Evidence that the transcriptional trans-activating function of the bovine papillomavirus type 1 E2 gene is not required for viral DNA amplification in division-arrested cells

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Amplification of bovine papillomavirus type 1 (BPV-1) DNA in growth-arrested mouse cell cultures appears to mimic the process of induction of vegetative BPV-1 DNA synthesis in cells of the stratum spinosum in productively infected bovine warts. In both cases, cells permissive for viral DNA amplification express large amounts of viral E2 protein which accumulates within the cell nucleus. Whereas in latently infected virus-transformed cells truncated transcriptional repressor forms of E2 predominate, our previous studies have demonstrated that the full-length E2 transcriptional trans-activator protein is preferentially expressed during the period of maximal BPV-1 DNA amplification in growth-arrested cell cultures. To investigate the role of the full-length E2 gene in the induction of viral DNA amplification in this experimental viral replication system we have used a mutant BPV-1 genome (BPVE2-ts1) containing an E2 gene which is temperature-sensitive (ts) for transcriptional trans-activation. This mutant genome has also been shown to be ts for stable viral plasmid DNA replication and for the induction of cell transformation. We show here that viral DNA amplification was not severely impaired when BPVE2-ts1-transformed cells were tested at the restrictive temperature, indicating that the transcriptional trans-activating function of E2 was not essential for viral DNA amplification in division-arrested cells and, moreover, that the trans-activation and replication functions of E2 were separable. Consistent with this hypothesis, amplification of the BPVE2-ts1 genome at the restrictive temperature was still associated with the accumulation of large amounts of nuclear E2 antigen, showing that the mutation did not disrupt nuclear transport or render the E2 protein highly unstable. Furthermore, C127 cells harbouring ts E2 and full-length E1 expression constructs supported transient plasmid replication of a BPV origin vector at the restrictive temperature. These observations imply that E2 functions primarily as a viral replication factor in the vegetative phase of BPV-1 DNA replication, and suggest a fundamental difference in the genetic regulation of stable BPV-1 plasmid DNA replication in mitotic cells and viral DNA amplification in post-mitotic cells.

Introduction

The papillomavirus early-to-late genetic switch is initiated in association with the onset of terminal differentiation of virus-infected keratinocytes in wart tissue (reviewed by Howley, 1991). Viral activation, which occurs in a subpopulation of cells in the spinous layers, is marked by a large amplification of viral DNA in the cell nucleus and, in the case of bovine papillomavirus type 1 (BPV-1), by expression of abundant viral E2 antigen (Burnett et al., 1990). Understanding the molecular mechanism of the switch from latent viral DNA replication in mitotic basal cells to viral DNA amplification in post-mitotic spinous cells represents a central problem in papillomavirus biology, and is also of more general interest as a model for differentiation-specific regulation of gene expression.

The BPV-1 genome persists as a stable multi-copy extrachromosomal nuclear plasmid in bovine or rodent cell lines transformed in vitro by virus infection (Law et al., 1981). It has been postulated that there is an initial transient amplification of the BPV-1 genome shortly after infection of a cell, followed by a period of stable plasmid DNA replication in which each viral plasmid copy replicates once per cell cycle in synchrony with cellular chromosomes (Berg et al., 1986; Botchan et al., 1986). Much effort has been aimed at understanding the mechanism of stable BPV-1 plasmid DNA replication as a model for chromosome replication regulated once per cell cycle. Recent research has defined some of the cis-
and trans-acting viral components involved in the initiation of viral DNA replication. Thus, it has been shown that the full-length viral E1 protein and the full-length viral E2 transcriptional trans-activator protein are both required to initiate BPV-1 DNA replication in a transient replication assay in murine C127 cells (Ustav & Stenlund, 1991), and in an in vitro replication system using a mouse cell nuclear extract supplemented with purified viral proteins (Yang et al., 1991). However, it is unclear, at present, whether these systems monitor the genetic requirements for viral DNA amplification, or the regulated replication of BPV-1 during stable viral plasmid DNA replication.

Amplification of BPV-1 DNA can be induced in virus-transformed murine C127 cell lines containing latent BPV-1 plasmid genomes after exposure of the cells to a prolonged period of growth arrest in confluent culture (Burnett et al., 1989). Viral genome amplification occurs predominantly in giant cells which are blocked in cell division, and which express very high levels of nuclear E2 protein (Burnett et al., 1990). This induction appears to parallel closely the induction of vegetative viral DNA amplification in spinous cells in vivo. It has been suggested that the mechanism of viral DNA amplification in post-mitotic cells may resemble the initial transient amplification of viral DNA which occurs in mitotic cells shortly after infection (Burnett et al., 1989). The latter stages of the vegetative BPV-1 replication phase, in which late proteins are synthesized in highly keratinized epidermal cells, are not reproduced in this culture system. Thus, although the C127 model system does not provide a fully permissive culture system for BPV-1, it does provide an opportunity to examine the regulation of the initial events of the early-to-late switch and to explore the genetic control of viral DNA amplification.

Since previous studies have implied a central role for the full-length E2 protein in BPV-1 DNA amplification in post-mitotic cells (Burnett et al., 1990), we have analysed the role of this gene in viral DNA amplification in this cell culture system. A classical approach to defining the role of specific gene products in complex regulatory pathways is to use conditionally defective mutants. The availability of a mutant BPV-1 genome, BPVE2-TS1, containing a temperature-sensitive (ts) E2 gene (DiMaio & Settleman, 1988) has allowed us to investigate the role of the viral E2 transcriptional trans-activator function in the induction of vegetative BPV-1 DNA amplification.

**Methods**

*Recombinant plasmids.* Mutant BPVE2-TS1 (DiMaio & Settleman, 1988), containing a ts E2 mutation cloned into p142-6 (the full-length BPV-1/pML recombinant plasmid; Sarver et al., 1982), and plasmid p407-Lac (Settleman & DiMaio, 1988), containing an E2-responsive β-galactosidase gene, were kindly provided for these studies by Dr Daniel DiMaio (Department of Human Genetics, Yale University, New Haven, Conn., U.S.A.). The E2 expression vector, pMSV-E2 (Haugen et al., 1988), containing the E2-E5 open reading frames (ORFs) of BPV-1 inserted downstream of the Moloney murine sarcoma virus (MSV) long terminal repeat (LTR), was a gift of Drs T. Haugen and L. Turek, Department of Pathology, University of Iowa, Iowa City, Ia., U.S.A. To induce the ts mutation into this expression vector, the BsrEII-BsXI fragment spanning the E2 gene in the BPVE2-TS1 plasmid was gel-purified and ligated to BsrEII- and BsXI-cleaved pMSV-E2 DNA. Sequence analysis by the method of Sanger et al. (1977), using an end-labelled BPV-1 primer [nucleotides (nt) 3271 to 3290] was done to confirm that the ts mutation was correctly inserted into the expression vector. The construct was named pMSVtsE2.

To construct a BPV-1 origin vector lacking E1 and E2 coding sequences, p142-6 plasmid DNA was digested with endonucleases XmnIII to delete BPV-1 sequences between nt 619 (XmnIII site) and 4450 (BamHI site), and was recircularized by ligiation prior to transformation of competent Escherichia coli HB101 cells. This procedure also resulted in deletion of 564 bp within the tetracycline resistance gene of pML-2 between the BamHI and XmnIII sites. The resulting plasmid (BPVori) retained a single BamHI site.

**Transfection of cells.** Murine C127 cells grown in 5 cm diameter dishes were transfected overnight with 2 μg of recombinant plasmid DNA in 2.5 ml Dulbecco's modification of Eagle's medium (DMEM) by the calcium phosphate coprecipitation procedure (Grham & van der Eb, 1973). The following day, the medium was removed and the cell monolayers were rinsed once with DMEM. The cultures were then incubated at the appropriate temperature (32.5 °C or 37 °C) in DMEM, which was renewed every third day. In transformation assays, cell cultures were fixed at 3 to 4 weeks after transfection and transformed foci were visualized by staining with methylene blue as described elsewhere (Burnett et al., 1988). To isolate transformed cells, foci were taken up with a sterile Pasteur pipette, and were dispersed in separate 2 cm diameter culture dishes. When transformed cells had grown out from these isolated foci, the cells were trypsinized and grown successively in 5 cm diameter dishes and then in 10 cm diameter dishes. For establishment of transformed cells containing monomeric viral plasmid genomes, the DNA for transfection had been cut with BamHI and then ligated at a concentration of 10 μg DNA/ml to promote recircularization.

**Southern blotting and hybridization.** Total cellular DNA was isolated by phenol extraction of SDS-proteinase K-treated cells, essentially as described earlier (Burnett et al., 1989). Purified cell DNA was digested to completion with the restriction endonucleases SacI (no sites in BPV-1) or BamHI (one site in BPV-1). Equal amounts of cleaved cell DNA were electrophoresed in adjacent lanes of a 0.8% (w/v) agarose gel. For two-dimensional (2D) gel electrophoresis, SacI-digested cell DNA was electrophoresed through 0.5% (w/v) agarose (first dimension) followed by electrophoresis through 1-0% (w/v) agarose (second dimension) as described by Dürst (1987). The DNA was then blotted onto a nylon filter by the method of Southern (1975). After u.v.-irradiation to cross-link the DNA to the filter, it was hybridized overnight with a BPV-1 virion DNA probe which had been labelled with 32P-dCTP by random priming. Subsequent washing of the filters was done using stringent conditions (65 °C for 2 h in 30 mM-NaCl, 30 mM-sodium citrate, 0.5% w/v SDS). Autoradiography was for 1 to 4 days using Kodak X-Omat film.

**Hybridization in situ.** As a hybridization probe for in situ hybridization to intact fixed cells, purified BPV-1 virion DNA was labelled with digoxigenin-dUTP by random priming using a kit obtained from
Boehringer Mannheim. Hybridization to fixed cells and subsequent detection of probe DNA was done as described previously (Burnett et al., 1989).

Indirect immunofluorescence analysis. Cells were fixed in situ in culture dishes by treatment for 10 min with methanol which had been pre-cooled to -20 °C. The cell monolayers were then rinsed three times in cold PBS, and 50 μl of rabbit antiserum was applied to the centre of the dish. Subsequent washing and detection steps were as described earlier (Burnett et al., 1990). Primary sera were anti-E2 fusion protein serum (Androphy et al., 1987), used at a dilution of 1:4000 in PBS, or anti-STTG E2 peptide serum at a dilution of 1:200 in PBS (the STTG E2 peptide corresponded to residues 181 to 203 of a full-length E2 protein). Detection of bound rabbit IgG antibody was achieved by using swine anti-rabbit immunoglobulins conjugated with fluorescein isothiocyanate (FITC), at a dilution of 1:80 in PBS. Fluorescence was preserved by mounting the cells in Citifluor antifadant (City University, London, U.K.).

Immunoprecipitation analysis. Confluent cultures of cells in 10 cm diameter dishes were incubated for 3 h at 32.5 °C or 37 °C with [35S]methionine and [35S]cysteine (sp.act. 1000 Ci/mmol) in 1.5 ml serum-free DMEM which lacked unlabelled methionine and cysteine. Immunoprecipitation of cell lysates and gel electrophoretic analysis of immunoprecipitated proteins were done as before (Burnett et al., 1990).

Reporter gene (β-galactosidase) assay. Exponentially growing cells in 10 cm diameter dishes were transfected with 2 μg p407-Lac DNA mixed with 18 μg carrier DNA (sonicated C127 cellular DNA). At 48 to 72 h post-transfection, cell lysates were prepared and β-galactosidase activity was determined by a previously described spectrophotometric assay (Sambrook et al., 1989).

Isolation of stable cell lines harbouring ts E2 and E1 expression vectors. C127 cell lines containing the MSVtsE2 expression vector were isolated after transfection of cells with pMSVtsE2 plasmid DNA and subsequent selection by morphological transformation induced by the linked BPV-1 E5 transforming gene. After establishment of cell lines from single transformed foci, expression of E2 was assessed by immunofluorescence and/or immunoprecipitation analysis as described above. To introduce the E1 gene into cells expressing the ts E2 protein, a representative pMSVtsE2-transformed cell line was transfected with an expression vector containing the full-length E1 gene under the control of the MSV LTR regulatory sequences (Schiller et al., 1989), and transfected cells were isolated by biochemical selection for the cotransfected neomycin resistance plasmid, pSV2-neo (Southern & Berg, 1982). Selection was done in medium containing G418 at a final concentration of 1 mg/ml. Neomycin-resistant cell lines were then screened for the presence of stably incorporated E1 expression vector by Southern blotting analysis of NsiI-cleaved total cellular DNA using a BPV-1 E1 region probe labelled with [32P]dCTP by random priming. A cell line (C127/E1/tsE2) containing multiple (> 20) copies of the intact E1 gene was selected for use in transient replication experiments.

Transient DNA replication assay. Exponential phase C127/E1/tsE2 cells in a 10 cm diameter culture dish were transfected with 2 μg BPVori plasmid DNA mixed with 18 μg carrier (sonicated C127 cell DNA) as described above but including a shock with 20% (v/v) glycerol in DMEM for 90 s at 4 h post-transfection. Parent C127/tsE2 or normal C127 cells were transfected in parallel as controls. At 12 to 16 h after transfection the cultures were then trypsinized and seeded into three 10 cm diameter dishes. Low Mₖ total cellular DNA was then prepared from parallel cultures by the method of Hirt (1967) at 24, 48 or 72 h after trypsinization. The low Mₖ DNA preparations were treated with a combination of the restriction endonucleases BamHI and DpnI prior to Southern blot analysis with a BPV-1 late gene region probe (BPV-1 nt 4450 to 6958), essentially as described by Ustav & Stenlund (1991).

Results

Transformation of C127 cells by BPVE2-ts1 DNA

The isolation and properties of the BPVE2-ts1 mutant genome have been described previously (DiMaio & Settlemen, 1988). The mutation consisted of an insertion of four amino acid residues (Pro-Arg-Ser-Arg) within the E2 coding sequence between residues 181 and 182 of the wild type (wt) protein. The full-length BPV genome harbouring this mutation has been reported to have wt transformation and stable plasmid DNA replication properties in C127 cells at the non-restrictive temperature (32.5 °C), and to be defective for both cell transformation and stable plasmid DNA replication at the restrictive temperature (37 °C).

In keeping with the data of DiMaio & Settlemen (1988), we found that there was a complete block to the induction of C127 cell transformation in transfection experiments with the ts mutant viral genome at 37 °C (data not shown). We observed neither microscopic nor macroscopic cell morphological transformation during observation periods of up to 6 weeks for BPVE2-ts1-transfected cell cultures at the restrictive temperature, indicating that there was a tight block to the expression of the E2 trans-activator function. In contrast, the ts mutant genome efficiently induced cell-transformation at 32.5 °C. Mutant BPV genomes harbouring deletion mutations within the E2 gene were also defective for cell transformation, confirming that a functional E2 trans-activator protein was necessary for the induction of cell transformation under our assay conditions (data not shown).

To isolate C127 cell lines in which the BPVE2-ts1 genome was established as a stable plasmid, cells were transformed by transfection with the mutant viral genome at 32.5 °C. To ensure that the resulting cell lines contained intact copies of the circular viral plasmid, the viral genome was first excised from the bacterial plasmid vector and then recircularized by ligation at low DNA concentration prior to transfection. Four independently arising foci of transformed C127 cells which developed at 32.5 °C after transfection with the recircularized ts mutant viral DNA were picked and established as cell lines. Cells transformed by the ts mutant viral genome had a transformed morphology indistinguishable from that of wt BPV-1-transformed cells (Fig. 1, panels a and c). When such ts mutant-transformed cells were shifted to the restrictive temperature (37 °C) there was complete reversion of the cell morphology within 24 h to a flat non-transformed shape (Fig. 1, panel b), similar to that of non-transfected C127 cells (Fig. 1, panel d). To analyse the physical status of the mutant viral genome in these transformed cell lines when grown at 32.5 °C, total cell
Fig. 1. Morphology of BPVE2-tsl-transformed cells. Murine C127 cells were transfected with BPVE2-tsl DNA (excised from the pML vector with BamHI and recircularized with DNA ligase) at 32.5 °C, and cell lines were established from individual transformed foci at this temperature. Panels a and b, ts-1 cell line grown at 32.5 °C (panel a) or incubated for 24 h at 37 °C (panel b). Panel c, control wt BPV-1-transformed C127 cell line; panel d, normal uninfected C127 cells.

DNA was isolated from each ts mutant-transformed cell line after an estimated 20 population doublings at the non-restrictive temperature, and was analysed by the Southern blot hybridization technique using a 32P-labelled BPV-1 virion DNA probe to detect virus-specific DNA sequences. The cell DNA was treated with a restriction endonuclease (SacI) that does not cleave the mutant viral DNA, to ascertain whether unrearranged circular viral genomes could be detected, or it was treated with a second enzyme (BamHI) that cuts the viral DNA at a single site, to compare the copy number with that of the wt genome in control cells. As shown in Fig. 2 (a, lanes 3 and 4), both ts mutant-transformed cell lines tested contained circular viral plasmid DNA forms without detectable rearrangements, and the copy number was similar to that of a control wt BPV-1-transformed cell line (lane 2).

We also analysed the replication properties of the mutant viral genome at the restrictive temperature (37 °C). These two ts mutant-transformed cell lines were grown at 37 °C for 2 weeks in exponential phase culture and were then analysed by Southern blotting to examine the physical status of the ts viral genome and the copy number relative to control cells grown at the non-restrictive temperature. As shown in Fig. 2(a), for one line (ts-3) there was a reduction in the copy number of the ts genome to an undetectable level (lanes 6 and 12), suggesting that these cells had been cured of the viral genome by passaging at the restrictive temperature. In the second line (ts-1), viral DNA was still present at approximately the same copy number as in cells grown at 32.5 °C, but the viral sequences now migrated predominantly as a diffuse band at approximately the same position as form II (nicked circular DNA) in this analysis (lane 5). It was thus unclear whether the viral DNA sequences were retained in these cells in an integrated or an extrachromosomal state at the non-permissive temperature.

To investigate further the physical status of the viral DNA in these ts-1 cells when grown at the permissive and non-permissive temperatures, we analysed SacI-digested DNA samples by 2D gel electrophoresis in combination with Southern blotting as described by Dürst (1987). As shown in Fig. 2(b), analysis of total cell DNA extracted from ts-1 cells grown at the permissive temperature revealed two distinct arcs, indicating the
Role of E2 protein in BPV-1 replication

The role of the E2 protein in BPV-1 replication was studied. Fig. 2 shows autonomous plasmid replication properties of the BPVE2-tsl genome. (a) Total cell DNA was cleaved with SacI (lanes 1 to 6) or BamHI (lanes 7 to 12) and was subjected to Southern blot hybridization analysis with a radioactively labelled BPV-1 DNA probe. Lanes 1 and 7, control C127 cell DNA; lanes 2 and 8, control wt BPV-1-transformed cell line; lanes 3 and 9, ts-1 cell line grown at 32.5 °C; lanes 4 and 10, ts-3 cell line grown at 32.5 °C; lanes 5 and 11, ts-1 cell line passaged at 37 °C; lanes 6 and 12, ts-3 cell line passaged at 37 °C. The positions of supercoiled (FI), nicked circular (FII) and linear (FIII) virus DNA are indicated at the right-hand side of the figure. (b) 2D gel electrophoretic analysis of viral DNA sequences in ts-I cells at 32.5 °C and 37 °C. Equal amounts of SacI-treated total cell DNA from cells grown at each temperature were run on a 0.5% (w/v) agarose gel. After visualization with ethidium bromide the first-dimension lanes were excised, and were then electrophoresed in the second dimension through a 1.0% agarose gel containing 200 μg/ml ethidium bromide. The separated DNAs were then blotted onto Pall nylon filter membranes and were hybridized with a 32P-labelled BPV-1 virion DNA probe prepared by random priming. Autoradiographic exposure time was 4 days.

The presence of both integrated and plasmid viral DNA sequences. The arc that migrated further in the second dimension was associated with the cellular chromosomal DNA as revealed by ethidium bromide staining (not shown). In sharp contrast to the situation at the permissive temperature, the viral DNA sequences present in ts-I cells after growth at the restrictive temperature (37 °C) were almost exclusively associated with the cellular DNA sequences, implying that there was a selection for cells containing a higher copy number of integrated viral DNA genomes during growth at 37 °C. The conclusion that the viral DNA was present predominantly in an integrated state in these cells after growth at 37 °C was also supported by restriction endonuclease analysis with additional no-cut enzymes (SacII, SalI and XhoI). For each enzyme a unique pattern of high Mr fragments was observed by Southern blotting analysis (data not shown). Our results therefore appear to confirm the previous finding that the ts mutant was defective for stable autonomous plasmid DNA replication at the restrictive temperature.

Amplification of the ts mutant at the restrictive temperature

We then determined whether the mutant genome was defective also for DNA amplification in post-mitotic cells. Our strategy to test for viral DNA amplification
Fig. 3. Amplification of BPVE2-tsl genome at the restrictive temperature. (a) In situ hybridization analysis with a digoxygenin-labelled BPV-1 DNA probe was done to examine the potential of the BPVE2-tsl genome to undergo amplification at the restrictive temperature. Mutant ts-transformed cells (line ts-1) were growth-arrested at the restrictive temperature (37 °C) or at the non-restrictive temperature (32.5 °C) by serum deprivation. At 12 days after removal of serum, the cultures were restimulated with serum for 48 h, after which time the cultures were fixed and processed for in situ hybridization. Panel a, ts-1 cell line, 32.5 °C; panel b, ts-1 cell line, 37 °C. Control cultures included wt BPV-l-transformed cells treated in parallel under the same conditions of serum deprivation and restimulation (panel d). Additional controls included ts-1 cells maintained in exponential phase at 32.5 °C (panel c), and non-transformed C127 cells (panel e). Note that the sensitivity of the in situ technique as used here is not sufficient to detect latent viral plasmid genomes in the majority of non-responsive cells within a culture. Positive cells contain > 1000 copies of the viral genome (Burnett et al., 1989). (b) Detection of mutant viral DNA amplification by Southern blot analysis. Total cell DNA was digested with BamHI (one site in BPV-1 DNA) and equal amounts of each sample were separated in an 0.8% agarose gel prior to transfer onto a nylon membrane and hybridization with a 32P-labelled BPV-1 DNA probe. Lanes 1 and 2, exponential phase and growth-arrested wt BPV-1-transformed cell line, respectively; lanes 3 and 4, exponential phase (32.5 °C) or induced (37 °C) ts-1 cell line; lanes 5, 6 and lanes 7, 8, ts-2 and ts-3 cell lines uninduced (lanes 5 and 7) and induced (lanes 6 and 8), respectively.
Role of E2 protein in BPV-1 replication

Fig. 4. Detection of E2 antigen at the restrictive temperature. (a) Induction of abundant E2 protein expression in giant cell nuclei at the restrictive temperature was monitored by indirect immunofluorescence analysis with polyclonal anti-E2 fusion protein serum. Cells containing the BPVE2-tsl or wt viral genomes were growth-arrested by serum deprivation and were fixed in situ with methanol after 12 days of growth arrest. The cells were incubated for 30 min at room temperature with E2 antiserum diluted 1:4000 in PBS. After extensive washing with PBS, specifically bound rabbit IgG antibodies were detected with FITC-conjugated anti-rabbit IgG antibody (Dakopatts) as described previously (Burnett et al., 1990). Panels a and b, positively stained giant cells induced by serum deprivation of ts-1 cell line at 32.5 °C and 37 °C, respectively. Panels c and d, giant cells induced by serum deprivation of wt BPV-1-transformed cell line at 32.5 °C and 37 °C, respectively. Panel e, control uninfected C127 cells tested with E2 serum. Panel f, serum-deprived ts-1 cell line tested with preimmune serum. (b) Immunoprecipitation analysis of ts E2 protein expressed at the restrictive temperature. Cell lines harbouring BPVE2-tsl (lanes 3 and 4) or wt BPV-1 (lanes 1 and 2) were serum-deprived for 12 days and were then labelled with [35S]methionine and [35S]cysteine for 3 h at 37 °C in serum-free medium. Cell lysates were then immunoprecipitated with antiserum to E2 peptide (STTG, lanes 2 and 4) or were tested with preimmune serum (lanes 1 and 3). The positions of Mr markers run in lane 5 are shown at the right-hand side of the figure.

Results for amplification were obtained in each of three ts-transformed cell lines tested by in situ hybridization analysis at the restrictive temperature.

To confirm and extend the results obtained from in situ hybridization experiments, we also performed Southern blotting analysis on total cellular DNA extracted from unfractionated cell cultures to attempt to quantify the level of ts mutant genome amplification at the restrictive temperature. We compared the viral genome copy number in ts mutant-transformed cell lines grown in exponential phase at 32.5 °C (as a base line) with the copy number after the period of serum deprivation and serum stimulation as used in the in situ experiments. Amplification of the tsE2 mutant genome was observed by this method for each of three ts-transformed cell lines tested (Fig. 3b, lanes 3 to 8). The level of amplification varied from three to fivefold for different cell lines. This level of amplification was 25 to 30% of that routinely observed for amplification of the wt BPV genome under the same assay conditions (lanes 1 and 2). There were probably several factors which contributed to this reduced level of amplification compared to wt virus-infected cells. Firstly, as demonstrated by in situ analysis, the level of DNA amplification attained in individual giant cells and the frequency of induction of permissive cells were reduced slightly relative to wt BPV-1 infection. Secondly, our assay conditions require a period of serum stimulation (36 to 48 h) to induce maximal viral DNA amplification. During this prolonged serum stimulation the majority of non-responding mitotic cells have undergone at least one complete cell cycle. As shown above, the copy number of the ts mutant may rapidly fall in mitotic cells at the restrictive temperature. Therefore, since we are measuring the average copy number in a Southern blot analysis (no attempt is made to purify the giant cell subpopulation), it is likely that the signal

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\caption{Detection of E2 antigen at the restrictive temperature. (a) Induction of abundant E2 protein expression in giant cell nuclei at the restrictive temperature was monitored by indirect immunofluorescence analysis with polyclonal anti-E2 fusion protein serum. Cells containing the BPVE2-tsl or wt viral genomes were growth-arrested by serum deprivation and were fixed in situ with methanol after 12 days of growth arrest. The cells were incubated for 30 min at room temperature with E2 antiserum diluted 1:4000 in PBS. After extensive washing with PBS, specifically bound rabbit IgG antibodies were detected with FITC-conjugated anti-rabbit IgG antibody (Dakopatts) as described previously (Burnett et al., 1990). Panels a and b, positively stained giant cells induced by serum deprivation of ts-1 cell line at 32.5 °C and 37 °C, respectively. Panels c and d, giant cells induced by serum deprivation of wt BPV-1-transformed cell line at 32.5 °C and 37 °C, respectively. Panel e, control uninfected C127 cells tested with E2 serum. Panel f, serum-deprived ts-1 cell line tested with preimmune serum. (b) Immunoprecipitation analysis of ts E2 protein expressed at the restrictive temperature. Cell lines harbouring BPVE2-tsl (lanes 3 and 4) or wt BPV-1 (lanes 1 and 2) were serum-deprived for 12 days and were then labelled with [35S]methionine and [35S]cysteine for 3 h at 37 °C in serum-free medium. Cell lysates were then immunoprecipitated with antiserum to E2 peptide (STTG, lanes 2 and 4) or were tested with preimmune serum (lanes 1 and 3). The positions of Mr markers run in lane 5 are shown at the right-hand side of the figure.}
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Fig. 5. Properties of ts E2 protein expressed from a heterologous promoter. (a) Immunofluorescence analysis with E2 antiserum was done on MSVtsE2- (panels a and b) or MSVwtE2- (panels c and d) transformed cell lines at 32.5 °C (panels a and c) or at 37 °C (panels b and d). Panel e shows control non-transfected parent C127 cells tested with E2 antiserum. (b) Immunoprecipitation analysis of ts E2 protein in cells stably transformed by MSVE2 vectors. MSVwtE2- or MSVtsE2-transformed cell lines grown at 32.5 °C (lanes 3 to 6) or at 37 °C (lanes 7 to 10) were analysed by immunoprecipitation analysis with TTSG anti-E2 peptide serum (lanes 4, 6, 8 and 10) or preimmune serum (lanes 3, 5, 7 and 9). Non-infected parent C127 cells were also subjected to immunoprecipitation with immune (lane 2) or preimmune (lane 1) serum. The sizes of Mr markers run in lane M are shown at the left-hand side. The arrow at the right-hand side indicates the immunoprecipitated full-length E2 protein. (c) E2 trans-activator activity in MSVtsE2-transformed cells. C127 cells stably
contributed by viral DNA amplification in giant cells is balanced partly by a concomitant downward fluctuation in the plasmid copy number in the majority of non-responsive small cells within the culture.

The data presented above thus revealed a remarkable property of the ts E2 mutant viral genome: whereas this mutant was defective for stable autonomous plasmid DNA replication at the restrictive temperature, it was still capable of significant amplification at this temperature. This result indicated that there was a fundamental difference in the regulation of expression of viral DNA replication factors between mitotic cells and post-mitotic permissive cells.

Expression of ts E2 protein at the restrictive temperature

In a previous study we have shown by indirect immunofluorescence analysis with E2 antisera that there is a profound induction of E2 protein expression in the permissive giant cell population in this replication system, implying a central role for E2 in viral DNA amplification (Burnett et al., 1990). Our results to this point appeared to rule out a requirement for the trans-activating function of E2 in viral DNA amplification in post-mitotic cells. However, a clear prediction of the hypothesis that full-length E2 protein was necessary for viral DNA amplification was that the ts E2 protein should still be produced at the restrictive temperature. The point of action of the ts E2 mutation has not been defined previously. In theory the mutation could inhibit expression of the trans-activator function at several levels. For example, the mutation might prevent efficient translation of E2 protein, or it might alter the stability of the E2 protein, its transport to the cell nucleus, or prevent E2 from adopting a conformation necessary for its function in transcriptional trans-activation. It was therefore important to ascertain whether the E2 protein was expressed in permissive giant cells at the restrictive temperature.

To examine expression of the ts E2 protein, immunofluorescence and immunoprecipitation analyses were done. We found that abundant nuclear E2 antigen was present in this cell subpopulation at the restrictive temperature (Fig. 4a, panel b). The patterns of nuclear E2 antigen distribution observed for cells harbouring the ts mutant were similar to those of giant cells containing the wt genome (Fig. 4a, panel d). These included homogeneous punctate staining throughout the nucleus and/or large aggregates of E2 antigen concentrated at one or several sites within the nucleus. Similar immunofluorescence patterns were observed with serum raised either to an E2 fusion protein containing the C-terminal two-thirds of the E2 ORF, or with serum raised against a synthetic peptide (STTG: amino acid residues 181 to 203 of E2) located within the central region of E2 upstream of the common early mRNA splice-acceptor signal at nt 3225 (McBride et al., 1988). As described previously, sera specific for the N terminus of E2 have not been informative in immunofluorescence analyses (Burnett et al., 1990). For this reason we have been unable to show unequivocally by this method that the abundantly expressed E2 protein in the giant cell subpopulation consists predominantly of full-length E2 protein rather than the 31K E2 transcriptional repressor protein initiated from an internal ATG codon.

As shown in Fig. 4(b), the 48K and 31K E2 protein species were detected by immunoprecipitation analysis under these assay conditions in both ts mutant (lane 4) and wt (lane 2) BPV-1-transformed cells. This result was consistent with the idea that synthesis of the ts E2 protein was not inhibited at the restrictive temperature. A slight difference in the electrophoretic mobility of the 48K and 31K E2 species was observed in these two cell lines, presumably due to the four amino acid residue insertion, and this mobility shift was marginally more pronounced for the shorter protein. As noted previously, the 28K E8/E2 protein was also immunoprecipitated with this anti-peptide serum, although the STTG epitope is located upstream of the E2 coding sequence present in this spliced gene product. This appears to demonstrate the existence of complex formation between different species of the E2 protein within the cell (Burnett et al., 1990).

Properties of ts E2 protein overexpressed from a strong heterologous promoter

The results described above suggested that the full-length ts E2 protein was synthesized at the restrictive temperature and accumulated in the cell nucleus, implying that the ts defect of the mutant E2 protein was due to something other than an effect on protein synthesis, half-

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*Contributed by viral DNA amplification in giant cells is balanced partly by a concomitant downward fluctuation in the plasmid copy number in the majority of non-responsive small cells within the culture.*

*The data presented above thus revealed a remarkable property of the ts E2 mutant viral genome: whereas this mutant was defective for stable autonomous plasmid DNA replication at the restrictive temperature, it was still capable of significant amplification at this temperature. This result indicated that there was a fundamental difference in the regulation of expression of viral DNA replication factors between mitotic cells and post-mitotic permissive cells.*

**Expression of ts E2 protein at the restrictive temperature**

In a previous study we have shown by indirect immunofluorescence analysis with E2 antisera that there is a profound induction of E2 protein expression in the permissive giant cell population in this replication system, implying a central role for E2 in viral DNA amplification (Burnett et al., 1990). Our results to this point appeared to rule out a requirement for the trans-activating function of E2 in viral DNA amplification in post-mitotic cells. However, a clear prediction of the hypothesis that full-length E2 protein was necessary for viral DNA amplification was that the ts E2 protein should still be produced at the restrictive temperature. The point of action of the ts E2 mutation has not been defined previously. In theory the mutation could inhibit expression of the trans-activator function at several levels. For example, the mutation might prevent efficient translation of E2 protein, or it might alter the stability of the E2 protein, its transport to the cell nucleus, or prevent E2 from adopting a conformation necessary for its function in transcriptional trans-activation. It was therefore important to ascertain whether the E2 protein was expressed in permissive giant cells at the restrictive temperature.

To examine expression of the ts E2 protein, immunofluorescence and immunoprecipitation analyses were done. We found that abundant nuclear E2 antigen was present in this cell subpopulation at the restrictive temperature (Fig. 4a, panel b). The patterns of nuclear E2 antigen distribution observed for cells harbouring the ts mutant were similar to those of giant cells containing the wt genome (Fig. 4a, panel d). These included homogeneous punctate staining throughout the nucleus and/or large aggregates of E2 antigen concentrated at one or several sites within the nucleus. Similar immunofluorescence patterns were observed with serum raised either to an E2 fusion protein containing the C-terminal two-thirds of the E2 ORF, or with serum raised against a synthetic peptide (STTG: amino acid residues 181 to 203 of E2) located within the central region of E2 upstream of the common early mRNA splice-acceptor signal at nt 3225 (McBride et al., 1988). As described previously, sera specific for the N terminus of E2 have not been informative in immunofluorescence analyses (Burnett et al., 1990). For this reason we have been unable to show unequivocally by this method that the abundantly expressed E2 protein in the giant cell subpopulation consists predominantly of full-length E2 protein rather than the 31K E2 transcriptional repressor protein initiated from an internal ATG codon.

As shown in Fig. 4(b), the 48K and 31K E2 protein species were detected by immunoprecipitation analysis under these assay conditions in both ts mutant (lane 4) and wt (lane 2) BPV-1-transformed cells. This result was consistent with the idea that synthesis of the ts E2 protein was not inhibited at the restrictive temperature. A slight difference in the electrophoretic mobility of the 48K and 31K E2 species was observed in these two cell lines, presumably due to the four amino acid residue insertion, and this mobility shift was marginally more pronounced for the shorter protein. As noted previously, the 28K E8/E2 protein was also immunoprecipitated with this anti-peptide serum, although the STTG epitope is located upstream of the E2 coding sequence present in this spliced gene product. This appears to demonstrate the existence of complex formation between different species of the E2 protein within the cell (Burnett et al., 1990).

**Properties of ts E2 protein overexpressed from a strong heterologous promoter**

The results described above suggested that the full-length ts E2 protein was synthesized at the restrictive temperature and accumulated in the cell nucleus, implying that the ts defect of the mutant E2 protein was due to something other than an effect on protein synthesis, half-
life or intracellular targeting. This would be compatible with the hypothesis that E2 acts as a viral replication factor during viral DNA amplification, and furthermore suggested that the transcriptional trans-activating and replication functions of E2 were separable. Nevertheless, it could still be argued that the transcriptional trans-activating defect of the ts E2 protein was overcome by expression of the mutant protein at high level. We therefore designed experiments to assess the effectiveness of the block to trans-activation when the full-length ts E2 protein was expressed in C127 cells in large amounts, and to examine its ability to support viral DNA replication in combination with an E1 replication factor.

To overexpress the ts E2 gene, we chose to modify a previously constructed E2 expression vector containing the MSV promoter upstream of the whole E2 gene (Haugen et al., 1988), since we have found that this vector efficiently expresses E2 in mouse cells (unpublished results). The ts mutation was inserted into the MSV-E2 vector as described in Methods.

We next developed stable C127 cell lines expressing ts E2 or wt E2 proteins from these MSV vectors. For this we exploited the ability of these vectors to induce morphological cell transformation by expression of the viral E5 transforming gene which is located immediately downstream of E2 and is retained in these vectors. Foci of transformed cells induced at 37 °C after transfection with MSVtsE2 or MSVwtE2 vectors were picked at 3 weeks post-transfection and were established as cell lines. Each of these cell lines expressed E2 protein at high level, as shown by intense staining of the cell nucleus in indirect immunofluorescence analyses with E2 antisera (Fig. 5a, panels a to d). We observed a slight reduction in the level of nuclear ts E2 antigen expressed in these lines when compared at the restrictive and non-restrictive temperatures (Fig. 5a, panels a and b). However, this effect was observed also for wt E2 protein (Fig. 5a, panels c and d) indicating that this apparently reduced level of expression was not due to the ts mutation. The results of an immunoprecipitation analysis on these cell lines confirmed that full-length ts E2 protein was synthesized efficiently at both 32.5 °C and at 37 °C (Fig. 5b, lanes 6 and 10, respectively). Furthermore, this analysis showed that no detectable truncated E2 species were expressed from the MSV expression vector, confirming that the nuclear E2 protein detected by immunofluorescence analysis in these cells consisted exclusively of the full-length ts E2 protein. These results constituted further evidence that the full-length ts E2 protein could be stably expressed at the restrictive temperature.

To test the effectiveness of the block to transcriptional trans-activation in C127 cell lines overexpressing ts E2 protein, transfection experiments were done with an E2-responsive reporter gene. Cells stably expressing ts E2 protein at 37 °C were transfected with p407-Lac (Settleman & DiMaio, 1988), which contains a bacterial β-galactosidase coding sequence regulated by the minimal simian virus 40 promoter and almost the entire upstream regulatory region of BPV-1 (nt 6938 to 1) containing the strong E2-dependent enhancer, E2RE1 (Spalholz et al., 1987). As shown in Fig. 5(c) there was almost complete inhibition of E2 trans-activator activity in these cells at the restrictive temperature, whereas at 32.5 °C the wt E2- and ts E2-expressing cell lines exhibited similar levels of trans-activation. Thus, there appeared to be a tight block to transcriptional trans-activation at 37 °C even when the ts E2 protein was overexpressed in C127 cells.

Finally, to test whether the ts E2 protein could function in combination with an E1 replication factor to support replication of a BPVori plasmid, we established a cell line containing both ts E2 and E1 expression vectors as described in Methods. These cells were then transfected at 37 °C with a BPVori plasmid which lacked E1 and E2 coding sequences (Methods) and replicated BPVori plasmid DNA was sought by assaying for resistance to cleavage with the endonuclease DpnI, essentially as described by Ustav & Stenlund (1991). As shown in Fig. 5(d), DpnI-resistant BPVori ori vector DNA accumulated in cells harbouring ts E2 and E1 expression vectors (C127/E1/tsE2), but was not detected in the parent ts E2-expressing cell line (C127/tsE2), nor in normal C127 cells. These results were therefore consistent with the proposal that the full-length ts E2 protein was functional as a viral replication factor in combination with E1, under conditions in which the trans-activator activity of E2 was inhibited.

Discussion

On the basis of the results presented in this paper we propose that the BPV-1 E2 protein functions primarily as a virus-specific replication factor during amplification of the viral genome in post-mitotic cells. Thus, we found only a moderate reduction in the frequency of giant cell formation and in the level of amplification of the BPV2-ts1 genome compared to wt BPV-1 at the restrictive temperature. Consistent with the above hypothesis, we found that the ts mutation did not affect nuclear targeting of E2 nor did it render E2 highly unstable at the restrictive temperature. Furthermore, we found no evidence for significant 'leakiness' of the transcriptional block when the ts E2 protein was overexpressed from a strong heterologous promoter. These results indicate that the role of E2 in viral DNA amplification does not require the transcriptional trans-activator function of E2. Moreover, our results indicate
that these functions of a full-length E2 protein can be dissociated.

The BPV-1 E2 factors have been shown to regulate BPV-1 early gene expression by binding to specific sequence elements within the viral genome (Spalholz et al., 1985, 1987; Androphy et al., 1987). The full-length E2 protein trans-activates transcription from multiple early promoters, whereas the truncated E2 factors (E8/E2 and E2TR) repress transcription, either by competing with full-length E2 for binding to viral DNA or by forming heterodimers inactive in transcription (McBride et al., 1989b). The role of E2 in viral DNA replication in mitotic cells may be complex: in addition to being an essential replication factor, E2 may also be required to induce expression of another viral replication factor (e.g. E1). The E2 trans-activator protein might also regulate its own expression (Hermonat et al., 1988; Spalholz et al., 1991). Our results imply that the inability of the BPVE2-tsl genome to continue replicating as a latent plasmid in mitotic cells when switched to the restrictive temperature may be due primarily to a requirement for the trans-activator function of E2 to induce expression of essential viral replication factors, rather than to the loss of an E2 replication function. In contrast, the capacity of the BPVE2-tsl genome to amplify at the restrictive temperature argues for a fundamentally different genetic regulation of viral DNA amplification when compared to latent viral DNA replication. In this case it appears that the trans-activator function is not necessary for the induction of expression of essential viral replication factors.

A total of 17 E2 binding sites with widely differing affinities for the E2 protein have so far been mapped within the viral early region (Li et al., 1989). A cluster of four E2-binding sites within the upstream regulatory region (E2RE1) constitutes a strong E2-responsive enhancer element. Indirect evidence for a role for E2 in viral DNA replication first came from the mapping of a latent viral plasmid origin of bidirectional replication within E2RE1 (Yang & Botchan, 1990) and from the detection of abundant E2 expression in cells permissive for viral DNA amplification (Burnett et al., 1990). The observation that the full-length E2 protein formed a specific complex with a full-length E1 protein (the presumed replication initiation protein of BPV-1) led to the hypothesis that E2 might function in replication by targeting the E1 replication factor to a viral replication origin via the DNA-binding specificity of E2, since E1 itself appeared to lack strong site-specific DNA-binding activity (Mohr et al., 1990). However, the position of an E1/E2-dependent replication origin did not correspond to the position of E2RE1. Instead a new origin region has been defined in recently developed in vivo and in vitro viral replication systems (Ustav et al., 1991; Yang et al., 1991). This origin region overlaps the HpaI restriction site at nt 1 in the BPV-1 sequence, and E1 has very recently been shown to bind to this site in vitro (Wilson & Ludes-Meyer, 1991; Ustav et al., 1991). The position of a viral replication origin(s) utilized during amplification of the viral genome in post-mitotic C127 cells has not been mapped, but it is interesting to note that amplification of BPV-1 DNA in this system is associated with a promoter-switch at a pair of closely spaced transcriptional promoters precisely bordering the HpaI site (Burnett et al., 1990). Further studies are required to determine whether viral DNA amplification and viral plasmid maintenance replication modes involve the utilization of functionally separate replication origins.

Previous studies have shown that E2 N-terminal amino acid sequences are necessary for E2 to function in transcriptional trans-activation and in viral DNA replication (Haugen et al., 1988; McBride et al., 1989b; Ustav & Stenlund, 1991), thus implying that the role of E2 in these processes may involve a common interaction(s). Consequently, it has been suggested that the requirement for E2 in BPV-1 DNA replication may mirror the role of cellular transcription factors in host cell chromosome replication (Yang et al., 1991). Our results strongly indicate that the full-length E2 protein has functionally distinct roles in the activation of transcription and of viral DNA replication. Several earlier characterized properties of E2 protein include homodimer formation (McBride et al., 1988), sequence-specific DNA-binding activity (Androphy et al., 1987), complex formation with the cellular transcription factor Sp1 (Li et al., 1991) or the viral E1 protein (Mohr et al., 1990), post-translational modification in vivo by phosphorylation (McBride et al., 1989a), the potential to stimulate looping between widely separated transcription factor binding sites (Knight et al., 1991), and to induce DNA bending (Moskaluk & Bastia, 1988). In theory, the ts defect of the mutant E2 protein could disturb any of these interactions. Further study of the ts E2 protein may therefore help to elucidate the mechanism of E2-induced transcriptional trans-activation, and to identify those specific properties of E2 required for its function as a viral replication factor.

Our findings highlight another fundamental question concerning the regulation of viral gene expression: what regulates E2 overexpression in the absence of a functional E2 trans-activator? This is a particularly intriguing question, since it has been proposed that E2 acts as a ‘master regulator’ of viral early gene expression (Szymanski & Stenlund, 1991). Alternatively, recent results suggest that E2 trans-activator expression in mitotic cells may be largely regulated by cellular factors via a ‘constitutive’ enhancer within the viral upstream regulatory region (Van de Pol & Howley, 1992).
results argue against autoinduction of viral early gene expression by an altered balance of E2 transcriptional repressor and activator factors in growth-arrested cells. Instead, the possibility is raised that E2 expression is induced by a change in the cellular transcriptional machinery in the subpopulation of division-arrested cells in which viral DNA amplification occurs. For example, this may involve a differentiation event analogous to that which occurs when transformed basal keratinocytes become committed to terminal differentiation in vivo. Further study of this experimental system may lead to the identification of cellular factors and viral cis-elements which regulate the viral early-to-late switch and may provide a means for identifying common host factors involved in the process of cellular differentiation.

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References


Role of E2 protein in BPV-1 replication


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