Analysis of the L1 Gene Product of Human Papillomavirus Type 16 by Expression in a Vaccinia Virus Recombinant

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(Accepted 3 March 1988)

SUMMARY

The L1 open reading frame of human papillomavirus type 16 (HPV16) has been expressed in vaccinia virus under the control of both the 7.5K early and late promoter, and the 4b major late promoter. Antibodies to a β-galactosidase fusion protein containing a C-terminal portion of the HPV16 L1 gene product were used to compare the levels of L1 expression in the two recombinants, and showed that greater levels of expression were obtained when the gene was placed under the control of the 4b late promoter. Immunofluorescence studies revealed a nuclear location of the L1 gene product when expressed in vaccinia virus. Antibodies to the β-galactosidase fusion protein detected a major polypeptide species of 57K and a minor species of 64K in Western blots of recombinant-infected cell lysates. The 64K species was not detected when cells were infected in the presence of tunicamycin, indicating that the primary translation product of the HPV16 L1 open reading frame is modified by N-linked glycosylation when expressed in vaccinia virus. Whereas antibodies to HPV16 L1 fusion proteins and to a peptide containing amino acids from the C terminus of HPV16 L1 reacted well in Western blots with the HPV16 L1 target expressed in vaccinia virus, no reactivity was observed with antibodies to bovine papillomavirus type 1 particles or to a HPV6b fusion protein.

INTRODUCTION

Human papillomaviruses (HPV), of which more than 40 types have now been reported, induce epithelial or fibroepithelial proliferations of skin or mucosa (zur Hausen, 1977). The DNA of several HPV types is found in a variety of genital lesions, ranging from benign condylomata acuminata, which often contain HPV6b or HPV11 DNA (Gissmann et al., 1983), to invasive squamous cell carcinomas of the cervix, which frequently harbour HPV16, -18, -35 or -39 genomes (Dürst et al., 1983; Beaudenon et al., 1986).

Understanding the life cycle of the papillomaviruses has been restricted by the lack of a tissue culture system suitable for their growth. Nucleotide sequence data (Chen et al., 1982; Schwarz et al., 1983; Seedorf et al., 1985; Cole & Streek, 1986) have, however, revealed considerable homology between papillomavirus genomes, and have shown similar organizations of open reading frames (ORFs). All sequenced papillomaviruses contain two large ORFs, designated L1 and L2, which by analogy with bovine papillomavirus type 1 (BPV1) are predicted to encode virus structural polypeptides. Analysis of late gene products of papillomaviruses to date has relied largely on a commercially available antisera (Dako, Copenhagen, Denmark), raised against detergent-disrupted BPV virions. The antisera reacts with capsid antigens from a broad range of papillomavirus types, staining the nuclei of those cells in the superficial layers of a lesion which are producing virus particles (Jenson et al., 1980, 1982; Lancaster & Jenson, 1981). However, specific reagents for individual HPV types are necessary, both for diagnostic
use and to allow analysis of the HPV structural polypeptides, particularly since most HPV-containing lesions are a poor source of virus particles.

We report the construction and characterization of a vaccinia virus recombinant containing the L1 ORF of HPV16. The recombinant virus has been used in conjunction with polyclonal antibodies raised against an HPV16 L1 fusion protein expressed in bacteria, to investigate the subcellular location and the nature of L1 polypeptide species produced in recombinant-infected cells.

METHODS

Cells. African green monkey kidney (CV-1) cells and human TK− 143 cells were maintained in Glasgow modified Eagle's medium (GMEM) containing 10% foetal calf serum and, in the case of TK− cells, 25 μg/ml 5-bromodeoxyuridine was included. Baby hamster kidney 21 clone 13 cells (BHK) were maintained in GMEM containing 10% tryptophosphate broth and 10% newborn calf serum.

Viruses. Vaccinia virus strain WR and the recombinant vaccinia viruses L1-Vacc and vL1RK were grown in BHK cells and titrated on CV-1 cells (Mackett et al., 1985). Recombinant gG-VAC was constructed by Sullivan & Smith (1987).

Antisera. Rabbit antisera raised against BPV1 particles were obtained from Dako (Dakopatts). Rabbit antisera raised against trpE fusion proteins containing part of the HPV16 L1 and HPV6 L1 amino acid sequences were gifts from Dr J. Firzlaff (Fred Hutchinson Cancer Center, Seattle, Wash., U.S.A.). The preparation of these sera is described in Firzlaff et al. (1987) and the regions of the coding sequences represented in the trpE fusion constructs are shown in Fig. 6. Mouse antiserum raised against an oligopeptide corresponding to the C-terminal 14 amino acids of HPV16 L1 was a gift from Dr L. Banks (Imperial Cancer Research Fund, London, U.K.).

Preparation of these antisera is described in detail in Banks et al. (1987). The construction of plasmids expressing β-galactosidase fusion proteins is described in the Results section. Mice were immunized with 15 μg of protein purified by acrylamide gel electrophoresis. Three further immunizations were given at 4 weekly intervals and animals were bled 8 days after the final immunization.

Western blot analysis. BHK monolayers infected at 30 p.f.u./cell with wild-type vaccinia virus or with L1 recombinant virus were harvested between 6 and 24 h after infection. Cell pellets were washed with cold phosphate-buffered saline (PBS), lysed in an equal volume of 2× Laemmli sample buffer (24 mM-Tris–HCl pH 6.8, 100 mM-dithiothreitol, 2% SDS, 20% glycerol, 0.02% bromophenol blue) and sonicated thoroughly. Boiled samples were subjected to SDS–PAGE. Proteins were transferred to nitrocellulose in transfer buffer (25 mM-Tris–HCl pH 8.3, 0.2 M-glycine, 20% methanol) at 250 mA for 16 h. Filters were incubated in 3% bovine serum albumin (BSA) in PBS at 37 °C for 1 h and then incubated with polyclonal or monoclonal antibody diluted in 3% BSA in PBS for 1 h at room temperature. After a 30 min wash in 1% NP40 in PBS, the filters were incubated with affinity-purified 125I-labelled Protein A, diluted to 0.2 μCi/ml in 1% BSA in PBS for 1 h at room temperature, then washed for 20 min in 1% NP40 in PBS, dried and autoradiographed.

Immunofluorescence. CV-1 cells were grown on 19 mm coverslips and infected at 30 p.f.u./cell. At 18 h post-infection cells were fixed in 5% formaldehyde and permeabilized by treatment with 1% Triton X-100, 10% sucrose and 1% foetal calf serum in PBS. Monolayers were reacted with antisera or hybridoma supernatant, followed by incubation with the appropriate fluorescein isothiocyanate conjugate.

RESULTS

Construction of vaccinia virus recombinants

HPV16 DNA cloned into the BamHI site of pBR322 was supplied by Dr H. zur Hausen. Since the BamHI site of HPV16 lies within the L1 coding region, it was necessary to reconstruct the intact ORF before insertion into the vaccinia virus genome. Fig. 1 illustrates the strategy used to clone L1 into two vaccinia virus insertion vectors. The KpnI–BamHI fragment containing the 5′ portion of the L1 ORF was ligated into the KpnI and BamHI sites of pUC18 to give pUC18L1a. The BamHI–SphI fragment of HPV16 containing the 3′ portion of the L1 ORF was ligated into the BamHI and SphI sites of pUC18L1a and the resultant plasmid was termed pUC18L1. The complete L1 ORF was obtained by partial EcoRI digestion of pUC18L1 and isolation of a 2.074 kb fragment. This fragment, containing 193 bp upstream and 304 bp downstream of the L1 ORF, was ligated into the EcoRI site of two vaccinia virus insertion vectors, pGS62 (Smith et al., 1987) and pRK19 (R. K. Kent & G. L. Smith, unpublished data). The resultant plasmids were termed pGSL1 and pRKL1 respectively. In pGSL1, the L1 ORF is cloned downstream of the 7.5K vaccinia virus promoter which is active both early and late in infection, whereas in pRKL1 it is downstream of the 4β late promoter. Plasmids pGSL1 and pRKL1 were used to construct
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Fig. 1. Construction of recombinant plasmids containing the HPV16 L1 ORF.

TK- vaccinia virus recombinants containing the HPV16 L1 coding region using established transfection, selection and purification methods (Mackett et al., 1984, 1985). The recombinant derived from transfection with pGSL1 was called L1-Vacc, and that derived from pRKL1 was called vL1RK. Correct orientation of the inserted fragment within the vaccinia virus genome was confirmed by Southern blot analysis of DNA extracted from purified recombinant virus, digested with appropriate restriction enzymes and hybridized to an HPV16 L1 DNA probe.

Production of HPV16 L1–β-galactosidase fusion protein

A BamHI–SphI fragment which contained part of the HPV16 L1 ORF from map position 6150, through the L1 termination codon at position 7153, to the SphI site at position 7464, was cloned from pUC18L1 into BamHI- (position 375) and SphI- (position 565) digested pBR322. The resultant plasmid, called pH2, was then digested with BamHI and SalI (position 650) and the fragment containing the C-terminal portion of the L1 ORF was cloned into BamHI- and SalI-digested pEX-1 (Stanley & Luzio, 1984). This plasmid was designated pHX2. On induction of bacterial cultures containing this construct, a β-galactosidase fusion protein of the predicted Mr was detected after SDS–PAGE and staining with Coomassie Brilliant Blue. Following gel purification, this fusion protein was used to immunize mice.
Expression of the L1 ORF in vaccinia virus

Antisera raised against the pHX2 fusion protein were used to investigate the expression of L1 in the vaccinia virus recombinants. The initial recombinant, L1-Vacc, contained the L1 ORF cloned downstream of the 7.5K vaccinia virus promoter. This promoter is active at both early and late times during infection. Immunoprecipitation of [\textsuperscript{35}S]methionine-labelled infected cell lysates showed weak expression of a 57K polypeptide by this recombinant (data not shown). This corresponded in \( M_\text{r} \) to that of the predicted L1 gene product but was present at too low a level for detailed analysis of the L1 polypeptide. Studies by Rohrmann et al. (1986), however, have recently indicated that 3' ends of mRNA of vaccinia virus early genes are formed by termination downstream of a regulatory signal, and that this control region includes all or part of the sequence TTTTTNTNT. The L1 coding region of HPV16 contains TTTTTTTTTT at positions 5626 to 5633, and TTTTTTTAT at positions 6375 to 6385, and Northern blot analysis of RNA produced in L1-Vacc-infected cells revealed that the majority of L1-specific transcripts terminated at the first of these consensus sequences (data not shown).

To overcome this problem, the L1 ORF was also cloned into vaccinia virus under the control of the 4b late promoter (Rosel & Moss, 1985) using the insertion vector pRK19, and this recombinant was termed vL1RK. Polyclonal sera raised against pHX2 were used to compare the expression of the L1 gene product in the two vaccinia virus constructs by \( \textsuperscript{125}I \)-labelled Protein A detection of antibody bound to methanol-fixed plaques as shown in Fig. 2. When this ORF was placed downstream of the 4b late promoter, as compared to the 7.5K promoter an approximately 10-fold increase in expression of the L1 gene product was seen. The vL1RK construct was therefore used in all subsequent experiments.
Fig. 3. Location of the L1 gene product in recombinant-infected cells. CV-1 cells grown on glass coverslips were infected at 30 p.f.u./cell with either vL1RK recombinant virus (a) or wild-type vaccinia virus (b). Eighteen h after infection, cells were fixed, permeabilized and incubated with polyclonal anti-pHX2 serum diluted 1:200.

Localization of the L1 gene product in recombinant-infected cells

To determine the subcellular location of the L1 gene product expressed in vaccinia virus, we examined recombinant-infected CV-1 cells by immunofluorescence with polyclonal sera raised against pHX2. In permeabilized cells infected with vL1RK, anti-pHX2 sera gave strong nuclear fluorescence as shown in Fig. 3 (a). No specific binding was detected with this antiserum on wild-type vaccinia virus-infected cells (Fig. 3b). Other hyperimmune polyclonal sera raised against unrelated β-galactosidase fusion proteins did not react with vL1RK-infected cells. This result implies that the L1 gene product migrates to the nucleus of the cell in the absence of any other HPV proteins, and may therefore contain a signal or signals for nuclear localization within its coding sequence.

Analysis of L1 polypeptides in recombinant-infected cells

Lysates of infected cells harvested 18 h after infection with either wild-type vaccinia virus or the vL1RK recombinant were resolved by electrophoresis on 12.5% polyacrylamide gels and analysed by Western blotting with polyclonal sera raised against pHX2. A major polypeptide species of 57K was detected in vL1RK- but not wild-type-infected cells. In addition, a minor species of 64K was detected in vL1RK-infected cells but was not present in cells infected with wild-type virus. Western blot analysis of infected cell lysates harvested at intervals of 2 h after infection with vL1RK, shown in Fig. 4, revealed that the 57K species is synthesized from 6 h onward, with the gradual appearance of the larger species between 10 and 12 h post-infection.
This suggested that the primary L1 gene product was undergoing some post-translational modification when expressed in vaccinia virus.

The amino acid sequence of the L1 gene product contains four potential sites for N-linked glycosylation: NAS at amino acid position 156, NKS at 241, NMS at 366 and NST at 420. To determine whether the 64K species was produced by such a modification, cells were infected with vL1RK or wild-type vaccinia virus in the presence or absence of tunicamycin and harvested 18 h later. Western blot analysis of these lysates, shown in Fig. 5, revealed that in cells infected with vL1RK in the presence of tunicamycin, only the 57K species was detected, indicating that the larger 64K species seen in the absence of tunicamycin represents an N-linked glycosylated form of the primary L1 translation product.

Reactivity of HPV16 L1 with other antisera

In addition to antisera raised against pHX2, other sera were tested for their ability to react in Western blots or by immunofluorescence with the L1 gene products expressed in vL1RK-infected cells.
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(a) (b)
1 2 3 4 1 2 3 4

Fig. 5. Western blot analysis of polypeptides synthesized in vL1RK- and gG-VAC-infected cells (lanes 1, 2 and 3, 4 respectively) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of tunicamycin. Tunicamycin (10 μg/ml) was added to monolayers of BHK cells for 20 min before infection with recombinant virus. The drug was present throughout the 18 h infection period. Lysates were resolved by electrophoresis on 12.5% SDS-polyacrylamide gels and nitrocellulose filters were probed with either (a) polyclonal rabbit anti-herpes simplex virus type 1 serum diluted 1:100 or (b) polyclonal anti-pHX2 serum diluted 1:200. ¹⁴C-labelled Mr markers were transferred to the filter and their Mr values are indicated.

Polyclonal rabbit sera were raised against HPV6 and HPV16 L1 trpE fusion proteins (Firzlaff et al., 1987). The HPV6 fusion protein was expressed from a construct containing a XhoII fragment (nucleotides 6013 to 7087) of HPV6 L1 as indicated in Fig. 6. The HPV6 antiserum did not react by Western blotting with either of the L1 polypeptide species that were detected with anti-pHX2, even at a dilution of 1:50 (Fig. 7), and also failed to react with vL1RK-infected cells by immunofluorescence (data not shown). A similar lack of reactivity with HPV16 L1 expressed in vaccinia virus was observed using anti-BPV1 serum (Dako) at a dilution of 1:50. The HPV16 L1 trpE fusion protein contains the amino acids encoded by a BamHI–XhoII (nucleotides 6150 to 7013) fragment of the ORF (Fig. 6), and therefore lacks 66 amino acids at the C terminus of the gene which are present in the β-galactosidase fusion protein pHX2. Antiserum raised against the HPV16 L1 trpE fusion protein reacted with both polypeptide species expressed in vL1RK-infected cells. Its reactivity in Western blots was equivalent to that seen with anti-pHX2 (Fig. 7). Antiseras raised against a synthetic peptide containing 14 amino acids from the C terminus of the L1 ORF of HPV16 (Banks et al., 1987) also reacted in Western blots with the two L1 polypeptide species which were expressed in recombinant-infected cells (Fig. 7); this antiserum was less reactive than either of the sera raised against the HPV16 L1 fusion proteins.
Fig. 6. Regions of the HPV16 and HPV6 L1 ORFs covered by β-galactosidase (pHX1 and pHX2) and trpE (trp16L1 and trp6L1) bacterial fusion proteins.

Fig. 7. Western blot analysis of the immunoreactivity of L1 polypeptides synthesized in vL1RK-infected cells. Lysates of cells infected with wild-type vaccinia virus (lane 1) and with vL1RK (lane 2) were separated by electrophoresis on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Filters (a) to (e) were incubated with antibodies as follows: (a) pre-immune rabbit serum, 1:200; (b) rabbit anti-HPV6 L1 trpE serum, 1:50; (c) rabbit anti-BPV1 (Dako) serum, 1:50; (d) rabbit anti-HPV16 L1 peptide, 1:50; (e) rabbit anti-HPV16 L1 trpE serum, 1:200.
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DISCUSSION

The inability to culture HPV in vitro, together with the fact that very low amounts of virus capsid proteins are present in clinical lesions, necessitates the use of alternative expression systems for the study of HPV late gene products. Bacterial expression systems have been widely exploited for the generation of antibodies to specific papillomavirus ORFs and subsequent identification of HPV proteins in HPV lesions and HPV-containing cell lines (Doorbar et al., 1986; Androphy et al., 1987; Smotkin & Wettstein, 1986; Li et al., 1987; Doorbar & Gallimore, 1987). Fusion proteins expressed in Escherichia coli, however, are of limited use for studying certain aspects of HPV gene products, since expression in a prokaryotic system may not allow certain post-translational modifications to take place. Antibodies raised against a β-galactosidase fusion protein containing a C-terminal portion of HPV16 L1 have therefore been used to study the expression of the L1 ORF in a vaccinia virus recombinant.

The observation that greater levels of expression of the L1 gene product were obtained using the 4b late vaccinia virus promoter compared with the 7-5K early and late promoter may be of relevance for the construction of further recombinants expressing HPV ORFs. Papillomavirus genomes have an A + T content of approximately 60% (Danos et al., 1984) and are therefore likely to contain the transcription termination sequence described by Rohrmann et al. (1986). Two such sequences are present in the L1 ORF of HPV16, each of which contains two stretches of the sequence TTTTTNT. These termination signals obviously reduce the amount of full-length L1 gene product expressed under the 7-5K promoter. Late promoters are therefore preferable for the expression in vaccinia virus of HPV ORFs that contain such sequences. The vL1RK construct, however, may prove less suitable than L1-Vacc for studies involving T cell recognition of the L1 gene product expressed in vaccinia virus. Studies using vaccinia virus recombinants in which the influenza haemagglutinin gene was expressed under the control of three different classes of vaccinia virus promoters (Coupar et al., 1986) have shown that antigens synthesized early and late in infection are recognized by both B and T cells, but that late antigens may not be recognized by cytotoxic T lymphocytes. However, only one strain of mice out of the two tests showed this aberrant generation of cytotoxic T cells.

The predominantly nuclear location of the L1 gene product expressed by a vaccinia virus recombinant is consistent with the location of this protein in HPV lesions; Dako antiserum and other antisera raised against HPV late proteins react with antigens in the nuclei of cells in the superficial layers of HPV-infected epithelium. Despite the assembly of poxviruses in cytoplasmic factories, nuclear proteins expressed by vaccinia virus recombinants exhibit a nuclear location (e.g. polyoma virus VP1; Stamatos et al., 1987), presumably due to the correct recognition of nuclear migration signals in poxvirus-infected cells. Putative nuclear migration signals have been identified in the simian virus (SV) 40 T antigen (Kalderon et al., 1984), polyoma virus T antigen (Richardson et al., 1986), the yeast mata2 protein (Hall et al., 1984) and in the human oestadiol receptor (Green et al., 1986). In addition, studies with SV40 capsid polypeptides VP1 (Wychowski et al., 1986) indicate that the sequence of the first eight N-terminal amino acids of VP1 appear to contain a nuclear migration signal that is sufficient to target the protein to the cell nucleus. All potential nuclear localization sequences identified to date appear to consist of clusters of basic amino acids, e.g. in SV40 T these are Pro-Lys-Lys-Arg-Lys-Val, and in SV40 VP1 these are Ala-Pro-Thr-Lys-Arg-Lys-Gly-Ser. The C terminus of the L1 polypeptide also contains a stretch of basic amino acids: Ala-Lys-Arg-Lys-Arg-Lys-Leu. Though the nuclear location of the L1 gene product expressed by a vaccinia virus recombinant is not unexpected, the presence of a minor L1 species of high Mr, whose synthesis is sensitive to tunicamycin is difficult to explain. There are four potential N-glycosylation sites in the predicted amino acid sequence of L1 and the protein has a hydrophobic N-terminus that might have the capacity to act as a signal peptide. It is conceivable that, at least in vaccinia virus-infected cells, a minor fraction of the L1 protein is synthesized on the endoplasmic reticulum and is glycosylated, although protein synthesized by this route would not be expected to achieve an intranuclear location. The suggestion that the L1 protein can be glycosylated is given support by Larsen et al. (1987) who reported the presence, in purified BPV particles, of minor electrophoretic forms whose mobility was sensitive to endoglycosidase treatment.
However, it is difficult to envisage synthetic pathways that would lead to the incorporation of glycosylated L1 into capsids, and similar minor components have not been detected in analyses of the L1 protein in clinical specimens containing HPV1 and HPV6b (Li et al., 1987; Doorbar & Gallimore, 1987).

The specificity of Dako antiserum for HPV late proteins has been examined largely by Western blot analysis of bacterial fusion protein targets (J. M. Firzlaff & D. A. Galloway, personal communication; Banks et al., 1987). It reacts strongly with HPV6 L1 fusion proteins by Western blotting. It does not react with two HPV16 L1 fusion proteins, pHX1 (which contains the amino acids encoded by the HphI–BamHI fragment of HPV16 L1 as shown in Fig. 6) and trp16L1 (expressed from a plasmid containing the BamHI–XhoII fragment), and reacts only weakly with a pHX2 target. These data, coupled with the observation that Dako antiserum failed to react by immunoblotting with the L1 gene product expressed in vaccinia virus recombinant-infected cells, suggest that the epitopes which are cross-reactive between papillomavirus types may not be so highly conserved between BPV1 and HPV16. Antibodies to an HPV6 L1 fusion protein also fail to recognize the L1 of HPV16 as expressed in vL1RK, both by Western blotting and immunofluorescence. This may be important when designing systems for the diagnosis of HPV infections by immunohistochemical means, and points towards the need for reagents that are specific for HPV16 structural proteins. To this end, the vaccinia virus recombinant expressing the L1 ORF of HPV16 has proved a suitable and convenient source of target material against which to screen hybridoma supernatants for the isolation of a monoclonal antibody to HPV16 L1 (L. McLean, unpublished data).

We would like to thank Dr Juliana Firzlaff and Dr Denise Galloway of the Fred Hutchinson Cancer Research Center, Seattle, for antibodies to trpE fusion proteins, and Dr Lawrence Banks of the Imperial Cancer Research Fund for antiserum to an HPV16 L1 peptide. This work was supported by the Medical Research Council, U.K.

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(Received 11 December 1987)