (see below). Therefore, pBOP replicates in the C127 lines as an autonomous plasmid, consistent with its replication being under the control of BPV.

SV40 replication requires the SV40 T antigen and a permissive mammalian cell environment. To determine whether the pBOP plasmids established as BPV replicons would switch to an SV40 replication mode, we provided T antigen and the permissive simian environment to two of the lines, BOP-6 and BOP-7, by fusion to COS-7 cells. COS-7 is a simian cell line containing an integrated, replication-defective copy of SV40 which produces T antigen constitutively (4). Plasmids in the hybrid C127/COS-7 cells could theoretically replicate either by the BPV or the SV40 mode. Approximately 10⁶ BOP-6 or BOP-7 cells were plated with a twofold excess of COS-7 cells on 60-mm-diameter dishes. Twenty-four hours later the cells were fused by a 45-s exposure to 50% polyethylene glycol (2, 14). Forty-eight hours after fusion the cells were harvested, and Hirt extracts were prepared. The extracted DNA was transformed into *E. coli*, and the resulting colonies were counted. An approximately 20-fold increase in colony number was detected by using DNA extracted from the fused BOP cells, compared with that detected when using unfused controls. The positive result in this preliminary experiment (data not shown) implied that the plasmids had switched from a BPV to an SV40 mode of replication and led us to undertake a more thorough analysis of the apparent amplification.

The BOP-6 line was fused to COS-7 cells and harvested at 12-h intervals over a time course of 96 h. To test for a requirement for T antigen in the replication, BOP-6 cells were also fused to CV-1 cells. CV-1 is the untransformed parent line of COS-7; it therefore does not contain an SV40 insertion and consequently does not produce T antigen. Requirement for the SV40 origin was examined by fusing

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FIG. 1. Structure of pBOP. The SV40 origin is contained on a 311-base-pair *EcoRI* fragment which was inserted with *EcoRI* linkers into the *EcoRI* site of pML, a deletion derivative of pBR322, producing pML-RIIG (8). The unique *Bam* HI site in the 8-kilobase BPV genome was ligated to a *Bam* HI-*Hind*III digest of pML-RIIG by using *Bam*HI linkers. Symbols: B, *Bam*HI; R, *Eco*RI; kb, kilobase.

COS-7 cells to BP/C127, a C127 line transformed with the plasmid pBP. This plasmid is identical to pBOP, except that pBP lacks the *EcoRI* fragment encoding the SV40 origin. As a positive control for SV40 replication, a C127 line containing integrated copies of a BPV-SV40 derivative was fused to COS-7 cells. The plasmid i2113-1 (10), which contains the SV40 origin inserted into the E1 gene of BPV, was used to construct this line. Since E1 function is required for autonomous BPV replication, the E1⁻ i2113-1 integrates into the host chromosomes (10). To control for effects related to fusion, BOP-6 was also fused to itself.

The degree of replication of pBOP and pBP after fusion to COS-7 cells can be assayed by the number of colonies in *E. coli* that resulted from transformation with DNA extracted at each time point. These data are represented graphically in Fig. 2. For the fusion of BOP-6 to COS-7, the colony number reproducibly rose steadily after fusion, reaching a peak representing amplification of 10- to 100-fold at 72 h. The number of colonies recoverable after 72 h decayed slightly, presumably owing to cell death. These kinetics resemble those observed after transfection of SV40 vectors into simian cells (8) or rescue of SV40 from an integrated position in transformed cells (2). When BOP-6 cells were fused to CV-1 cells instead of to COS-7 cells, no amplification was observed. Only a modest rise in colony number occurred, doubtless owing to proliferation of the BOP-6 cells over the 3-day time course. Furthermore, BP/C127, carrying the pBP plasmid lacking the SV40 origin, did not amplify upon fusion to COS-7.

To corroborate these results, a direct analysis of plasmid DNA during the replication was done. Figure 3 shows a Southern blot of the plasmid DNA present over a time course encompassing the 3 days after fusion. Panel A shows the pBP-containing line BP/C127 fused to COS-7 cells. No amplification was observed. In panel B the BOP-6 line was fused to itself. Again, no amplification was observed, ruling out the fusion procedure as the source of the increased replication. Strong amplification was detected in the fusion of BOP-6 to COS-7 cells (panel C). However, fusion of BOP-6 to CV-1 cells did not produce amplification (panel D). This result, together with the one depicted in panel A, demonstrates the requirement for the SV40 origin of replication and T antigen in obtaining amplification. Although we

have not demonstrated that replication initiates at the SV40 origin, the requirement for the SV40 origin and T antigen, as well as the kinetics of the reaction, argues strongly that after fusion the plasmids are switching from replication governed by BPV to replication driven by the SV40 origin. A fusion between the line containing integrated copies of i2113-1 and COS-7 cells was also performed. Amplification of material hybridizing to pBOP was detected (data not shown), presumably representing *in situ* replication and excision of i2113-1 (2).

A theoretical amplification of 100- to 1,000-fold would be expected in this experiment if the 100 copies per cell typical of BPV increased to the 10^4 to 10^5 copies expected for SV40. We obtained amplification values in the range of 100-fold. These values would perhaps be higher but for the inefficiency and toxicity of the cell fusion process. It is also possible that not all BPV molecules in the cell are accessible to switch to an SV40 mode of replication when T antigen and a permissive environment are provided or that the BPV sequences provide some impediment to SV40 replication. However, it is clear that in both the mouse and the monkey environment, replication of the molecules by the SV40 mode predominates.

It is likely that the foregoing amplification scheme will be applicable to other low-copy-number mammalian plasmids. This system provides a methodology whereby a stably maintained plasmid such as BPV can be amplified at a moment chosen by the investigator. Furthermore, amplification methods that avoid cell fusion can be entertained. For example, a recently developed simian line which produces a temperature-sensitive SV40 T antigen (15) could be used to amplify chimeric plasmids with a simian host range; the plasmid copy number could theoretically be modulated by temperature shifts. The ability to amplify mammalian plas-

FIG. 2. Amplification kinetics. A fraction (1/12) of the DNA extracted from one 60-mm-diameter dish at each time point was transformed into *E. coli* MC1061 F'150kan *recA* (11), and ampicillin-resistant colonies were counted. The data from two independent experiments are shown (experiment 1, solid lines; experiment 2, dashed lines). The colony numbers are normalized to the maximum value, which was the 72-h point of the fusion of BOP-6 and COS-7 cells in each experiment. In experiment 1 this value was 4,400 colonies from the 60-mm-diameter dish, representing a 10-fold amplification over the fusion of BOP-6 and CV-1 at 72 h. For experiment 2 the dish containing the fusion of BOP-6 and COS-7 yielded 40,800 colonies, corresponding to an amplification of at least 100-fold. It is likely that the increased amplification frequency in experiment 2 was due to more efficient cell fusion.

FIG. 3. DNA accumulation after cell fusion. One-third of the DNA extracted from a 60-mm-diameter dish at each time point was subjected to electrophoresis uncut on a 0.8% agarose gel. The gel was blotted to GeneScreen Plus (New England Nuclear Corp.) and hybridized to ^{32}P -labeled pBOP DNA as described previously (7). (a) BP/C127 fused to COS-7 cells; (b) BOP-6 fused to BOP-6 cells; (c) BOP-6 fused to COS-7 cells; (d) BOP-6 fused to CV-1 cells. Time points indicate hours after fusion.

mids will be an aid in experiments requiring DNA recovery from mammalian cells. Possibly, amplification could also increase the yields of gene products encoded on chimeric vectors.

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