Loss of Bovine Papillomavirus DNA Replication Control in Growth-Arrested Transformed Cells

STANLEY BURNETT,* UDO KIESSLING,† AND ULF PETTERSSON

Department of Medical Genetics, Biomedical Centre, Box 589, University of Uppsala, S-751 23 Uppsala, Sweden

Received 4 October 1988/Accepted 17 January 1989

The bovine papillomavirus type 1 (BPV-1) genome replicates as a plasmid within the nuclei of BPV-1-transformed murine C127 cells at a constant multiple copy number, and spontaneous amplification of the viral DNA is rarely observed. We report here that a mutant BPV-1 plasmid within a contact-inhibited C127 cell line replicated as a stable multicopy plasmid in exponentially growing cells but amplified to a high level in confluent cell culture. In situ hybridization analysis revealed that most of the mutant viral DNA amplification occurred in a minor subpopulation of cells within the culture. These consisted of giant nondividing cells with greatly enlarged nuclei, a cell form which was specifically induced in stationary-phase cultures. These observations indicated that expression of a viral DNA replication factor was cell growth stage specific. Consistent with this hypothesis, considerable amplification of wild-type BPV-1 DNA associated with characteristic giant cell formation was observed in typical wild-type virus-transformed C127 cultures following a period of growth arrest achieved by serum deprivation. Further observations indicated that induction of the giant-cell phenotype was dependent on BPV-1 gene expression and implicated a viral E1 replication factor in this process. Moreover, heterogeneity in virus genome copy numbers within the giant-cell population suggested a complex regulation of induction of DNA synthesis in these cells. It appears that this process represents a mechanism employed by the virus to ensure maximal viral DNA synthesis within a growth-arrested cell. Fundamental questions concerning the integration of the virus-cell control circuitry in proliferating and resting cells are discussed.

The bovine papillomavirus type 1 (BPV-1) replicon (6), a circular double-stranded DNA genome containing 7,945 base pairs (11), has attracted considerable interest in recent years because of its ability to replicate autonomously as a stable nuclear plasmid in susceptible animal cells. In vitro tumorigenic transformation of murine C127 cells by BPV-1 follows single-hit kinetics (14), yet transformed cell clones contain multiple (50 to 100) plasmid copies of the viral genome (18). Hence, amplification of the BPV-1 genome occurs following infection of a C127 cell, and this is an early event in the process of transformation, determined by expression of a viral replication factor, *R*, encoded by a large segment of the E1 open reading frame (ORF) (20, 21). Mutations within this region of E1 almost invariably result in a complete loss of replication potential (20). In established BPV-1-transformed cell lines, the viral plasmid is maintained at a constant copy number by expression of a viral replication repressor, or modulator factor, *M*, encoded by a separate region of the E1 ORF localized to the N terminus (3, 22, 37). In addition to these *trans*-acting replication factors, the viral replication control system includes *cis*-acting elements, termed plasmid maintenance (PMS) sequences or PMS elements (19). Two such elements have been mapped within the BPV-1 genome: one within the viral upstream regulatory region coinciding with the position of the viral origin of DNA replication previously mapped in BPV-1-transformed hamster cells (38) and a second within the E1 ORF. There is cogent experimental evidence that the viral *M* factor interacts with sequences adjacent to the PMS elements to ensure stable

plasmid replication (29, 30); however, specific DNA-binding activity has not yet been demonstrated for this phosphoprotein.

Most studies on BPV-1 replication have thus been confined to an investigation of factors involved in persistence of the viral DNA as a stable plasmid, and cell culture conditions have not been established which permit vegetative BPV-1 DNA replication to take place—a process which occurs *in vivo* only in nondividing bovine papilloma cells committed to terminal differentiation. Indeed, no *in vitro* culture system capable of supporting vegetative viral replication has been developed for any wart virus, despite persistent efforts (7, 10, 16; reviewed in reference 36). It is consequently widely believed that this latter phase of viral DNA synthesis and late-gene expression is dependent on cellular factors expressed only in permissive papilloma cells of the natural host species.

In this report we describe the replication properties of a spontaneous mutant BPV-1 plasmid within a C127 cell clone (cl.2) which was previously isolated on the basis of its minimal transformed characteristics (9). The mutation was a 277-base-pair deletion encompassing the common early-gene mRNA polyadenylation signal and several base pairs of the “distal” enhancer element and did not affect any of the translational ORFs or regulatory elements known to be involved specifically in viral plasmid replication control. As detailed below, we observed a dramatic increase in copy number of this mutant viral plasmid when cl.2 cells were maintained as confluent cultures rather than as exponentially growing cells. In situ hybridization analysis was used to identify cells permissive for vegetative mutant viral DNA synthesis within stationary-phase cl.2 cultures. Furthermore, we show that wild-type (wt) BPV-1 DNA within typical virus-transformed C127 cells can similarly be induced

* Corresponding author.

† Permanent address: Central Institute of Molecular Biology, Academy of Science of the German Democratic Republic, 1115 Berlin-Buch, German Democratic Republic.

to amplify to uniquely high levels following a period of arrested cell growth. These observations form a basis for the hypothesis that expression of a viral DNA replication factor is cell growth stage specific.

MATERIALS AND METHODS

Cells. Subcloning of the cl.2 cell line has been described previously (9). The mutant viral genome copy number of cl.2 subclone e² was estimated to be 50 per cell by titration of total cellular DNA, in Southern blot hybridization analysis, against known amounts of cloned mutant viral DNA. Cell clones (a³, b², and wh.2) transformed by wt BPV-1 were isolated by dilution cloning of a C127 culture transformed by infection with purified BPV-1 virions, and their multiple wt viral genome content was confirmed by Southern blot hybridization analysis. Transformed C127 cells harboring integrated BPV-1 genomes were obtained as described in Results. All cell lines maintained in exponential culture were grown in Dulbecco modified Eagle (DME) medium supplemented with 10% fetal bovine serum (FBS; Gibco). Confluent cl.2 cell cultures were refed with DME medium containing 10% FBS every fourth day. In serum deprivation experiments, wt BPV-1-transformed C127 cultures which were one-half to three-quarters confluent were changed to serum-free DME medium. The serum-free DME medium was thereafter renewed every fourth day, until the reintroduction of DME containing 10% FBS after various periods of time.

Preparation of cell DNA and Southern blot hybridization analysis. Total cellular DNA was purified from sodium dodecyl sulfate (SDS)-lysed, proteinase K-treated cell cultures by phenol extraction followed by ethanol precipitation. Cell DNA (approximately 50 µg) was digested overnight at 37°C with a mixture of the endonucleases *Eco*RI and *Bam*HI (100 U of each enzyme in a reaction volume of 100 µl). Contaminating RNA was removed by simultaneous digestion with RNase A (50 µg/ml). Equal amounts of each DNA sample were then subjected to electrophoresis in 1% (wt/vol) agarose gels, followed by blotting onto nitrocellulose (33). Southern blots were hybridized with BPV-1 virion DNA labeled with ³²P by random priming with reagents obtained from Amersham International. The hybridization solution contained 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (wt/vol) bovine serum albumin, 0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) Ficoll 400, 0.1% (wt/vol) SDS, 100 µg of denatured salmon sperm DNA per ml, 10 mM EDTA, and 15 ng of probe DNA per ml. Filters were washed with four changes of a solution of 0.2× SSC–0.5% SDS for 2 h at 65°C prior to autoradiography.

Hybridization in situ to fixed cells. In situ hybridization analysis was carried out with nonradioactive BPV-1 DNA or control pBR328 DNA probes labeled with digoxigenin-dUTP by random priming with a novel DNA labeling and detection kit purchased from Boehringer Mannheim (catalog no. 1093 657). The hybridization procedure was modified from the established protocol (B. Heiles, E. Genersch, R. Neumann, C. Kessler, and H. J. Eggers, Bio/Technology, in press) to enable screening of cells grown in 9-cm-diameter tissue culture dishes as follows. Culture dishes were rinsed three times with phosphate-buffered saline (PBS) and then fixed with ice-cold 100% ethanol for 5 min. Following stepwise rehydration of the cultures with 70%, 50%, and 30% (vol/vol) ethanol (5 min per step), they were rinsed twice for 10 min each in PBS–5 mM MgCl₂. This was followed by immersion of the cultures in 0.2 N HCl for 20 min at room temperature.

Dishes were then washed twice in 2× SSC–5 mM EDTA for 30 min at 50°C. At this stage, proteinase K treatment (1 µg/ml in PBS, 15 min at 37°C) was carried out to increase the sensitivity of the hybridization step. The dishes were thereafter washed in 0.2% glycine in PBS for 10 min at room temperature. Cultures were then fixed with paraformaldehyde (4%, vol/vol) for 15 to 30 min at room temperature. Prehybridization was for 1 h at 42°C in 10 ml of 6× SSC–45% formamide–5× Denhardt solution (1× is 0.02% bovine serum albumin [wt/vol], 0.02% Ficoll [wt/vol], 0.02% polyvinylpyrrolidone)–100 µg of denatured salmon sperm DNA per ml. Denaturation of cellular DNA was carried out by incubating the cultures in prehybridization solution at 65°C for 20 min and then placing the dishes on an aluminium hot plate at 100°C for 1 min. The prehybridization solution was then discarded, and the dishes were placed on ice. Hybridizations were performed under plastic circles cut from polythene hybridization bags to fit into the bottom of the culture dishes. The hybridization solution was 150 to 200 µl of 6× SSC–45% formamide–5× Denhardt solution–8% dextran sulfate containing 0.5 to 1 µg of digoxigenin-labeled probe DNA per ml, which had previously been denatured by heating for 4 min at 100°C and quenching on ice. Hybridizations were carried out at 42°C in a moist chamber for 14 to 18 h. The cultures were washed as follows: twice for 15 min each at 42°C with 6× SSC–45% formamide; twice for 5 min each at room temperature with 2× SSC; twice for 15 min each at 50°C with 0.2× SSC; and finally once for 30 min at 65°C with 0.2× SSC. Detection of specific hybridization was performed by enzyme immunoassay with polyclonal sheep antidigoxigenin Fab fragments conjugated to alkaline phosphatase, as described by the manufacturers of the DNA labeling and detection kit. Cells were photographed with a phase-contrast photomicroscope.

RESULTS

Amplification of a mutant viral genome in confluent cell culture. Preliminary observations indicated that the mutant viral genome content of cl.2 cultures maintained at confluence was unusually high. To examine this phenomenon further, cellular DNA was prepared from cl.2 cultures at various times after the cells reached confluence or from cells maintained in exponential culture, and the viral DNA content was analyzed by Southern hybridization in combination with restriction endonuclease digestion (Fig. 1). After a period of 7 weeks, the average mutant viral DNA content per cell within the confluent culture had risen to a level at least eightfold higher than the initial plasmid maintenance level of approximately 50 copies. In contrast, cl.2 cells passaged continuously for the same time period did not contain amplified levels of the viral plasmid (Fig. 1B, lane C; compare with lane U). Similar results were obtained with each of five cl.2 subclones tested, and levels of amplification between 10-fold and 40-fold have been recorded after 4 to 8 weeks at confluence. No significant amplification of the wt BPV-1 genome occurred during aging of a wt virus-transformed C127 clone when maintained as confluent transformed cultures in the same growth medium (Fig. 1A, WT). Much of the amplified mutant viral DNA existed in a high-molecular-weight form, as revealed by its low migration in agarose gels within cl.2 cellular DNA treated with endonucleases which did not cleave the BPV-1 genome (data not shown). This might represent complex viral concatemers or large rolling-circle viral DNA replication intermediates. Evidence that BPV-1 can replicate by a rolling-circle mecha-

FIG. 1. Amplification of a mutant BPV-1 plasmid in confluent cell culture. (A) Increase in copy number of the mutant plasmid (M) with time at confluence (left to right, lanes represent 1, 2, 3, 5, and 7 weeks). Cl.2 cells (subclone e^2) and a wt virus-transformed C127 line (clone b^2) were grown to confluence in 6-cm-diameter culture dishes and maintained without further passaging in standard growth medium (DME medium supplemented with 10% FBS). Dishes were harvested for extraction of cellular DNA at 1, 2, 3, 5, and 7 weeks (subclone e^2) or at 1, 2, 3, and 5 weeks (clone b^2 ; these wt virus-transformed cultures could not be maintained for longer periods due to detachment of cells from the substratum). Purified total cellular DNA was digested with a combination of the single-cut endonucleases *EcoRI* and *BamHI*, and equal amounts of cellular DNA from each time point were fractionated by electrophoresis in a 1.0% agarose gel prior to Southern hybridization analysis with a ^{32}P -labeled BPV-1 DNA probe. The arrow at the right-hand side indicates a faint signal resulting from the appearance of a new mutant plasmid (see Results). (B) Copy number of the mutant plasmid in cl.2 cells maintained at confluence for 7 weeks (lane U) compared with cl.2 cells passaged continuously for the same time period (lane C). Cellular DNA cleaved with *EcoRI* plus *BamHI* was analyzed by Southern hybridization. Serial twofold dilutions (shown above lanes) of cl.2 cell DNA (sample U) indicate the level of viral DNA amplification.

nism in the vegetative phase of virus multiplication within natural wart tissue has recently been uncovered (9a).

Two types of cellular morphological change developed in parallel with viral DNA amplification in confluent cl.2 cultures. The first notable alterations, beginning about 1 week after the cultures became confluent, were the formation of large (giant) cells (Fig. 2), which occurred singly or in clusters and were scattered throughout the entire area of the culture dish. The numbers of these cells in cl.2 cultures rose to between 4 and 6% of the total cell population at 4 to 8 weeks postconfluence. The increased cell size involved an increase not only in the cytoplasmic volume, but also in the size of the nucleus. Most giant cells contained a single giant

nucleus, the diameter of which often exceeded that of an entire neighboring cell. Other giant cells were multinucleate, and many giants displayed perinuclear or cytoplasmic vacuolation. In addition, a pronounced clearing of the peripheral cytoplasm, as observed by phase-contrast microscopy, was seen frequently in developing giant cells as well as in many small cells within confluent cl.2 cultures.

Extensive observation of cl.2 cultures by light microscopy over a period of 1 year indicated that giant cells were unable to divide or occasionally underwent abortive mitoses. Thus, they developed by expansion of the smaller cells. Evidence that these cells resemble a differentiated cell state is reviewed in the Discussion. Although unable to divide, there was a net accumulation of these cells in the cultures since they remained attached to the substratum for many days before cell death and eventual shedding into the medium occurred. Some lysis of small cells was also noted when cl.2 cultures became confluent. Giant cells were not detected in normal untransformed C127 cultures maintained under identical conditions and were seen in wt BPV-1-transformed C127 clones only at very low levels (<0.1% of cells) except when induced by specific culture conditions, described below. Such giant cells were described previously in wt virus-transformed C127 cultures as rare sporadic "jackpot" cells, a term used to indicate their high viral DNA content (30).

Further observations of another cell system indicated that giant-cell formation was specific for cell lines which contained the viral genome. Both characteristic giant-cell formation and outgrowth of foci of transformed cells (see below) in confluent culture were observed in a contact-inhibited bovine fibroblast cell clone harboring the mutant viral plasmid, which was obtained following transfection of primary bovine conjunctival fibroblasts with a molecularly cloned BPV-1 genome containing the identical deletion mutation (T. S. Burnett, unpublished observations). Such changes were not seen in control uninfected bovine fibroblast cultures maintained at confluence for up to 3 months.

Development of foci of neoplastic transformation in confluent cl.2 cultures. A second type of morphological change associated with mutant viral DNA amplification within confluent cl.2 cultures was the development of foci of transformed cells (Fig. 2, inset). Clonal transformed cell populations isolated from neoplastic foci had unusual properties, including a variable giant-cell content and a tendency to revert to a nontransformed flat shape. Southern hybridization analysis of three such lines, isolated from large transformed foci which had developed within confluent cultures of three different cl.2 subclones, was carried out after 12 to 14 population doublings in exponential-phase culture following their isolation. In each case there was a modest (two- to sixfold) amplification of viral DNA. Restriction endonuclease analysis in combination with Southern hybridization revealed, furthermore, that each of these cell lines contained a new spontaneous mutant plasmid in combination with the original mutant genome. The new mutants were present at a copy number similar to that of the original mutant plasmid in these cell lines. The emergence of one such de novo mutant plasmid in a cl.2 subclone with increasing time at confluence can be seen as a faint signal in the autoradiogram shown in Fig. 1A (arrow). Mapping analysis of these new mutant plasmids established that they were derived by further mutation of the original mutant genome, since they retained the same short deletion at the distal end of the transforming gene region. Moreover, they appeared to have a replication-defective genotype, since each had suffered a deletion, different for each new mutant, affecting the R region of the

FIG. 2. Morphological changes in confluent cl.2 cultures. Phase-contrast photomicrographs, at the same magnification, of a contact-inhibited cl.2 culture at 1 week postconfluence (left) and a cluster of giant cells in a culture maintained at confluence for 7 weeks (right). Insets: Tissue culture dishes for the corresponding time points. Dishes were fixed with isopropanol (70%, vol/vol) and stained with methylene blue (1%, wt/vol) to visualize areas of neoplasia.

E1 ORF, and in one case the R region was almost entirely deleted. From the above observations, as well as from *in situ* hybridization analyses described below, we concluded that the majority of the mutant viral DNA amplification observed in confluent cl.2 cultures was not accounted for by the outgrowth of transformed cells containing a high viral plasmid copy number. Precise mapping analysis of these evolutionary mutant plasmids and experiments to investigate their relevance for viral DNA replication control and neoplastic cellular transformation are in progress and will be published at a later date.

Identification of cells with amplified mutant viral DNA in confluent cl.2 cultures. In order to establish which cells within a confluent cl.2 culture contained amplified mutant viral DNA, *in situ* hybridization analysis was performed (Fig. 3). The sensitivity of the *in situ* hybridization system used under the conditions described here was not sufficient to detect the plasmid maintenance level in this cell line. However, *in situ* hybridization analysis of a wt BPV-1-infected bovine fibroblast culture with a high plasmid maintenance level, estimated to be 500 to 1,000 copies per cell (8; T. S. Burnett, unpublished results), showed that this level was readily detected. In confluent cl.2 cultures, cells positive for amplified viral DNA included (i) abundant giant cells and less-abundant small or intermediate-sized cells scattered at random or in small clusters throughout the culture dish and (ii) small cells localized within foci of neoplastic transformation. There was a large variation in intensity of the intranuclear signal detected within the giant-cell population, and rare giant cells contained no visible amplification of viral DNA sequences. The randomly occurring cells of small and intermediate size exhibited lower average copy numbers than the giant cells, as judged from their consistently weaker nuclear staining, and probably represented precursor giant

cells at an early stage of development. The mutant viral copy numbers within transformed cell foci which developed in cl.2 cultures were comparable to the plasmid maintenance level of the wt virus-infected bovine fibroblasts (500 to 1,000 copies) when tested under identical conditions. By far the highest copy numbers occurred in rare giant cells with abnormally enlarged nuclei, which showed extremely intense signals. Although it was not possible to measure the precise copy numbers attained in these cells, it is likely in some cases to be in excess of 100,000 copies.

By adapting the *in situ* technique so that an entire confluent culture dish could be screened, we estimated that the relative fraction of total amplified viral DNA contributed by the giant-cell population was between 60 and 80% in a cl.2 culture maintained at confluence for 5 weeks, and the remainder was contributed by the emerging transformed-cell population. We stress that this figure underestimates the relative amount of viral DNA synthesized within giant cells compared with that in transformed cells in a cl.2 culture over a period of weeks, since giant cells accumulated only slowly because of continual cell death and shedding into the medium, whereas transformed cells accumulated exponentially.

Amplification of wt BPV-1 DNA in transformed C127 cells following a period of growth arrest. To test whether wt BPV-1 DNA replication control was also lost in growth-arrested cultures, C127 cells transformed by the wt BPV-1 genome were maintained under conditions intended to inhibit their division. This was accomplished by incubating cells in tissue culture medium without serum. After the growth medium of near-confluent cultures was replaced with serum-free medium, there was a rapid reduction in the frequency of mitotic cells, and cells undergoing division were only rarely observed after 3 to 4 days of serum deprivation. Inhibition of cell division was accompanied by a

FIG. 3. In situ hybridization analysis to identify cells containing amplified mutant viral DNA. Cultures of cl.2 cells (subclone e²) maintained at confluence for 5 weeks were subjected to in situ hybridization analysis with a BPV-1 probe (A–C) or a control pBR328 probe (D) as described in Materials and Methods. Three different areas of the same cl.2 culture tested with the viral probe are shown. Cells predominantly positive for hybridization include randomly occurring giant cells, as well as cells localized to foci of neoplasia. The edge of a focus of transformed cells can be seen in panel C (arrows).

marked flattening of the cells and limited cell death. Southern hybridization analysis of total cellular DNA prepared from unfractionated cell cultures was used to investigate the wt viral DNA content of serum-deprived cell cultures before and after the readdition of medium containing serum. In the analysis shown in Fig. 4, cells maintained in the absence of serum for 12 days contained an approximately fourfold-higher copy number (lane 0h.) over the plasmid maintenance level (lane C). Following the reintroduction of medium containing serum, viral DNA copy levels increased further to as much as 8- to 16-fold that of the plasmid maintenance level within 36 h and remained high for up to 15 days poststimulation. This high level of viral replication was only transient, however, since transformed cells regrown from parallel 15-day cultures following trypsinization and reseeded at low density contained the same plasmid copy number as control cells (lane P; compare with lane C). Similar high levels of viral DNA amplification were obtained with a

second independently isolated wt virus-transformed C127 clone.

There was limited formation of giant cells (0.5 to 1% of cells) in wt virus-transformed cultures maintained in serum-free medium. However, remarkable cellular morphological changes accompanied the reintroduction of medium containing serum to growth-arrested wt virus-infected cultures, with the development of many nondividing giant cells, frequently with peripheral cytoplasmic clearing (Fig. 5). These constituted 4 to 6% of the cells at 48 h poststimulation but soon declined again to a low level due to the continued growth of rapidly dividing transformed cells as well as the death and detachment of many giant cells, which consequently accumulated in the medium. Some death of small cells was also noted within the first 2 days of serum stimulation. Transformed cells regrown from serum-deprived cultures were morphologically indistinguishable from control cells which had not been deprived of serum, and they could be induced

FIG. 4. wt BPV-1 DNA amplification following a period of serum deprivation. BPV-1 DNA levels in wt virus-transformed C127 cells (clone a³) maintained in DME medium containing 10% FBS (lane C) are compared with those in cultures deprived of serum for 12 days (lane 0h.) or following the release of growth arrest (6 to 36 h or 2 to 15 days [d]) and transformed cells regrown from low density (lane P). Equal numbers of transformed cells (10⁶) were seeded in each 9-cm-diameter culture dish and grown in DME medium containing 10% FBS. When the dishes were one-half to two-thirds confluent, the medium of test cultures was changed to serum-free DME. Equal amounts of total cellular DNA from each time point were digested with *Eco*RI plus *Bam*HI prior to hybridization analysis with a ³²P-labeled BPV-1 DNA probe. Serial twofold dilutions of the 9-day time point sample at the right-hand side of the figure indicate the level of viral DNA amplification (U, undiluted).

to undergo a second round of giant-cell formation after another period of serum deprivation. The extent of giant-cell formation after the reintroduction of serum increased with longer periods of serum deprivation, reaching a level of between 15 and 20% of the cells at 1 month (data not shown).

In situ hybridization analysis was performed on wt BPV-1-transformed cells maintained in the absence of serum or 36 h following the reintroduction of medium containing serum to cultures which had been deprived of serum for 12 days (Fig. 6A and D, respectively). This analysis confirmed that most if not all of the wt viral DNA amplification induced following serum stimulation took place in the subpopulation of developing nondividing giant cells. This explained the transient nature of the wt viral DNA amplification seen by Southern hybridization analysis of total cellular DNA. Moreover, the continued high level of viral replication following the peak in number of giant cells, as described above, indicated that viral DNA replication proceeded in an exponential manner in many of these nondividing cells. However, heterogeneity in the giant-cell population was evidenced in this case also by the identification of rare giant cells (2 to 4% of developing giants) which contained no detectable amplification of viral DNA sequences. It appeared, furthermore, that many of the precursors to the giants were already present within the cell population prior to stimulation with serum. These were identified as cells with very slightly enlarged nuclei which displayed weak positive staining. Cells with intense nuclear staining were very rare in serum-starved cultures (<0.1% of cells). No giant cells developed in normal uninfected C127 cells following an identical period of serum deprivation.

The potential for giant-cell formation was limited to virus-transformed cells harboring extrachromosomal BPV-1 genomes. Thus, neither giant-cell formation nor viral DNA amplification was detected in cultures of BPV-1-transformed C127 cells containing integrated BPV-1 DNA (Fig. 6C and F). Many cell lines containing integrated viral DNA have been isolated following transfection of C127 cultures with wt

BPV-1 DNA linearized by digestion with *Eco*RI, which cuts the viral genome at nucleotide 2113, i.e., within the *R* region of the E1 ORF. In each case, the transformed lines contained a low copy number of integrated viral genomes, and the *Eco*RI site was invariably destroyed (T. S. Burnett, unpublished observations).

DISCUSSION

In this study we have established that the rigid control of BPV-1 plasmid copy numbers in virus-transformed C127 cells grown in vitro is relaxed under certain cell culture conditions. Specifically, we found that a mutant BPV-1 plasmid within a minimally transformed C127 cell line which retained the response of density-dependent growth inhibition (contact inhibition) replicated as a stable multicopy plasmid in exponentially growing cells, but amplified to a high level within growth-arrested cells in confluent culture. Based on this observation, we were subsequently able to devise cell culture conditions, involving a period of growth arrest achieved by serum deprivation, which induced amplification of wt BPV-1 DNA in typical wt virus-transformed C127 cells. It is clear from these findings why wt BPV-1 DNA amplification has not been readily observed previously in established BPV-1-transformed cells, since they are not contact inhibited and are thus continuously cycling when maintained under standard growth conditions. The present work has thereby uncovered a fundamental relationship between cell growth stage and viral DNA replication potential in BPV-1-transformed cells grown in vitro. The regulation of viral DNA copy numbers in this experimental model system closely parallels that in a natural infection, where vegetative viral DNA synthesis is initiated following differentiation of transformed basal papilloma cells within a wart.

Several fundamental questions arising from a consideration of the phenomena disclosed by this investigation are discussed below.

To what extent do the nondividing giant cells represent a permissive differentiated cell state? Vegetative papillomavirus DNA synthesis occurs within the hyperkeratinizing epithelium of a wart in a variable fraction of cells of the spinous and granular layers, differentiated cells where DNA synthesis is totally repressed in normal epidermis or in chemically induced epidermal papillomas (26, 28). It is consequently believed that papillomaviruses have evolved a mechanism to derepress cellular genes essential for viral DNA synthesis in these nondividing cells (reviewed in reference 25). Characteristic histopathological features associated with vegetative papillomavirus replication include cellular enlargement, often in combination with cytoplasmic vacuolation, and rare suprabasal mitoses. Viral capsid antigens are detected at a late stage within the nuclei of cells of the granular layer, whereas vegetative viral DNA synthesis may be initiated as early as one cell layer removed from the proliferative basal layer. The amount of virus production within a wart may depend on the type of infecting virus, the anatomical site of the target epithelium, or—in the case of an experimental in vivo infection—the genetic constitution of the host animal species (25, 28, 32).

The giant C127 cells described in this paper resemble permissive terminally differentiated cells within a wart in their enlarged size, frequent cytopathic effects, occasional abortive cell divisions, and unscheduled DNA synthesis. However, whereas permissive papilloma cells are continuously generated as a consequence of the terminal differentiation program of the keratinocyte, this does not appear to be

FIG. 5. Morphological changes in wt virus-infected C127 cells following the release of growth arrest. (A) Flattened appearance of growth-arrested wt virus-infected C127 cells 12 days after removal of serum from the culture medium. (B) Morphological changes 48 h after release of growth arrest by readdition of medium containing serum. Arrows indicate giant cells, with enlarged nuclei and peripheral cytoplasmic clearing, which developed by expansion of smaller cells.

FIG. 6. Identification of cells containing amplified wt BPV-1 DNA in virus-transformed C127 cultures following a period of serum deprivation. Cultures of wt virus-transformed C127 cells (subclone wh.2) were tested by in situ hybridization after 12 days of serum deprivation (A and B) or 36 h after the readdition of medium containing serum (D and E) with either a BPV-1 probe (A and D) or a control pBR328 probe (B and E). C127 cells containing the BPV-1 genome as integrated, replication-defective genomes were also tested with the BPV-1 probe before and after serum stimulation (C and F, respectively). Following serum stimulation, positive nuclear hybridization was detected only in cultures containing the viral DNA in plasmid form (D). Only a minor fraction of the cells exhibited positive hybridization, and these were predominantly cells with greatly enlarged nuclei. Prior to serum stimulation, only weakly positive cells with slightly enlarged nuclei were detected in these cultures (A, arrow).

the case in the nonkeratinocyte experimental cell systems which we have studied. The complete absence of giant cells from control uninfected murine C127 or bovine fibroblast cultures indicates that these represent a differentiated cell state induced or maintained by expression of a viral gene(s). This conclusion is further supported by transfection experiments with cloned BPV-1 DNA, in which we observed giant cells with characteristic enlarged nuclei and cytopathic features within developing primary transformed C127 cell foci (unpublished observations). The observation of rare giants with typical giant nuclei but no detectable amplification of viral DNA sequences suggested that cellular DNA synthesis alone was induced in these cells and therefore implied a complex mechanism for the induction of viral and cellular DNA synthesis.

In giant C127 cells, unlike fully permissive differentiated papilloma cells, we have not detected intranuclear capsid antigens by using immunocytological techniques (unpublished results). A possible explanation for the failure to complete the BPV-1 replication cycle in C127 cells is a block to activation of the viral major late promoter (2), which directs synthesis of mRNAs for the major capsid polypeptide, as well as the E4 gene product, which may be involved in the process of viral maturation (13, 24). Transcriptional

analyses are currently being performed in order to resolve this issue.

What is the molecular mechanism of viral DNA amplification, and how might induction of cellular genes involved in DNA synthesis be controlled in growth-arrested cells? Previous genetic mapping experiments have defined viral replication factors (*M*, *R*) encoded by the E1 ORF, which have been proposed to act in concert to ensure stable plasmid replication in established virus-transformed C127 cells (3, 22). Based on the premise that the viral *M* and *R* factors are expressed constitutively in proliferating transformed cells, a simple model to explain the phenomenon of cell growth arrest-dependent viral DNA amplification might propose that there is a shut-off or a qualitative change in viral gene expression in cells at growth arrest, resulting in the disappearance or inactivation of the replication repressor, *M*. According to such a "passive" model, viral DNA amplification would occur spontaneously by continued expression of an *R* factor when the level of a functional *M* peptide fell below a threshold level. However, unlike the primary infection by a single virus particle, amplification would begin from the relatively high plasmid maintenance level. Thus, there would be a lower probability that amplification came again under control of the modulator or repressor function,

and viral DNA amplification leading to eventual cell death—analogueous to the situation proposed for M^- BPV-1 mutants (3)—would be more likely to occur than stable cell transformation.

A number of considerations suggest that the above model is not sufficient to explain the phenomena described in this paper. In particular, the dramatic induction of DNA synthesis coupled with pronounced nuclear enlargement and the potential for prolonged viral DNA replication to uniquely high levels are more likely to be the result of a complex active process involving multiple cellular and viral genes, with the aim of maximizing viral DNA synthesis within a division-arrested cell. Undoubtedly an E1 factor encoded by the *R* region performs a fundamental role in this process, in view of its absolute requirement for plasmid replication. This is further indicated by the complete absence of giant-cell formation in C127 cells harboring replication-defective (integrated) BPV-1 genomes, as described in this paper. At present we know nothing of the molecular mechanisms by which an *R* factor stimulates replication of BPV-1 DNA. However, the striking amino acid sequence homology between multiple domains of a putative BPV-1 E1 polypeptide and the intensively studied large T antigens of simian virus 40 and polyomavirus (12) suggests a common mechanism of action for these proteins in the initiation of viral DNA replication and the induction of cellular DNA synthesis in growth-arrested cells.

Several additional observations lead us to believe that the control of expression of BPV-1 E1 factors is more complicated than predicted from previous genetic complementation studies. Whereas candidate mRNAs for an *M* factor can be detected in proliferating transformed C127 cells, mRNAs from the *R* region are essentially undetectable in these cells in the absence of an inhibitor of protein synthesis (8, 35). Furthermore, the conservation of E1 as an uninterrupted ORF between papillomaviruses of different species (reviewed in reference 27) implies that it is translated intact as a large *M/R* polypeptide during some stage of the viral replication cycle. Indeed, a large mRNA species apparently covering the entire E1 ORF has been detected in virus-infected bovine conjunctival fibroblast cultures at early passage levels after infection (8). Together these observations indicate the existence of multiple E1 factors and are consistent with the hypothesis that an E1 *R* factor(s) is expressed specifically at growth arrest but repressed in proliferating virus-transformed cells.

Another BPV-1 function which has been implicated in viral plasmid copy number control is an E6/E7 fusion polypeptide. It has been reported that the ability of an expressed E6/7 cDNA to complement a low-copy-number BPV-1 mutant with a disrupted E7 ORF and thus restore copy numbers to wt levels is conditional upon simultaneous introduction of these plasmids into C127 cells (4). Transfection of this cDNA into cells previously transformed with the E7 mutant did not lead to restoration of a high plasmid copy number. These data are consistent with a cooperative action of viral E1 and E6/7 factors in the induction of viral DNA synthesis during a period of growth arrest prior to the establishment of transformation. Thus, it is conceivable that there is a set of BPV-1 genes specifically expressed during growth arrest which are involved in this process, and it will be interesting to investigate a possible common mechanism of regulation of these genes with recently described cellular growth arrest-specific genes (31). The BPV-1 E5 gene has previously been shown to be capable of inducing DNA synthesis in quiescent C127 cells (15) and may therefore also play a role in the process of

growth arrest-dependent viral DNA amplification in addition to its recognized role in cell transformation. The requirement for serum for maximal wt BPV-1 DNA synthesis following a period of serum deprivation indicates that the activity of viral and/or cellular genes which cooperate in this process is modulated in growth-arrested cells by a serum factor-induced intracellular signal.

What is the molecular basis for the apparent transformation defect of the mutant viral genome central to the development of this work? In the case of the mutant BPV-1 plasmid, prolonged cell-cell contact in confluent cl.2 cultures was sufficient to permit the full response of giant-cell formation associated with viral DNA synthesis without the need for a period of serum deprivation. Thus, there appears to be a fundamental influence on the expression of viral replication factors by cellular signals which control cell proliferation and maintenance of growth arrest. Other observations of the growth properties of cl.2 cells underline the complex nature of such interactions. These cells exhibit a surprisingly high colony-forming efficiency in soft agar, not expected for a contact-inhibited cell line. Furthermore, when viewed by light microscopy, cl.2 cells in exponential culture are not easily distinguishable from wt BPV-1-transformed C127 cells, and newly confluent cl.2 cultures exhibit extensive overlapping of cellular processes characteristic of overtly transformed cells. However, after several days at confluence, the cultures revert to a flat contact-inhibited monolayer. Thus, it seems that cellular growth control mechanisms which repress proliferation dominate over the viral transforming functions. We point out that this minimal or "recessive" transformation phenotype of cl.2 cells is similar to the latent type of interaction of BPV-1 observed with other cell lines (1, 23) or in C127 cells transfected with certain BPV-1 recombinant plasmids (17). We have previously discussed possible explanations for the apparent minimal transformed properties of cl.2 cells, including a possible dysregulation of expression of viral transforming genes caused by the utilization of an inefficient cryptic common early mRNA polyadenylation signal (9). Interestingly, although flat cell lines similar to cl.2 cells can be obtained by transfection of C127 cultures with the molecularly cloned mutant plasmid, it is also possible to obtain stable overtly transformed C127 lines with properties similar to those of wt virus-transformed C127 cells with the same mutant genome. This heterogeneity in transformation phenotypes suggests that the deletion mutation in this spontaneous mutant viral genome is a true regulatory mutation, which would appear to affect a switch between minimal and overt transformation states of the virus. Perhaps this results in some way from an alteration in the "distal" enhancer element, which was partially deleted in the mutant. Further comparative analyses of viral transcriptional patterns and viral DNA content in minimally and overtly transformed C127 cell lines are being performed in order to investigate the nature of this putative switch.

In conclusion, this study forms a basis for the hypothesis that expression of a BPV-1 replication factor is cell growth stage specific and provides an explanation for the concept that neoplastic cellular transformation is incompatible with vegetative papillomaviral replication. Several observations described in this paper imply a complex regulation of the process of cell growth stage-dependent BPV-1 DNA amplification and point to a fundamental interaction between cellular growth control signals and the expression of viral genes involved in cell transformation and viral DNA replication. Furthermore, this work leads us to the conclusion

that the transforming genes of BPV-1, a natural tumor-inducing agent, have evolved to perform a conceptually different role than those of other papovaviruses. Whereas simian virus 40 and polyomavirus have incorporated genes with transforming potential which appear to function as replication factors in permissive cells of the natural host, maximizing vegetative viral DNA synthesis in growth-arrested cells (reviewed in references 5, 15, and 34), the BPV-1 transforming genes—by keeping an infected cell proliferating continuously—instead effectively delay the onset of vegetative viral replication. In this case, viral DNA synthesis is maximized by maintaining an expanded population of non-permissive transformed cells which are capable of generating an essentially limitless number of permissive differentiated cells.

ACKNOWLEDGMENTS

Support for this study was provided by the Swedish Cancer Society, the Swedish National Board for Technical Development, and the University of Uppsala. Stanley Burnett was supported in part by an EMBO fellowship.

ADDENDUM

In keeping with the hypothesis formulated in this paper, we have recently detected an E1 antigen in the nuclei of giant cells, using antisera raised against E1 polypeptides expressed in *Escherichia coli*. Antisera to E1 proteins encoded both by the N-terminal half of E1 (nucleotides 860 to 1471) (37) as well as the C-terminal half of E1 (nucleotides 1515 to 2663) identified this antigen.

Rabbit polyclonal antiserum to the C-terminal E1 polypeptide was provided by Tiit Palpsept and Mart Ustav (Laboratory of Molecular Biology, Tartu University, Tartu, Estonia).

LITERATURE CITED

1. Amtmann, E., and G. Sauer. 1982. Activation of non-expressed bovine papilloma virus genomes by tumour promoters. *Nature (London)* **296**:675–677.
2. Baker, C. C., and P. M. Howley. 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and in productively infected wart tissues. *EMBO J.* **6**:1027–1035.
3. Berg, L., M. Lusk, A. Stenlund, and M. R. Botchan. 1986. Repression of bovine papilloma virus replication is mediated by a virally encoded *trans*-acting factor. *Cell* **46**:753–762.
4. Berg, L., K. Singh, and M. Botchan. 1986. Complementation of a bovine papilloma virus low-copy-number mutant: evidence for a temporal requirement of the complementing gene. *Mol. Cell. Biol.* **6**:859–869.
5. Bishop, J. M. 1985. Viral oncogenes. *Cell* **42**:23–38.
6. Botchan, M. R., L. Berg, J. Reynolds, and M. Lusk. 1986. The bovine papillomavirus replicon. *Ciba Found. Symp.* **120**:53–67.
7. Burnett, T. S., and P. H. Gallimore. 1983. Establishment of a human keratinocyte cell line carrying complete human papillomavirus type 1 genomes: lack of vegetative viral DNA synthesis upon keratinization. *J. Gen. Virol.* **64**:1509–1520.
8. Burnett, S., J. Moreno-Lopez, and U. Pettersson. 1987. Messenger RNAs from the E1 region of bovine papillomavirus type 1 detected in virus-infected bovine cells. *Nucleic Acids Res.* **45**:8607–8620.
9. Burnett, S., J. Moreno-Lopez, and U. Pettersson. 1988. A novel spontaneous mutation of the bovine papillomavirus-1 genome. *Plasmid* **20**:81–84.
- 9a. Burnett, S., J. Zabielski, J. Moreno-Lopez, and U. Pettersson. 1989. Evidence for multiple vegetative DNA replication origins and alternative replication mechanisms of bovine papillomavirus type 1. *J. Mol. Biol.* **206**:239–244.
10. Butel, J. S. 1972. Studies with human papilloma virus modeled after known papovavirus systems. *J. Natl. Cancer Inst.* **48**:285–299.
11. Chen, E. Y., P. M. Howley, A. D. Levinson, and P. H. Seeburg. 1982. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature (London)* **299**:529–534.
12. Clertant, P., and I. Seif. 1984. A common function for polyoma virus large-T and papillomavirus E1 proteins? *Nature (London)* **311**:276–279.
13. Doorbar, J., D. Campbell, R. J. A. Grand, and P. H. Gallimore. 1986. Identification of the HPV-1a E4 gene products. *EMBO J.* **5**:355–362.
14. Dvoretzky, I., R. Shober, S. K. Chattopadhyay, and D. R. Lowy. 1980. A quantitative *in vitro* focus assay for bovine papilloma virus. *Virology* **103**:369–375.
15. Green, M., and P. M. Loewenstein. 1987. Demonstration that a chemically synthesized BPV1 oncoprotein and its C-terminal domain function to induce cellular DNA synthesis. *Cell* **51**:795–802.
16. La Porta, R. F., and L. B. Taichman. 1982. Human papilloma viral DNA replicates as a stable episome in cultured epidermal keratinocytes. *Proc. Natl. Acad. Sci. USA* **79**:3393–3397.
17. Law, M., J. Byrne, and P. M. Howley. 1983. A stable bovine papillomavirus hybrid plasmid that expresses a dominant selective trait. *Mol. Cell. Biol.* **3**:2110–2115.
18. Law, M. F., D. R. Lowy, I. Dvoretzky, and P. M. Howley. 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proc. Natl. Acad. Sci. USA* **78**:2727–2731.
19. Lusk, M., and M. R. Botchan. 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. *Cell* **36**:391–401.
20. Lusk, M., and M. R. Botchan. 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. *J. Virol.* **53**:955–965.
21. Lusk, M., and M. R. Botchan. 1986. Transient replication of BPV-1 plasmids: *cis* and *trans* requirements. *Proc. Natl. Acad. Sci. USA* **83**:3609–3613.
22. Lusk, M., and M. R. Botchan. 1986. A bovine papillomavirus type 1-encoded modulator function is dispensable for transient viral replication but is required for establishment of the stable plasmid state. *J. Virol.* **60**:729–742.
23. Meneguzzi, G., B. Binétruy, M. Grisoni, and F. Cuzin. 1984. Plasmidial maintenance in rodent fibroblasts of a BPV1-pBR322 shuttle vector without immediately apparent oncogenic transformation of the recipient cells. *EMBO J.* **3**:365–371.
24. Neary, K., B. Horwitz, and D. DiMaio. 1987. Mutational analysis of open reading frame E4 of bovine papillomavirus type 1. *J. Virol.* **61**:1248–1252.
25. Orth, G., F. Breitburd, M. Favre, and O. Croissant. 1977. Papillomaviruses: a possible role in human cancer, p. 1043–1068. *In* H. H. Hiatt, J. D. Watson, and J. A. Winsten (ed.), *Origins of human cancer*, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Orth, G., P. Jeanteur, and O. Croissant. 1971. Evidence for and localization of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumors induced by Shope papilloma virus. *Proc. Natl. Acad. Sci. USA* **68**:1876–1880.
27. Pettersson, U., H. Ahola, A. Stenlund, and J. Moreno-Lopez. 1987. Organization and expression of papillomavirus genomes, p. 67–107. *In* N. P. Salzman and P. M. Howley (ed.), *The papovaviridae*, vol. 2. Plenum Publishing Corp., New York.
28. Rashad, A. L., and C. A. Evans. 1967. A difference in sites of DNA synthesis in virus-induced (Shope) and in chemically induced epidermal tumors of rabbit skin. *Cancer Res.* **27**:1639–1647.
29. Roberts, J. M., and H. Weintraub. 1986. Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. *Cell* **46**:741–752.
30. Roberts, J. M., and H. Weintraub. 1988. *cis*-Acting negative control of DNA replication in eukaryotic cells. *Cell* **52**:397–404.

31. **Schneider, C., R. M. King, and L. Philipson.** 1988. Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**:787-793.
32. **Shope, R. E.** 1935. Serial transmission of virus of infectious papillomatosis in domestic rabbits. *Proc. Soc. Exp. Biol. Med.* **32**:830-832.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
34. **Spindler, K. R., C. Y. Eng, and A. J. Berk.** 1985. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. *J. Virol.* **53**:742-750.
35. **Stenlund, A., G. L. Bream, and M. R. Botchan.** 1987. A promoter with an internal regulatory domain is part of the origin of replication of bovine papilloma virus. *Science* **236**:1666-1671.
36. **Taichman, L. B., F. Breitburd, O. Croissant, and G. Orth.** 1984. The search for a culture system for papillomavirus. *J. Invest. Dermatol.* **83**:2s-6s.
37. **Thorner, L., N. Bucay, J. Choe, and M. Botchan.** 1988. The product of the bovine papillomavirus type 1 modulator gene (*M*) is a phosphoprotein. *J. Virol.* **62**:2474-2482.
38. **Waldeck, W., F. Rösl, and H. Zentgraf.** 1984. Origin of replication in episomal bovine papillomavirus type 1 DNA isolated from transformed cells. *EMBO J.* **3**:2173-2178.