Expression in *Escherichia coli* of Seven DNA Fragments Comprising the Complete L1 and L2 Open Reading Frames of Human Papillomavirus Type 6b and Localization of the 'Common Antigen' Region

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(Accepted 15 November 1988)

**SUMMARY**

Molecular cloning was used to express human papillomavirus type 6b (HPV-6b) antigens in *Escherichia coli*. Seven genomic DNA fragments of HPV-6b which together comprise the complete L1 and L2 open reading frames, known to code for capsid proteins, were cloned and expressed in *E. coli* as both β-galactosidase and TrpE fusion proteins. Western blots of HPV-6b β-galactosidase fusion proteins using 'genus-specific' antisera produced by immunization of rabbits with disrupted bovine papillomavirus type 1 (BPV-1) showed that polypeptides encoded by two DNA fragments from the mid portion of L1 of HPV-6b were cross-reactive. Only one of these two polypeptides reacted with antisera raised against disrupted HPV-1, directly demonstrating that this polypeptide contains the papillomavirus 'common antigen'. The cross-reactive region was confirmed by reversing antigen and antibody. Polyclonal antisera were raised against the seven HPV-6b β-galactosidase fusion proteins and tested against BPV-1 virion proteins on Western blots. Only antiserum against the mid portion of L1 of HPV-6b reacted with the BPV-1 major capsid protein. HPV-6b fusion proteins were also used to test human sera for antibodies reactive in Western blots. Serum samples from 38 patients with documented HPV-6 infections and from 22 presumably uninfected controls were tested. Antibodies were not detected in any of the sera to any of the seven fusion proteins. HPV-6b β-galactosidase fusion proteins are antigenic and can be used on Western blots to localize immunologically reactive sub-regions of proteins by reacting protein fragments with antisera from immunized animals. However, alternative methods will be required to detect anti-HPV antibodies in human sera.

**INTRODUCTION**

Human papillomaviruses (HPVs) infect cutaneous and mucosal stratified epithelial tissues. More than 50 different types have now been cloned and can be differentiated on the basis of DNA homology. Under natural conditions, papillomavirus (PV) types show species specificity and most exhibit definite tropism for specific anatomical sites (Pfister, 1984). HPV type 6 (HPV-6) preferentially infects genital and oral mucosa and causes as much as 60% of condyloma acuminatum, a sexually transmitted disease of increasing incidence (Centers for Disease Control, 1983; Chuang, 1987). The large number of PV types, the absence of a tissue culture system that can be productively infected with PVs, and the low yield of viral particles in most naturally occurring lesions, especially those associated with human oral and anogenital warts, have hampered studies of PV antigens and immune responses to PV infection.

Papillomaviruses share common antigens. For example, rabbit antisera produced against disrupted virions of HPV-1 or bovine papillomavirus (BPV) cross-react broadly with multiple HPVs (Jenson *et al.*, 1980; Orth *et al.*, 1978). One such 'genus-specific' antiserum raised against
disrupted BPV-1 is available commercially (Dako) and is often used for immunochemical confirmation of PV infections. SDS-PAGE of purified BPV-1, HPV-1a and cottontail rabbit PV (CRPV) virions demonstrates a major and often a minor capsid protein (Favre et al., 1975; Meinke & Meinke, 1981; Pass & Maizel, 1973). Common antigens have been localized to the major capsid proteins in studies of cross-reactivity between CRPV and HPV-1 using polyclonal antibodies (Orth et al., 1978) and also between BPV and several untyped human viruses using monoclonal antibodies (Nakai et al., 1986).

Recently, several laboratories have used specific antibodies raised against PV polypeptides expressed in *Escherichia coli* from cloned PV DNA open reading frame (ORF) segments to provide direct evidence that the L1 and L2 ORFs encode virion structural proteins. Antibodies against an *E. coli*-expressed L1 protein of BPV-1 neutralized BPV-1 in a transformation inhibition assay and reacted with BPV-1 particles in ELISA (Pilacinski et al., 1984). Antisera against a recombinant L1 protein of HPV-1 specifically identified the corresponding major capsid proteins of *M*, 57K in Western blots using HPV-1 virions or tissue extracts (Doorbar & Gallimore, 1987). Similar antibodies against *E. coli*-expressed HPV-6b L1 proteins identified a protein of *M*, 56K consistent with the major capsid protein in dissociated condylomata acuminata (Tomita et al., 1987b), and cross-reacted with the 57K major capsid protein of HPV-1 (Li et al., 1987). Minor capsid proteins estimated at 78K (Doorbar & Gallimore, 1987) or 76K (Komly et al., 1986) were identified on Western blots of purified HPV-1 virions using antisera against HPV-1 L2 recombinant proteins. Reactive proteins of identical *M*, were recognized in extracts of HPV-1-induced warts in each case. Similarly for HPV-6b, extracts of genital condylomas contained a 70K to 76K protein that reacted with anti-HPV-6b L2 antiserum (Komly et al., 1987a) and presumably represented the minor capsid protein. The apparent *M*, of the minor capsid protein in each case is greater than that predicted from the size of the L2 ORF. The reason for this discrepancy has not been determined. It has been speculated that aberrant migration may be a result of mRNA splicing (Danos et al., 1984; Roseto et al., 1984; Doorbar & Gallimore, 1987) or post-translational modifications such as glycosylation (Tomita et al., 1987b). Komly et al. (1986) noted that an *E. coli*-produced L2 fusion protein of HPV-1 missing 106 N-terminal amino acids migrated at 70K rather than the 51-2K calculated from the nucleotide sequence. These investigators suggested that the aberrant migration may be due to an intrinsic property of the protein.

For the present studies polypeptide subregions of the L1 and L2 proteins of HPV-6b were individually expressed in *E. coli*. Bacterial fusion proteins containing these polypeptides were used in Western blots to localize regions of cross-reactivity with BPV-1 and HPV-1 antisera. Fusion proteins were also used to raise anti-HPV-6 antibodies, and to test human sera for PV antibodies by Western blots. A method is presented for confirmation of anti-HPV-6 activity of antibodies raised against fusion proteins. Antisera to HPV-6 polypeptides were tested against BPV-1 particles to confirm the location of cross-reactive epitopes.

**METHODS**

_Cloned HPV-6b DNA, bacterial cells, plasmids, virus and antisera._ HPV-6b DNA cloned at the _BamHI_ site into pBR322 was supplied by Dr L. Gissmann, German Cancer Virus Research Center, Heidelberg, F.R.G. pop2136 cells were supplied by Dr O. Raibaud, Institut Pasteur, Paris, France. pEX vectors were provided by Dr K. Stanley, European Molecular Biology Laboratory, Heidelberg, F.R.G. and pATH vectors were obtained from Dr M. Crivellone, Columbia University, New York, N.Y., U.S.A. Antisera against HPV-1 were provided by Dr K. Shah, Johns Hopkins University, Baltimore, Md., U.S.A. and by Drs L. Taichman and D. Baker, SUNY, Stonybrook, Long Island, N.Y., U.S.A. BPV-1 was purified from one of several naturally occurring bovine fibropapillomas provided by Dr R. Smith and P. Frasca, Rochester, N.Y., U.S.A.

_Cloning strategy._ Two different expression vectors were chosen, each capable of expressing cloned ORF DNA as the corresponding polypeptide fused to a different bacterial protein. Seven genomic DNA ORF fragments from the late region of HPV-6b (Fig. 1) were cloned in pEX vectors (Stanley & Luzio, 1984) for expression as polypeptides fused to the carboxy terminus of a 116K _cro-β_ galactosidase protein. The same ORF fragments were also cloned in pATH vectors (Dieckmann & Tzagoloff, 1985; Firzlaff et al., 1987) which, upon induction, express cloned DNA ORF fragments as corresponding polypeptides fused to the carboxy terminus of a 37K truncated TrpE polypeptide under the control of the _trp_ operator/promoter sequence.
HPV 6b late proteins

(a)

![Diagram of HPV-6b genomic map and origins of restriction fragments. The genome has been linearized and the scale is in bp. ORFs are represented by boxes (b) and the broken vertical line in each box represents the position of the first potential start codon. Restriction fragments derived from L2 and L1 are labelled by size, and the names and sites of restriction endonucleases generating the fragments are indicated. Potential ATG start codons for L1 and L2 are at nucleotides 5789 and 4423 respectively. Nucleotides preceding the stop codons for L1 and L2 are 7288 and 5799 respectively. The length of HPV-6b DNA fragments in bp followed by the predicted Mr for HPV portions of fusion proteins are as follows: 300 (11K), 690 (25K), 601 (14K), 215 (8K), 480 (18K), 594 (22K), 4731 (7K).](image)

HPV-6b DNA was digested with BamHI, purified from vector DNA by agarose gel electrophoresis, and electrophoretically transferred to activated DE81 paper (Whatman). The resulting genomic DNA was digested with XhoII and PstI to produce six fragments, five of which could potentially express portions of L1 and L2 proteins (Fig. 1). DNA fragments are referred to by length in bp, as fragments 806, 690, 601, 480, 594 and 4731, and corresponding constructs in expression vectors are named using fragment lengths. Three fragments (690, 480 and 4731) were inserted at the PstI and BamHI/XhoII sites of pEX3. Transformed pop2136 cells were plated at 30 °C on LM agar plates containing ampicillin, and colonies containing appropriate constructs were identified by mini-isolation and restriction analysis of plasmids. Fragments 601 and 594, because of reversal of PstI and XhoII ends, required reversal of orientation for cloning. This was accomplished by adding new PstI sites at the 3' ends by a two-step procedure using pUC9 plasmids (Vieira & Messing, 1982) (Bethesda Research Laboratories) and DH5 α cells (Bethesda Research Laboratories) for transformation. First, fragments were inserted into pUC9 at the PstI site and the XhoII-compatible BamHI site. The DNA was then digested at the 5' flanking HindIII site and blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I. Digestion with XhoII produced fragments with 5' blunt and 3' BamHI-compatible ends. The fragments were inserted into pUC9 at the BamHI and Smal sites, removed from pUC9 by PstI digestion and cloned into pEX2.

The final two fragments (300 and 215) were derived from pEX constructs. Fragment 300 was obtained from a pEX construct containing an 806 bp PstI–BamHI fragment (Fig. 1). The construct was digested with NcoI, filled in using Klenow fragment, and digested with BamHI. The resulting 300 bp fragment was cloned into the Smal and BamHI sites of pUC9. This intermediate construct was digested with EcoRI and BamHI and the resulting fragment was ligated into pEX2 for expression. Fragment 215 was obtained by digesting pEX601 with PstI and HaeIII to produce a blunt/PstI fragment which was ligated into pUC9 (AccI/Klenow/PstI), then excised with BamHI/PstI and ligated into pEX3. All cloned fragments were verified by cleavage at internal restriction sites. Fragment 601 was also verified by DNA sequencing of the L2 stop codon region.

All seven cloned DNA segments were transferred directly from pEX to pATH vectors for expression as TrpE fusion proteins in *E. coli* HB101 cells.

**Production of antigens.** Plasmid-containing pop2136 cells were grown to mid-log phase and plasmid expression was induced by thermal inactivation of constitutively expressed cI857 repressor at 42 °C for 2 h. Cells were pelleted, resuspended in lysis buffer (25% sucrose, 10 mg/ml lysozyme, 50 mM Tris–HCl pH 8) and incubated on ice for 15 min. Triton X-100 was added to a final concentration of 0.1% and the mixture was held on ice for 30 min. Insoluble cellular material including fusion proteins was pelleted by centrifugation at 14000 g for 30 min in an Eppendorf microcentrifuge. The pellet was solubilized in Laemml buffer (Laemmli, 1970) with 8 M-urea by
grown in 5 ml of M9 medium with 5% casamino acids, L-tryptophan (20 μg/ml) and ampicillin (50 μg/ml). Overnight cultures were diluted 1:10 with M9 medium containing ampicillin and no tryptophan. Indoleacrylic acid (Sigma) was added to a final concentration of 10 μg/ml and cells were grown for an additional 4 h and pelleted.

Fusion protein preparations for SDS-PAGE and Western blots were prepared as follows. Cell pellets were resuspended in 10 ml cold TE buffer (50 mM-Tris, 5 mM-EDTA, pH 8.0) with 20 μg of lysozyme for 30 min. NaCl and Nonidet P40 were added to 0.3 M and 0.7% final concentrations, respectively, and incubation was continued for an additional 30 min on ice. Samples were passed three times through an 18-gauge needle and incubated on ice for an additional 30 min. Insoluble proteins including the fusion proteins were pelleted and then resuspended in 10 ml 10 mM-Tris–HCl pH 8 and 1 M- NaCl for 10 min on ice. Proteins were re-pelleted and resuspended in 10 mM-Tris–HCl pH 8 and the final pellet was dissolved in 1 ml Laemmli buffer and stored at −20°C.

**Purification and typing of BPV**. A bovine fibropapilloma was ground with a Polytron tissue grinder (Brinkman Instruments) and submitted to several centrifugation steps with intervening treatments with trypsin (Gibco) and collagenase (Sigma) according to the protocol of Favre et al. (1975). Negative staining of the resulting preparation revealed clean viral particles by electron microscopy. BPV protein concentration was determined by the Bio-Rad protein assay kit using bovine serum albumin as an standard.

DNA recovered from virus by proteinase K treatment and phenol/chloroform extraction (Kirby, 1956) was digested with restriction endonucleases. The DNA restriction pattern was consistent with that of BPV-1 (Campo et al., 1981; Lancaster, 1979).

**Rabbit immunizations**. Fusion proteins and vector-produced cro-β-galactosidase were excised from unstained polyacrylamide gels (see earlier). Gel slices and purified BPV-1 virions disrupted by SDS and 2-mercaptoethanol (Jenson et al., 1980) were mixed 1:1 (v/v) with complete Freund's adjuvant (Difco), emulsified, and injected intradermally at multiple sites on the shaven backs of New Zealand rabbits (Vaitukaitis, 1981) (Hazleton Research Animals). A booster dose in incomplete Freund's adjuvant (Difco) was administered 1 month later as a single intramuscular injection in the thigh. Pooled plantar warts were used as a source of HPV-1 virions, and production of HPV-1 antisera was carried out in a similar fashion.

**Confirmation of HPV-6 infection**. Excision biopsies of exophytic condylomata were obtained from patients attending a sexually transmitted diseases clinic and specimens were divided into two portions. One portion was fixed in 10% formalin for in situ hybridization, and the other was immersed in liquid nitrogen for Southern blots.

Southern blots were performed using total cellular DNA extracted from tissue by the phenol/chloroform method. Recovered DNA was treated with selected endonucleases known to give characteristic patterns for HPV types commonly found in the genital tract. DNA digests were electrophoresed in 1.2% agarose gels, transferred to nylon membranes (Gene Screen Plus), and hybridized to a whole genomic, 32P-labelled HPV-6 probe at high stringency (Tm = 5°C). The probe was labelled by nick translation (Rigby et al., 1977).

**In situ hybridization** was performed as previously described using whole genomic RNA probes directed against HPV-6, -11, -16, -18 and -31 (Stoler & Broker, 1986). Diagnosis of HPV-6 infection was made if HPV-6 DNA was identified by Southern and/or in situ hybridization. Immunocytochemical assays were performed with a modification of an avidin–biotin technique (Wilbur et al., 1988).

**Human sera**. Sera were obtained from 38 patients (mean age 25.1 years, range 19 to 43 y) with genital warts in various stages of resolution. All patients had HPV-6 infection confirmed as described above. In addition, 15 of 34 (44%) biopsy specimens also contained papillomavirus antigens as detected by immunocytochemistry. Control sera were obtained from 22 nuns (mean age 50-2 years, range 30 to 72 years). None had ever been sexually active or had had genital warts. At the time of blood drawing, eight nuns had active non-genital warts, and eight gave no history of HPV lesions.

**Western blots**. For human serology, all seven β-galactosidase fusion proteins were electrophoresed in SDS–polyacrylamide 6% stacking/12% separating gels (Laemmli, 1970) and electrobotted overnight into nitrocellulose membranes (Kirkegaard & Perry Laboratories). After quenching for 2 h in 5% non-fat dry milk (Carnation) in 6.7 mM-phosphate-buffered saline (Blotto buffer) (Johnson et al., 1984), and washing with 0.5% Tween-PBS buffer, the membranes were cut into strips, and placed in separate wells; immunostains were carried out for each of the 60 sera in a total volume of 2.5 ml/well.

Sera (1:100 dilution in Blotto buffer) were incubated with strips for 90 min at room temperature (primary antibody step). After washing, a goat anti-human γ chain antibody conjugated to alkaline phosphatase (Tago) was added for 90 min (1:5000 dilution in Blotto buffer). A final wash was followed by a 7 to 10 min incubation with the substrate, naphthol AS-MX (0-1 mg/ml)/Fast Red TR (1 mg/ml) in 0.1 mM-Tris–HCl buffer pH 8.4, with 1% N,N,N-dimethylformamide (Mason & Sammons, 1978). To indicate the position of each fusion protein on nitrocellulose,
an antigen control strip was incubated with an affinity-purified mouse anti-β-galactosidase antibody (Jackson ImmunoResearch) and then a rat anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch).

For Western blots using rabbit sera, the protocol was identical. Sera were incubated for 2 h and the antisera against the seven fusion proteins and β-galactosidase were detected by goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Tago). Buffer solutions for quenching, dilution of sera and washing, and the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, were obtained from Kirkegaard & Perry.

RESULTS

Production of β-galactosidase fusion proteins and localization of the 'common antigen' region of HPV-6b L1 polypeptide

All β-galactosidase fusion proteins produced from pEX vectors were readily identified by SDS-PAGE of the insoluble portion of E. coli cell extracts (Fig. 2a). The fusion proteins were not seen in cells grown at non-permissive temperatures (data not shown). The peptide from fragment 4731 was consistently produced at a lower level than the other fusion proteins, presumably because of the large size of the cloned DNA segment (Stanley & Luzio, 1984). The peptide expressed from fragment 601 (lane 3) migrated aberrantly on polyacrylamide gels, in that it comigrated with the fusion protein produced from the 594 fragment (lane 6), a peptide predicted to be 7-6K larger.

When the seven peptides were tested in Western blots using antibody to disrupted BPV-1 (Dako) at 1:5000 dilution, only polypeptides produced from fragments 480 and 594 were reactive (Fig. 2 b). These segments were derived from the mid portion of the L1 ORF. The lower bands from the strongly reactive 480 bp segment presumably represented breakdown products. The same pattern was reproduced using a second antisera prepared in our laboratory against disrupted BPV-1 and again using Dako antiserum at 1:1000 dilution (data not shown). Three antisera, prepared against detergent-disrupted HPV-1 extracted from pooled plantar warts, were similarly tested. Two of the three reacted with only the polypeptide produced from fragment 480 (Fig. 2c). The third reacted also with the polypeptide encoded by the 300 bp fragment from the 5' end of L2 (see Discussion; data not shown).

Production of TrpE fusion proteins, and Western blots confirming anti-HPV-6 activity of antibodies raised against β-galactosidase fusion proteins

TrpE fusion proteins produced from pATH vectors and corresponding to the seven DNA fragments were readily identified on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels when compared to non-induced cells in whole cell lysates (Fig. 3 and data not shown). Five of the seven TrpE fusion proteins migrated to the position of the predicted Mr values, supporting translation in the appropriate reading frames. As with the β-galactosidase fusion protein from fragment 601 the product of pATH601 again showed aberrantly slow migration (lane 3) and comigrated with the protein from pATH594 (lane 6). DNA sequencing of the 3' end of fragment 601 reconfirmed that the HPV-6 insert was authentic. If the reading frame had been altered, a smaller rather than a larger product would be expected and therefore the reading frame appeared to be correct. The slow migration may be due to intrinsic properties of the polypeptide. Similar considerations apply to the TrpE fusion protein expressed from restriction fragment 215, which also appears to migrate more slowly than expected. Both the levels of expression and the solubilities of different TrpE fusions were variable. Therefore, when concentrations of fusion proteins were made equal by loading different quantities of fusion protein preparations in each lane, differing 'backgrounds' were seen. The extra 'background' bands presumably represent E. coli proteins or breakdown products of fusion proteins.

Antibodies against the seven β-galactosidase fusion proteins were tested for reactivity with the corresponding TrpE fusion proteins on Western blots. For each TrpE fusion protein, reactivity was seen at the appropriate position and was not present on the vector control strips, suggesting that antibodies directed against the HPV portion of the β-galactosidase fusion protein are present (Fig. 4). In addition, several reactive bands of lower Mr, were seen that were
Fig. 2. (a) β-Galactosidase fusion proteins (see asterisks) expressed in *E. coli* from HPV-6b L1 and L2 ORF DNA segments (7.5% SDS-PAGE, Coomassie Brilliant Blue stain). Lane V, vector-expressed β-galactosidase marker; lanes 1, 2 and 3, L2 antigens expressed from plasmids pEX300, pEX600 and pEX601 respectively; lanes 4, 5, 6 and 7, L1 antigens expressed from plasmids pEX215, pEX480, pEX594 and pEX4731 respectively. Concentrations of fusion proteins have been adjusted to similar levels. A second band was consistently seen at 116K from two independent isolates containing pEX594 (lane 6). The band was present only on induction, reacted with anti-β-galactosidase antibody, and probably represented premature termination of transcription or translation, or degradation of the fusion protein. (b) Western blot of HPV-6b β-galactosidase fusion proteins with antiserum raised against disrupted BPV-1 (Dako). Lanes V and 1 to 7 are as described in (a). Although this antiserum reacted with both L1 and L2 gene products of BPV-1, only the HPV-6b antigens in lanes 5 and 6 corresponding to fragments 480 and 594 of L1 were cross-reactive. Dako and conjugate antisera were both used at 1:5000 dilution. (c) Western blot of same fusion proteins with antiserum raised against disrupted HPV-1. Lanes as in (a). Only the polypeptide encoded by HPV-6b fragment 480 was reactive. Antiserum was used at 1:1000 dilution and conjugate at 1:5000.
Fig. 3. TrpE fusion proteins corresponding to L1 and L2 ORF DNA segments, expressed in E. coli (10% SDS-PAGE, Coomassie Brilliant Blue stain). Lane V, total E. coli lysate from pATH vector with no insert, expressing 37K TrpE protein; lanes 1 to 7, insoluble fraction of E. coli lysates containing fusion proteins expressed from pATH300, pATH690, pATH601, pATH215, pATH480, pATH594 and pATH4731 plasmids respectively. Positions of vector-encoded TrpE and fusion proteins are shown by asterisks. Concentrations of fusion proteins have been adjusted to approximate equality. Mr markers of 95.5K, 55K, 43K, 36K and 29K are indicated on the left.

Fig. 4. Western blots showing reactivity of antibodies raised against β-galactosidase fusion proteins, with corresponding TrpE fusion proteins. Grouped strips in (a) to (g) correspond to TrpE fusion proteins in lanes 1 to 7, respectively, of Fig. 3. For each group, strip 1 shows reactivity of antiserum with an E. coli lysate from cells containing the pATH vector with no insert, strip 2 shows reactivity of preimmune serum with lysate expressing the TrpE fusion protein, and strip 3 shows reactivity of postimmune serum with lysate from cells expressing the TrpE fusion protein. Primary antibodies were used at 1:1000 dilution and conjugate antibody was diluted 1:5000. Positions of Mr markers of 55K, 43K and 36K are indicated on the left. The positions of TrpE fusion proteins are indicated by arrows.

not present on vector controls and presumably represent breakdown products. The antibody against pEX4731 was consistently very weakly reactive although several different rabbits were immunized using various schedules for immunization.

**Reactivity of fusion protein antibodies on Western blots with BPV-1 capsid proteins**

Antisera against the seven HPV-6b fusion proteins were tested in Western blots for cross-reactivity against purified BPV-1 particles (Fig. 5). The antiserum raised against the 480 polypeptide was strongly reactive, while antiserum against the 594 polypeptide was only weakly
reactive (although the band was poorly reproduced photographically). Antisera against the other two L1 polypeptides did not cross-react. This result is reciprocal to observations using 'genus-specific' antibody against fusion proteins (Fig. 2b). The same immunologically cross-reactive region was identified by both methods. A band presumably representing the BPV-1 minor capsid protein was clearly present, as shown in Fig. 5(h and j), but did not react with antibodies produced against L2 polypeptides of HPV-6b.

Western blots using fusion proteins and human sera

The seven fusion proteins and plasmid-expressed β-galactosidase were each tested against 38 sera from patients with HPV-6 infections and from 22 uninfected controls. An example of Western blots using two different fusion proteins is shown (Fig. 6). The antigen control lanes demonstrated excellent transfer of proteins onto the nitrocellulose membrane for pEX480 and pEX594 and also for the other six antigens tested (data not shown) with broad, homogeneous bands that migrated at the expected positions. The upper margin of the protein is clearly seen and lower bands and smear presumably represent degradation products. Conjugate controls (reacted without primary antibody) were negative.

Most of the sera showed reactivity with multiple E. coli proteins, which was indicated by the higher and/or lower bands; this result was not surprising. For a given serum, the pattern of reactivity against E. coli proteins was generally conserved between experiments. However, neither sera from HPV-6-infected patients nor sera from presumably seronegative controls reacted with β-galactosidase alone or with any of the β-galactosidase fusion proteins. Occasionally reactivity was seen with an E. coli protein of high Mr (i.e. lanes 6, 23, 25, 36, 39, 50 and 56), but this protein migrated more slowly than the fusion proteins. This was most clearly evident when the entire battery of sera was tested against a preparation of E. coli cells transformed by pEX that had no foreign DNA insert and consequently did not express HPV-derived proteins (vector controls; data not shown). The same high Mr proteins were present,
Fig. 6. Western blots of human sera against *E. coli* lysates containing the fusion protein product of pEX480 (a) and pEX594 (b). All seven fusion proteins migrate more slowly than most *E. coli* proteins so purification to remove the *E. coli* proteins was not necessary. Lanes A, antigen control; lane C, conjugate control; lanes 1 to 8, patients with no history of warts; lanes 9 to 14, patients with a past history of non-genital warts; lanes 15 to 22, patients with a present history of non-genital warts; lanes 23 to 41, absence of the PV 'common antigen' on biopsy sections; lanes 42 to 56, presence of the PV 'common antigen' on biopsy sections; lanes 57 to 60, PV 'common antigen' status unknown. The arrowheads indicate the position of the fusion protein.
clearly indicating that they did not represent HPV proteins. The proteins were readily differentiated from plasmid-expressed β-galactosidase by their positions on these blots. Occasional faint bands were visible in the Mr range of the fusion proteins (e.g. lanes 26 and 36), but these bands were judged too thin to represent reactivity with fusion proteins.

**DISCUSSION**

We have shown that antisera raised against disrupted BPV-1 virions cross-react strongly in Western blots with antigens encoded within the 480 bp XhoI/PstI fragment of L1 of HPV-6b and to a limited extent with the 594 bp PstI/XhoII fragment, but do not cross-react with peptides from the amino or carboxy termini of the major capsid protein of HPV-6b, nor with any peptides from the L2 ORF. Similarly, antibodies against disrupted HPV-1 consistently show strong reactivity with the 480 bp XhoII/PstI fragment. Conversely, we have also shown that among antibodies raised against the seven HPV-6b β-galactosidase fusion proteins only those elicited by the mid portion of L1 react with BPV-1 capsid proteins. Immunogenicity of the HPV portion of all β-galactosidase fusion proteins was confirmed by testing antisera for reactivity with corresponding TrpE fusion proteins, although low antibody levels were induced by the expression product of pEX4731. Finally, anti-HPV antibodies were not detected in sera of patients infected with HPV-6 using β-galactosidase fusion proteins in Western blots.

Antigenic cross-reactivity has previously been demonstrated for several PV types. Antibodies against disrupted PVs have been shown to react with viral proteins in tissue sections from warts caused by multiple different PV types (Jenson et al., 1985). Similar cross-reactivity has not been seen when intact virions have been used to raise antibodies in rabbits (Gissmann et al., 1977; Orth & Favre, 1985). Cross-reactivity has therefore been attributed to internal or masked epitopes that are revealed by disruption of viruses and are shared by most human and animal PVs. In a comparative study of animal PVs, epitopes that are type-specific, cross-reactive, broadly cross-reactive and 'genus-specific' have been identified using monoclonal antibodies (Cowsert et al., 1987). The location of epitopes was not determined. Several recently reported studies have shown that commercial 'genus-specific' anti-BPV antibody reacts with L1 but not with L2 recombinant peptides of HPV-6 (Banks et al., 1987; Li et al., 1987; Thompson & Roman, 1987; Tomita et al., 1987b). In these studies, only large L1 peptides of HPV-6b were tested, and the particular domain of L1 responsible for cross-reactivity was not identified. Very recently, studies reported by Jenison et al. (1988) presented indirect evidence suggesting that the activity of Dako antiserum is limited to the product of the 480 bp XhoII/PstI fragment of HPV-6b. Reactivity was shown with a polypeptide encoded by the 1074 bp XhoII/XhoII fragment (fragments equivalent to our 480 plus 594) and with a polypeptide encoded by the 480 bp XhoII/PstI fragment. Reactivity to the larger polypeptide appeared to be removed completely by preabsorbing the Dako antiserum with the smaller polypeptide, suggesting that the polypeptide encoded by the 594 bp fragment does not react with Dako antiserum. However, as seen in Fig. 2(b), direct assay demonstrates that the polypeptide from fragment 594 clearly cross-reacts with Dako antiserum. The same region, however, does not react with anti-HPV-1 serum (Fig. 2c), indicating that antibodies reactive towards the polypeptide encoded by fragment 594 are not broadly cross-reactive. Taken together these experiments provide direct evidence that the PV 'common antigen' region is limited to the 480 bp XhoII/PstI fragment. Dako antiserum has also been shown to react with a large carboxy-terminal polypeptide of HPV-16 representing more than half of the L1 ORF (Jenison et al., 1988; Banks et al., 1987). Alignment of the L1 ORFs of HPV-6b and HPV-16 show that the large HPV-16 polypeptide overlaps with part of the 480 bp fragment of HPV-6b, suggesting that reactive regions of the two capsid proteins could be collinear (Jenison et al., 1988). Reactivity of Dako antiserum with HPV-16 was shown to be weak relative to reactivity with HPV-6b (Banks et al., 1987). Precise identification of reactive sequences for both HPV-6b and HPV-16 will undoubtedly be of interest and may ultimately help to predict reactivity of 'group-reactive' antisera with other virus types.

Neither the BPV-1 isolate used by Dako for production of group-reactive antiserum nor the second BPV-1 isolate, which we purified and used both to raise antibodies and as the substrate for Western blots, have been sequenced. Nevertheless, comparison of the putative L1 amino
acid sequence, as published for BPV-1 (Chen et al., 1982), which is probably almost identical to sequences for the isolates used (Ahola et al., 1983), with that of the HPV-6b DNA used for these studies (Schwarz et al., 1983), is of interest. Few uninterrupted homologous regions can be identified. There are five segments of five or six amino acids within the polypeptide derived from the HPV-6b 480 bp fragment that are homologous with the corresponding region of BPV-1. Two additional homologous segments are encoded by the 594 bp fragment. It is tempting to speculate that these homologous regions may participate in the reciprocal reactivity of these polypeptides on Western blots. However, two additional homologous sequences are also located within the polypeptide encoded by the 215 bp fragment from the 5'-end of L1. This polypeptide does not appear to cross-react with Dako antiserum or to induce antibodies that are cross-reactive with BPV-1. Two other completely conserved sequences of 12 and seven amino acids located near the amino terminus of the polypeptide derived from the 300 bp fragment at the 5'-end of L2 also failed to react with Dako antiserum or with a second antiserum raised against disrupted BPV-1. These domains also failed to induce antibodies cross-reactive with BPV-1 capsid proteins. Therefore, these results suggest that antigenic cross-reactivity cannot be predicted simply by identification of short regions of amino acid homology.

The conserved regions at the amino terminus of L2 are the longest continuous shared sequences in the late region, and are identical for multiple PV types (Chen et al., 1982; Cole & Streeck, 1986; Dartmann et al., 1986; Potter & Meinke, 1985; Schwarz et al., 1983) and very similar for most other PV types for which sequences have been published. It has therefore been speculated that these regions, if antigenic, could cause cross-reactivity of L2 antibodies for different virus types (Banks et al., 1987; Komly et al., 1986). Our data show that antisera against BPV-1 do not cross-react with shared sequences of HPV-6b L2 on Western blots, although the antisera clearly contain some antibodies that react with the minor capsid protein of BPV-1 as shown in Fig. 5(). Conversely, antiserum raised against the amino terminus of HPV-6b L2 does not cross-react with the minor capsid protein of BPV-1. Only one of three antisera raised against disrupted HPV-1 reacted with the amino terminus of L2 of HPV-6b. Taken together these data suggest that the highly conserved sequences at the amino terminus of L2 are not strongly antigenic in rabbits.

Antibodies to HPVs have been detected in human sera by a number of investigators using single or pooled antigen sources and a variety of methods, such as immunodiffusion, immune electron microscopy, complement fixation, ELISA, haemagglutination inhibition or radioimmunoprecipitation (for reviews, see Kirchner, 1986; Spradbrow, 1987). To date, two published reports have employed PV capsid proteins synthesized in E. coli for antibody detection in human sera using Western blots. Using a protein containing 92% of the HPV-6b L1 protein fused to a 14 amino acid vector-derived leader peptide and sera preabsorbed with an E. coli lysate, Li et al. (1987) found that 18 of 30 sera from a colposcopy clinic and two of 20 sera from children under the age of 5 were reactive at low dilution. In the second study reactivity of several human sera was shown with an L1-encoded polypeptide corresponding to the 1074 bp XhoI/Y(holI fragment of HPV-6b, but not with the product of the 480 bp XhoII/PsrI fragment (Jenison et al., 1988). In that study, HPV-6b polypeptides were expressed as TrpE fusion proteins.

The studies reported here, using β-galactosidase fusion proteins to test sera from patients with documented HPV-6 infections and adult controls, showed that serum antibodies were not detected by these methods. When bacterially produced fusion proteins are used to screen sera for specific antibodies, preabsorption of sera with bacterial lysates is often performed to reduce background. Because of the high M, of β-galactosidase fusion proteins relative to other E. coli proteins such preabsorption was not necessary here. It is possible that minor breakdown products of fusion proteins could be obscured by serum reactivity with E. coli proteins, although previous experiments have shown clearly that such products would not be expected to predominate. There are two possible explanations for the lack of reactivity observed. First, the fusion proteins used may not be recognized by antibodies to native PV proteins. This may be due to the disruption of the primary, secondary or tertiary structures of the antigenic determinants caused by the division of L1 and L2 proteins into short fragments, or to the denaturation process used in the Western blots. It may also be possible that important epitopes are masked by the
large β-galactosidase portions of fusion proteins. We think that such blocking is unlikely, however, because of the strong reactivity shown by the 480 and 594 fragments with group-reactive antisera. Another possibility to explain the lack of reactivity is that the Western blot methods that are described simply lack the necessary sensitivity for detection of antibodies in human sera. Based on previous studies, we believe that the complete absence of antibodies in the sera is very unlikely, but we report these preliminary results simply to document difficulties with the fusion proteins described. Studies of sera using the corresponding TrpE fusion proteins are currently in progress.

In summary, we have used β-galactosidase fusion proteins containing polypeptide domains of the HPV-6b major and minor capsid proteins to identify a region within the L1 protein which carries the 'group-specific' antigenic region. Our preliminary data suggest that β-galactosidase fusion proteins may have limited usefulness as antigens for serological studies.

We thank Drs M. H. Stoler and D. C. Wilbur for performance of in situ hybridization and immunocytochemical studies. We also thank J. Strusenberg and R. Syniewska for technical assistance, Drs Louise Chow, Tom Broker and David Merz for helpful discussion, and Margaret E. Aldrich for typing the manuscript. This work was supported by Public Health Service grant AI-23418 and contract AI-32510 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., U.S.A. Dr William Bonnez is the recipient of a Wilmot Cancer Research Fellowship.

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(Received 1 August 1988)