Immunofluorescent detection of bovine papillomavirus E4 antigen in the cytoplasm of cells permissive in vitro for viral DNA amplification

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The E4 gene of several human papillomavirus types is expressed in association with vegetative viral DNA synthesis in differentiated epidermal cells. To develop reagents to study expression of the bovine papillomavirus type 1 (BPV-1) E4 gene in warts and in virus-transformed cell lines, rabbit polyclonal antiserum was raised to the BPV-1 E4 antigen produced as a fusion polypeptide in *Escherichia coli*. By immunoblotting analysis of productively infected bovine fibropapilloma tissue, E4-related proteins of 16K, 21K, 30K and 42K were detected. In some but not all C127 cell lines transformed by BPV-1 or by a replication-competent BPV-1 deletion mutant, cytoplasmic E4 antigen with a predominantly perinuclear localization was detected by immunofluorescence analysis in a subpopulation of cells in stationary-phase cultures. The E4-expressing cells were identified by their grossly enlarged size to represent the same cell subpopulation shown earlier to support BPV-1 DNA amplification. The observation of synthesis of the E4 protein in association with viral DNA amplification in this system provides further evidence that there is a switch in viral early region gene expression in a subpopulation of division-arrested cells, which may accurately reflect events occurring during the vegetative phase of BPV-1 replication in terminally differentiated cells in vivo.

Papillomaviruses undergo vegetative replication in the nuclei of terminally differentiated cells within a wart. Little is known about the regulation of the late phase of replication of these viruses, since it has not yet been possible to obtain vegetative growth of any papillomavirus in virus-infected cells in vitro. The E4 gene of human papillomavirus type 1 (HPV-1) is expressed to an extremely high level (up to 30% of total detergent-soluble proteins) in plantar wart tissue, wherein the E4 antigen was localized to the cytoplasm of cells permissive for vegetative HPV-1 DNA synthesis, implying a role for this early region gene in the late phase of the viral replication cycle (Doorbar et al., 1986; Breitburd et al., 1987). The function of this gene is unknown, yet it seems unlikely that the E4 protein is a component of the mature virion, in view of its predominantly cytoplasmic localization. A similar pattern of E4 antigen expression was observed for other HPV types, and it is therefore probable that this gene has a common function in most, if not all, papillomaviruses (Doorbar et al., 1989). Consistent with its proposed role as a 'late' gene, mutations designed to abolish expression of the bovine papillomavirus (BPV-1) E4 gene had no apparent effect on either the transforming activity of the viral DNA, or on its ability to replicate as a latent plasmid in mouse C127 cells (Neary et al., 1987). A spliced BPV-1 E4 mRNA species was identified as a major viral transcript in a cDNA library prepared using RNA from a productively infected bovine fibropapilloma (Baker & Howley, 1987). The E4 mRNA was transcribed from the same promoter as a spliced L1 (major capsid protein) mRNA. This promoter was utilized neither in the fibroma portion of the wart nor in BPV-1-transformed C127 cell lines, and was therefore denoted the late promoter, P_l. Its activity was proposed to be dependent upon the differentiation state of the cell (Baker & Howley, 1987). We describe here the preliminary characterization of the BPV-1 E4 gene products expressed in naturally infected bovine fibropapilloma tissue, and we also report the detection of cytoplasmic E4 antigen associated with the induction of vegetative-like BPV-1 DNA amplification in virus-transformed mouse C127 cell lines.

To obtain purified E4 protein for immunization of rabbits, its gene was expressed in *Escherichia coli* as a fusion polypeptide. A 511 bp *Sau3A* fragment (BPV-1 nucleotides 3226 to 3737) containing almost the entire E4 ORF coding sequence (100 of 118 amino acids) was cloned into the *BamHI* site of the prokaryote expression vector pEX-2 (Stanley & Luzio, 1984) to create a β-galactosidase (β-gal)–E4 fusion gene. Following thermal induction, bacterial clones were screened for expression of fusion proteins by denaturing PAGE and Coomassie blue staining of detergent-
insoluble bacterial proteins. A bacterial clone which expressed a fusion polypeptide of approximately 120K was identified (Fig. 1a, arrow). Restriction endonuclease analysis of plasmid DNA isolated from this clone was performed to confirm that the Sau3A fragment was inserted in the correct orientation (data not shown). The fusion protein was purified in bulk from a 1 1 culture of thermally induced bacteria by gel filtration, as described by Doorbar et al. (1986).

Rabbits were immunized by intramuscular injection of 100 μg β-gal–E4 fusion protein dispersed in Freund’s complete adjuvant at 3-week intervals for a total of five immunizations. To test for the presence of E4-specific immunoglobulins in sera from immunized rabbits, Western blot analysis was performed on an E4 fusion protein expressed in a heterologous vector. The same 511 bp E4 region Sau3A fragment used to construct the β-gal–E4 vector was cloned into the BamHI site of pGEX-3 (Smith & Johnson, 1988) to create a fusion gene between the Schistosoma japonicum glutathione S-transferase gene, which encodes a 26K protein (Sj26), and the BPV-1 E4 ORF. Partial DNA sequence analysis of this recombinant plasmid was performed to confirm that the BPV fragment was inserted in the correct translational reading frame (data not shown). As shown in Fig. 1(b), immune serum from one of the immunized rabbits showed a strong reactivity against the Sj26–E4 protein (lane 5), but did not react with the native Sj26 protein (lane 4), whereas preimmune serum from the same rabbit failed to react with the Sj26–E4 protein (lane 7). As seen both by Coomassie blue staining (lane 3) and by Western blot analysis with the anti-β-gal–E4 serum, multiple forms of the Sj26–E4 protein were observed, indicating that this fusion protein might be subject to partial proteolysis in bacterial cells.

To characterize the E4 protein products expressed in naturally infected bovine fibropapilloma tissue, immunoblotting analysis was performed on a total protein lysate prepared from a cutaneous fibropapilloma obtained from a local farm. Analysis of viral DNA isolated from this wart by restriction endonuclease analysis with the enzymes EcoRI and BamHI indicated the presence of BPV-1 DNA (data not shown). The protein lysate was obtained by briefly homogenizing minced wart tissue suspended in PBS containing 100 units/ml Trasylol protease inhibitor (Bayer). Proteins
antigenically related to E4 were identified using anti-E4 serum (Fig. 2). Four putative E4 gene products with approximate Mr values of 16K, 21K, 30K and 42K were detected with immune serum (lane 2, arrows) but not with preimmune serum (lane 3). The 16K species appeared to represent the major E4 protein as judged by the intensity of the band in several immunoblotting analyses. The mass of this species was slightly greater than that expected for the translation product of the entire E4 ORF calculated from its predicted amino acid composition (12.5K). The 16K and 21K E4 species were not detectable by staining of the total fractionated fibropapilloma proteins with Coomassie blue (data not shown), implying that the BPV-1 E4 gene is not expressed to such a high level as the E4 gene of HPV-1. The composition of the minor BPV-1 E4-related species of larger Mr, is unknown. A similar multiplicity of E4 protein species has been found in HPV-1-induced warts, which contain major E4 species of 10/11K and 16/17K, and minor species of 21/23K and 32/34K (Doorbar et al., 1986).

We have recently developed cell culture conditions which induce BPV-1 DNA amplification in a process which may parallel events of the in vivo vegetative replication phase occurring prior to capsid protein synthesis (Burnett et al., 1989, 1990). Viral amplification is activated in contact-inhibited cultures of the mouse C127 cell line, cl. 2, harbouring a mutant BPV-1 plasmid with a 277 bp deletion downstream of ORF E5 (Burnett et al., 1988), or in wild-type (wt) BPV-1-transformed C127 cell lines under conditions of serum deprivation. Amplification of viral DNA in these cell lines is confined to a subpopulation of grossly enlarged division-arrested cells (giant cells) which express abundant viral E2 antigen (Burnett et al., 1990). To assess expression of the BPV-1 E4 gene under specific culture conditions we performed immunoprecipitation, immunoblotting and immunofluorescence analyses on cl. 2 cells maintained under various growth conditions. In several experiments we failed to detect an E4-specific protein product using immunoprecipitation or immunoblotting techniques. By immunofluorescence analysis, however, positive staining was observed in a fraction of cl. 2 cells within confluent growth-arrested cultures (Fig. 3, c to e). These cells were readily identifiable by their enlarged size to represent the same subpopulation shown earlier to be permissive for mutant viral DNA amplification (Burnett et al., 1989). The E4 antigen had a diffuse staining pattern and was normally observed with a perinuclear localization. Expression of E4 in this cell line was frequently associated with a pronounced c.p.e. consisting of perinuclear vacuolization (data not shown). The antigen often surrounded the nucleus or was concentrated asymmetrically to one side of it. Preimmune serum failed to react with this subpopulation of cl. 2 cells (Fig. 3b). Cells in uninfected C127 cultures maintained at confluence were reproducibly negative when tested with the E4 antiserum (Fig. 3a).

We also examined whether the BPV-1 cytoplasmic E4 and nuclear E2 antigens were coexpressed in cl. 2 cells by performing immunofluorescence tests using a mixture of E4 and E2 antisera (f and g). The majority of giant cells (approx. 75%) expressed both E4 and E2 antigens [arrows in (f) indicate aggregates of intranuclear E2 antigen; see also Burnett et al. (1990)]. However, we also observed cells (approx. 20% of giant cells) which were E2-positive but did not contain detectable E4 antigen (e.g. cell arrowed in g). Only rare cells exhibited cytoplasmic E4 staining but were negative for nuclear E2 antigen (not shown). There was a large variation in the patterns of E2 nuclear distribution, as noted previously (Burnett et al., 1990). E2 was observed either as a few dense aggregates or as multiple smaller granules. E4 was most often detected in cells which contained rare isolated

![Fig. 2. Detection of E4 antigens in a BPV-1-induced wart by Western blot analysis. A total protein lysate was prepared from a cutaneous bovine fibropapilloma by homogenizing the tissue briefly in PBS containing 100 units/ml Trasylol after which it was mixed with one volume of 2 x SDS sample buffer (100 mM-Tris–HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM-DTT) and boiled for 5 min. The extract was analysed for the presence of E4 protein by electrophoresis through a 15% (w/v) denaturing gel, followed by electrotransfer and immunodetection by Western blotting, using anti-β-gal-E4 serum (1:400 dilution) (lane 2) or preimmune serum (1:400) (lane 3). Sizes of Mr markers in lane 1 are indicated on the left. Arrows indicate putative E4 proteins in lane 2.](image-url)
dense aggregates of E2 within the nucleus, whereas the E4-negative cells tended to contain abundant intranuclear E2 granules, often concentrated at the nuclear membrane. The basis for the heterogeneity in E2 staining patterns is at present unclear. It is nevertheless conceivable that these different patterns reflect preferential expression of specific forms of E2 protein (transactivator, repressor). We have, however, been unable to test this possibility as sera specific for E2 amino-terminal epitopes were reproducibly negative in immunofluorescence studies (Burnett et al., 1990; unpublished results).

We sought to determine whether the E4 antigen was also expressed in C127 cells permissive for amplification of wt BPV-1 DNA. To do this, wt virus-transformed cells of the wh.1 or wh.2 subclones, transformed by virus infection (Burnett et al., 1989), were growth-arrested by serum deprivation and then restimulated with serum after 12 days of growth arrest, as described previously (Burnett et al., 1989). In these two independently transformed cell lines no E4 antigen was detected by immunofluorescence, although giant cells expressing abundant nuclear E2 antigen developed in these cultures (data not shown). These preliminary observations implied that there might be a difference in the regulation of E4 gene expression between the cl.2 deletion variant and the wt BPV-1 genome. However, analysis of additional transformed cell lines obtained by transfection with cloned viral DNA revealed a heterogeneity in the potential for E4 expression for both the wt genome as well as the cl.2 genome in independently isolated transformed cell lines. It was found that three of four wt BPV-1-transformed cell lines obtained by transfection were positive for E4 antigen expression in serum-free medium. An identical pattern of E4 antigen localization as observed in cl.2 cells was seen in these lines (data not shown). Similarly, two of three cell lines transformed by transfection with molecularly cloned cl.2 genomic viral DNA expressed the E4 antigen under conditions of growth arrest, as determined by immunofluorescence analysis. In view of the small number of cell lines tested to date, it would be premature to speculate that this variation might be due to the source of the transforming viral nucleic acid (virion or cloned DNA).

Several attempts have been made to characterize further the BPV-1 E4 antigen expressed in confluent cl.2 cultures. We have performed immunoprecipitation of growth-arrested cl.2 cells after labelling the cultures with either [35S]methionine and [35S]cysteine, or with [32P]phosphate. However, we were unable unequivocally to identify an E4 protein by this approach. It remains formally possible that the antiserum, which was raised to the denatured E4 antigen, was unable to immunoprecipitate the native E4 protein, and that the failure to detect E4 in cl.2 extracts by immunoblotting was due to the lower sensitivity of this technique. Further work is in progress to address these points. We consider it highly unlikely that the E4 antigen detected in cl.2 cells consisted of an E6–E4 fusion polypeptide, the hypothetical translation product of a spliced mRNA species previously identified in wt BPV-1-transformed C127 cells (Yang et al., 1985), for the following reasons. Firstly, immunoprecipitation analysis with polyclonal E6 antiserum demonstrated that only a single E6 species, with the M, of the normal full-length E6 protein (Androphy et al., 1985), was expressed in both cl.2 and wt virus-transformed cells under conditions of growth arrest (N. Jareborg & S. Burnett, unpublished data). Secondly, the polyclonal E6 antiserum failed to react with the cytoplasmic E4 antigen in cl.2 cells in immunofluorescence studies (data not shown). In keeping with the detection of the E4 antigen in confluent cl.2 cultures, we observed specific, albeit limited, induction of P, under conditions of cell growth arrest (Burnett et al., 1990). It is, however, possible that E4 could be expressed from promoters other than P,. For example, a colinear transcript encoding the 31K E2 transcriptional repressor is produced from the P3080 promoter, and this could conceivably function as a bicistronic mRNA by initiation of translation at the E4 AUG codon.

In conclusion, this paper describes the preliminary characterization of the BPV-1 E4 proteins expressed in bovine fibropapilloma tissue, and in addition reports the
Short communication

Detection of the BPV-1 E4 antigen in the cytoplasm of cells in vitro which support amplification of the viral genome. This is the first report of expression of a papillomavirus E4 gene in cultured cells. Furthermore, the regulation of expression of E4 and its cellular localization in this cell line appear to reflect those in a natural infection. Our results therefore suggest that expression of E4 is not strictly dependent on factors expressed only in differentiated keratinocytes, although it is possible that host- or cell-specific factors are required for efficient expression of this gene. It is, moreover, possible that the action of E4 is specifically tied to the metabolism of the host differentiated epidermal keratinocyte, and its function might therefore be lost or obscured in a different cell type. Nevertheless, these E4-expressing cell lines may provide a useful experimental system for studying aspects of E4 gene expression and function. It will be interesting to investigate whether E4 interacts with other virus-encoded proteins or with specific components of the host cell to promote or regulate in some way the process of viral replication.

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References


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