JOURNAL OF VIROLOGY, Feb. 1989, p. 809–818
0022-538X/89/020809-10$02.00/0

Vol. 63, No. 2

Human Antibodies React with an Epitope of the Human Papillomavirus Type 6b L1 Open Reading Frame Which Is Distinct from the Type-Common Epitope

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Received 3 August 1988/Accepted 26 October 1988

Recombinant proteins encoded by the human papillomavirus type 6b (HPV6b) L1 open reading frame react with sera from patients with condylomata acuminata and also react with rabbit antiserum raised against sodium dodecyl sulfate-disrupted bovine papillomavirus type 1 (BPV1) virions. To map the immunoreactive epitopes, a series of procaryotic expression plasmids was made which contained a nested set of 3′ to 5′ deletions in the HPV6b L1 open reading frame. The deleted plasmids expressed a set of carboxy to amino terminus truncated fusion proteins. Regions containing the immunoreactive epitopes were mapped by determining which of the deleted fusion proteins retained reactivity with sera in Western immunoblot assays. The coding sequence for a human antibody-reactive linear epitope mapped between HPV6b nucleotide coordinates 7045 and 7087, and the rabbit anti-BPV1-reactive epitope coding sequence mapped between coordinates 6377 and 6454. Synthetic peptides derived from the epitope mapping were reacted with sera in enzyme-linked immunosorbent assay. Human sera reacted with synthetic peptide QSQAITCQKPTPEKEKPDPYK (HPV6b L1 amino acids 417 through 437). Rabbit anti-BPV1 and rabbit antisera raised against HPV6b and HPV16 L1 recombinant proteins reacted with the synthetic peptide DGDMVDTGFGAMNFADLQTNKSDVPIDI (HPV6b L1 amino acids 193 through 220). Human sera which reacted with HPV6b L1 fusion proteins cross-reacted with an HPV11 L1 fusion protein but did not react with fusion proteins encoded by HPV1a, HPV16, or HPV18. Rabbit anti-BPV1 reacted with L1 fusion proteins encoded by all of these HPV types. In contrast to the type-common (rabbit anti-BPV1-reactive) epitope, the human antibody-reactive epitope appears to be relatively HPV type specific.

Human papillomavirus (HPV) types 6 and 11 are associated most frequently with benign proliferative epithelial lesions, including common genital warts (condylomata acuminata) (13, 19, 20, 38) and squamous laryngeal papillomas (18, 19, 35). The association of these HPV types with certain squamous cell carcinomas of the genital tract has been reported (3, 17, 19, 37, 45, 53). Condylomata acuminata develop most commonly on the vulvar, vaginal, penile, or perianal skin of sexually active adults (39). It usually has been assumed that the associated HPV infections are transmitted sexually (40). However, genital condylomata acuminata have also been reported in children (47), often without a history or suspicion of sexual abuse (12). Squamous laryngeal papillomas due to HPV6 and HPV11 often appear within the first five years of life (18, 19, 35). In these cases, the associated HPV infections have been presumed to be transmitted perinatally, during passage of the newborn through an infected birth canal. It is not unusual, however, for HPV-associated laryngeal papillomas to appear first in adulthood (35). These lesions could conceivably result from reactivation of a latent infection acquired at birth or could arise from a recently transmitted HPV infection. No serological assays are generally available for detecting antibodies directed against HPVs. Serological tests would be useful in clarifying modes of HPV transmission, in estimating relative rates of asymptomatic versus symptomatic disease, and in better characterizing the association between HPVs and squamous cell carcinomas.

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Human antibodies which react with HPV proteins have been detected by using purified HPV virions as targets in serological assays (41, 42). Pass et al. found that sera from 7 of 33 patients with plantar warts contained antibodies which reacted with HPV virions purified from plantar warts. Sera which reacted with whole virions also reacted with the major viral capsid (54-kilodalton [kDa]) protein, purified from sodium dodecyl sulfate (SDS)-disrupted virions by polyacrylamide gel electrophoresis (41). Using type-specified HPV1 virions as targets in a radioimmunoassay, Pfister et al. detected HPV1-reactive antibodies in 52 of 110 patients with plantar or common skin warts or both (42). Similar antibody prevalences were observed in age-matched populations selected without regard for history of plantar or skin warts. The authors concluded that HPV1 infections induced low levels of antibodies which were detectable in approximately 50% of serum samples from patients with warts and that the similar antibody prevalences in nonselected subjects reflected a high rate of asymptomatic infection in those populations.

The detection of human antibodies directed against HPV6b proteins is more problematic because of difficulties in obtaining HPV6b proteins from human tissue sources. HPV6b virions have not been isolated from condylomata acuminata in sufficient quantities for use in serological assays (20, 21, 38, 44). HPV6b has not been propagated in vitro, nor have HPV6b virions been produced by the in vivo technique used by Kreider et al. to propagate an isolate of HPV11 (28). As an alternative source of HPV6b-encoded proteins, several investigators have expressed segments of HPV6b DNA in *Escherichia coli* by means of procaryotic

TABLE 1. HPV fusion protein constructs

HPV type	L1 ORF boundaries (first ATG)	Expression construct	Restriction enzyme sites (map coordinates)	pATH vector (polylinker insertion site)
1a	5347–6936 (5413)	p1L1BB1	*Bgl*II(5582)-*Bgl*II(6358)	pATH1 (*Bam*HI-*Bam*HI)
		p1L1BH1	*Bgl*II(6358)-*Hpa*II(7308)	pATH11 (*Bam*HI-*Cla*I)
6b	5678–7288 (5789)	p6L1XH1	*Xba*I(5903)-*Hpa*II(7862)	pATH11 (*Xba*I-*Cla*I)
		p6L1XX1	*Xho*II(6013)-*Xho*II(7087)	pATH10 (*Bam*HI-*Bam*HI)
		p6L1XP1	*Xho*II(6013)-*Pst*I(6490)	pATH10 (*Bam*HI-*Pst*I)
		p6L1NP1	*Nsi*I(6244)-*Pst*I(6490)	pATH10 (*Pst*I-*Pst*I)
		p6L1RP1[a]	*Rsa*I(6314)-*Pst*I(6490)	pATH1 (*Eco*RI-*Hin*dIII)
		p6L1HP1	*Hinc*II(6377)-*Pst*I(6490)	pATH10 (*Sma*I-*Pst*I)
		p6L1HX42	*Hae*III(7045)-*Xho*II(7087)	pATH10 (*Sma*I-*Bam*HI)
		p6L1XH1K[a]	*Xba*I(5903)-*Hpa*II(7862)	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex0	*Xba*I(5903)- (7266)[b]	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex4	*Xba*I(5903)- (7118)[b]	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex5	*Xba*I(5903)- (7054)[b]	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex12	*Xba*I(5903)- (6454)[b]	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex13	*Xba*I(5903)- (6382)[b]	pATH11 (*Xba*I-*Cla*I)
		p6L1Ex14	*Xba*I(5903)- (6312)[b]	pATH11 (*Xba*I-*Cla*I)
11	5660–7273 (5771)	p11L1XN1[a]	*Xba*I(5885)-*Nsi*I(232)	pATH11 (*Xba*I-*Pst*I)
16	5527–7152 (5560)	p16L1BN1[a]	*Bst*NI(5529)-*Nsi*I(253)	pATH11 (*Sma*I-*Pst*I)
		p16L1BX3	*Bam*HI(6150)-*Xho*II(6951)[c]	pATH11 (*Bam*HI-*Bam*HI)
18	5418–7133 (5430)	p18L1XX1	*Xba*I(5730)-*Xba*I(321)	pATH11 (*Xba*I-*Xba*I)

[a] Refer to Materials and Methods for a more detailed discussion of the plasmid construction.
[b] This plasmid construct was made by exonuclease III and nuclease S1 deletions of the HPV6b LI DNA insert in p6L1XH1K as described in the text.
[c] The presence of a *Xho*II site at HPV16 DNA coordinate 6151 is not consistent with the published HPV16 sequence (49). We find that the T at coordinate 6950 of the published sequence is in fact an A, based upon DNA sequence analysis. The numbering of the HPV16 L1 nucleotide coordinates has also been modified to reflect the presence of an extra T in the E5 ORF at coordinate 3903 (23) which is not present in the published sequence (49).

plasmid expression vectors (1, 15, 27, 31–33, 50, 51). Using an HPV6b L1-encoded fusion protein as a target in Western immunoblot assays, Li et al. detected HPV6b L1-reactive antibodies in 18 of 30 serum samples from patients with condylomata acuminata and in 2 of 20 children less than 5 years of age (31). The children were participants in a study of measles immunization and had not been selected for history of wart virus infection. We tested sera from patients with condylomata acuminata and their sexual partners for antibody reactivities to recombinant proteins encoded by the L1, L2, E1, E2, E4, E6, and E7 open reading frames (ORFs) of HPV6b (27). The most common antibody reactivity detected was to the HPV6b L1 fusion protein. Sera which reacted with the L1 protein of HPV6b did not cross-react with homologous L1 recombinant proteins encoded by HPV16. Human antibodies reacted with a segment of the HPV6b L1 fusion protein distinct from the segment which reacted with rabbit anti-bovine papillomavirus type 1 (BPV1) antiserum.

The L1 ORF of HPV6b encodes a 56,000 dalton (56-kDa) protein which is present in HPV6b-associated human genital warts (51). The protein is localized within keratinocyte nuclei in superficial layers of the wart epithelium (16, 31, 51). Analogies to HPV1 and BPV1 suggest that the HPV6b L1 product is the major viral capsid protein. Antisera generated against HPV1 and BPV1 L1-encoded recombinant proteins stain cellular nuclei of HPV1-associated plantar warts and BPV1-associated bovine fibropapillomas, respectively. These L1 antisera also react with the major proteins (the 54-kDa protein of BPV1 virions and the 54-kDa protein of HPV1 virions) of purified viral capsids (31, 43, 51).

In this report, we have precisely mapped the regions of the HPV6b L1 ORF which encode the human antibody-reactive epitope and the type-common (or genus-specific) epitope. The HPV type specificity of antibodies which react with these epitopes was evaluated by reacting sera with L1 fusion proteins from HPV1a, HPV6b, HPV11, HPV16, and HPV18. Synthetic peptides were derived from the epitope mapping and were shown to react appropriately with sera in an enzyme-linked immunosorbent assay (ELISA). These results contribute to understanding the immunologic response to HPV infection and provide needed reagents for serologic assays to measure exposure to defined HPV types.

MATERIALS AND METHODS

Expression plasmid constructs. Methods for expressing HPV DNA fragments in *E. coli* by means of the procaryotic plasmid expression vector, pATH, have been described previously (15, 27). pBR322-based bacterial plasmids which contain HPV1a (KLuG19 and KLuG28) (5, 9, 10, 48), HPV6b (pHPV6b) (13, 48), HPV11 (pHPV11) (11, 18), HPV16 (pHPV16) (14, 23, 49), and HPV18 (pHPV18) (2, 7) DNA sequences were kindly provided by L. Gissmann and H. zur Hausen (Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany). Expression vectors pATH1, pATH10, and pATH11 were gifts from T. J. Koerner (Duke University Medical Center). HPV DNA restriction fragments derived from the pBR322-based plasmids were ligated to pATH DNA through restriction enzyme sites within the pATH polylinker sequences (see reference 15 for a description of the vectors). The identification of the expression constructs is shown in Table 1. Some of the constructions required multiple cloning steps. p11L1XN1 was made by ligating two HPV11 DNA fragments, *Xba*I(5885)-*Bam*HI(7072) and *Bam*HI(7072)-*Nsi*I(232), to pATH11 which had been digested with *Xba*I and *Pst*I. p16L1BN1 contains two HPV16 DNA fragments, *Bst*NI(5529)-*Bam*HI(6151) and *Bam*HI(6151)-*Nsi*I(253), ligated to pATH11 which had been digested with *Sma*I and *Pst*I. These two-insert ligations reconstituted the HPV11 and HPV16 L1 ORFs, which are interrupted at *Bam*HI sites in pHPV11 and pHPV16, respectively. p6L1RP1 was made by ligating the HPV6b *Rsa*I(6314)-*Pst*I(6490) fragment into pUC19 which had been digested with *Sma*I and *Pst*I. The HPV DNA insert, together with nucleotides derived from

the pUC19 polylinker, was digested with *Eco*RI and *Hin*dIII and ligated to pATH1 DNA which had been digested with *Eco*RI and *Hin*dIII. The 16 added nucleotides at the 5′ end of the HPV6b L1 fragment sense strand placed the *Rsa*I(6314)-*Pst*I(6490) fragment in the desired reading frame for expression in pATH1. Recombinant DNAs were sequenced across the pATH-HPV junction to confirm that the HPV DNA fragments were inserted in the desired reading frame orientations.

Exonuclease III and nuclease S1 deletions. p6L1XH1K is a derivative of p6L1XH1 and contains the synthetic oligonucleotide dGGGTACCC (*Kpn*I linker, New England BioLabs Inc., Beverly, Mass.) ligated into the *Oxa*NI (7841) site of the HPV6b DNA insert (34). The oligonucleotide insertion created a unique *Kpn*I restriction site in p6L1XH1K at coordinate 7841, which was utilized in making the unidirectional exonuclease III (ExoIII) deletions. Unidirectional 3′ to 5′ deletions were made in the HPV6b L1 insert of p6L1XH1K by using a combination of ExoIII and nuclease S1 digestions (46), following the protocol of Henikoff (25) with minor modifications. p6L1XH1K DNA was digested with restriction endonucleases *Bss*HII (7270) and *Kpn*I (*Kpn*I linker sequence inserted in the *Oxa*NI site at position 7841). The linearized DNA was incubated with ExoIII (New England BioLabs) at 25°C, and aliquots were removed from the reaction at 30-s intervals. Single-stranded DNA segments were digested with nuclease S1 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), the digested DNA ends were made blunt with Klenow polymerase (Promega Biotec, Madison, Wis.), and the DNA ends were religated with T4 ligase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (24, 25). The plasmid DNAs were used to transform *E. coli* HB101 bacteria. Selected DNAs were sequenced to evaluate the extent of the deletions (see Table 1).

Synthesis of fusion proteins, SDS-polyacrylamide gel electrophoresis, and Western immunoblot assays. The expression of fusion proteins, SDS-polyacrylamide gel electrophoresis, and Western immunoblot assays were performed as described previously (27).

Peptide synthesis. Peptides 6L1-H1 (QSQAITCQKPTPE KEKPDPYK) and 6L1-X1(DGDMVDTGFGAMNFADLQ TNKSDVPIDI) were made by solid-phase synthesis on an automated peptide synthesizer (model 430A, Applied Biosystems, Foster City, Calif.) (6). Synthetic peptides were purified by high-performance liquid chromatography.

Enzyme-linked immunosorbent assay. Synthetic peptides (100 μl per well at 10 μg/ml in 0.1 M sodium borate buffer, pH 9.5) were incubated in 96-well, flat-bottomed plates (Immulon 2, Dynatech Laboratories, Inc., Chantilly, Va.) overnight at 37°C. The peptide solution was decanted, and the wells were washed four times with 0.5% sodium deoxycholate–0.1 M NaCl–10 mM sodium phosphate (pH 7.5)–0.5% Triton X-100 (wash buffer). Peptide-coated and blank wells were preincubated with 300 μl of 5% nonfat dry milk–0.9% NaCl–0.1% Antifoam-A (Sigma Chemical Co., St. Louis, Mo.)–0.1% sodium azide (milk buffer) for 1 h at 37°C, and then the milk buffer was decanted. Twofold serial dilutions of human sera (final concentrations of 1:50 to 1:800) and rabbit antisera (final concentrations of 1:200 to 1:6,400) were prepared in milk buffer containing 0.1% sodium deoxycholate and 0.1% Nonidet P-40 (Sigma). One hundred microliters of each dilution was added to peptide-coated and blank wells, and incubation was continued for 2 h at 37°C. The wells were rinsed four times with wash buffer. One hundred microliters of alkaline phosphatase-conjugated goat anti-human immunoglobulin G or goat anti-rabbit immunoglobulin G (Boehringer Mannheim) at a 1:2,000 dilution in phosphate-buffered saline containing 0.05% Tween 20 (Sigma) was added to each well and incubated for 2 h at 37°C. After four rinses with wash buffer, 100 μl of alkaline phosphatase substrate solution (*p*-nitrophenyl phosphate disodium at 4.3 mg/ml in 0.1 M sodium bicarbonate [pH 9.5]–10 mM $MgCl_2$) was added and incubated at 22°C for 30 min. The reaction was stopped by the addition of 25 μl of 3 N NaOH, and optical densities were measured at 405 nm with an automated microplate spectrophotometer (model EL310, Bio-Tek Instruments, Winooski, Vt.). Final optical density values were calculated by averaging duplicate assays and subtracting the values obtained from blank wells from values obtained from peptide-coated wells.

Human and rabbit sera. Human sera included sera from 65 patients with genital condylomata acuminata and their sexual partners, 19 female patients with uterine cervical dysplasia, and 34 women attending a sexually transmitted disease clinic (all sera were collected from patients attending clinics at Harborview Medical Center, Seattle, Wash.). Rabbit antiserum prepared against SDS-disrupted BPV1 particles was obtained from a commercial source (rabbit anti-BPV-1, Dako Corp., Santa Barbara, Calif.). Rabbit antisera generated against the p6L1XX1 (HPV6b L1 ORF), p16L1BX3 (HPV16 L1 ORF), p6L2NX1 (HPV6b L2 ORF), and p16L2XX5 (HPV16 L2 ORF) fusion proteins have been described previously (15, 16).

RESULTS

Mapping of antibody-reactive regions of the HPV6b L1 fusion protein. Previous studies (27) showed that the immunoreactive region of the HPV6b L1 fusion protein recognized by human sera mapped to a region bounded by the *Pst*I site at position 6490 and the *Xho*II site at position 7087 and that type-common antisera reacted with a different region of the HPV6b L1 ORF which mapped between the *Xho*II site at position 6013 and the *Pst*I site at position 6490. A strategy was developed to map the immunoreactive regions precisely. A nested set of unidirectional 3′ to 5′ deletions was made in a plasmid expressing the HPV6b L1 ORF fusion protein by digestion with ExoIII and S1 nuclease (24, 25, 46). The deleted plasmids expressed a nested series of truncated fusion proteins, deleted sequentially from the carboxy to the amino terminus. The series of fusion proteins was reacted with sera on Western blots to identify the smallest protein which retained the immunoreactive epitope. DNA sequence analysis of the plasmids identified the 3′ boundary of the immunoreactive region coding sequence. To determine the 5′ boundaries, restriction enzyme sites upstream of the C-terminal boundary were selected, appropriate fragments were recloned into pATH vectors, and the fusion proteins were assayed in Western blots.

ExoIII deletions into the HPV6b L1 DNA insert of p6L1XH1K proceeded from a unique *Bss*HII restriction site at HPV6b DNA coordinate 7270, just 5′ to the L1 stop codon at 7289. *Bss*HII digestion generated a 4-base, 3′-recessed end which was susceptible to ExoIII digestion (22, 24, 46). The 3′ terminus of the HPV DNA insert was protected from ExoIII digestion by cleavage with *Kpn*I endonuclease, which cut within the synthetic *Kpn*I recognition sequence inserted at coordinate 7841 (*Oxa*NI site). *Kpn*I digestion generated a 4-base, 3′-overhanging end which was resistant to ExoIII digestion (22, 24). The *Kpn*I end protected from deletion the three pATH polylinker translation termination

codons, which are located just 3′ to the HPV DNA insert. The extent of DNA deletion was controlled by stopping the ExoIII digestions at progressive time points. After religating the deleted DNAs, the resulting plasmids were used to transform *E. coli* HB101 bacteria. Transformed bacteria were induced to express plasmid-encoded fusion proteins by bacterial growth in tryptophan-depleted medium (15). Selected ExoIII-deleted expression plasmids derived from p6L1XH1K and the fusion proteins which they encode are displayed in Fig. 1. The top two panels show the plasmid DNAs analyzed on agarose gels either uncut (panel 1) or cleaved with *Hpa*II to release a fragment containing the deleted DNA segment (panel 2). Panel 3 displays the truncated fusion proteins expressed by the plasmids in an SDS-polyacrylamide gel stained with Coomassie blue.

Mapping of a human antibody-reactive region encoded by the HPV6b L1 ORF is displayed in Fig. 2. The upper panel is a Coomassie blue-stained SDS-polyacrylamide gel of bacterial lysates containing the deleted L1 fusion proteins (ExoIII deletions) and L1 fusion proteins (p6L1) encoded by four different restriction enzyme fragments (p6L1XH1K, p6L1XX1, p6L1HP1, and p6L1HX42). The middle panel is a replicate Western blot reacted with serum from a patient with condylomata acuminata. The human serum reacted with the p6L1Ex4 fusion protein and with the less extensively deleted fusion proteins (Ex0 and Ex3) but failed to react with the p6L1Ex5 fusion protein and with the more extensively deleted fusion proteins (Ex6 and Ex12 through Ex15). The small amount of reactivity to Ex12 was not reproducible and may have been due to nonspecific sticking. DNA sequence analysis of the plasmids placed the 3′ boundary of the immunoreactive region coding sequence between nucleotides 7054 and 7118. The human serum also reacted with p6L1XX1 (3′ end at 7087), further specifying the 3′ boundary of the epitope coding sequence to the 7054 to 7087 region. The subclone p6L1HX42 contains a 42-base-pair restriction enzyme fragment, *Hae*III(7045)-*Xho*II(7087), located just 5′ to the *Xho*II site at 7087 that expresses only 14 amino acids encoded by the HPV6b L1 ORF. The human serum used in the Western blot in Fig. 2 and 30 of 30 serum samples which reacted with p6L1XHL1K also reacted with p6L1HX42. Therefore, sequences essential for coding the human antibody-reactive epitope mapped between HPV6b nucleotides 7045 and 7087.

The region of the HPV6b L1-encoded fusion protein which reacts with the broadly cross-reactive antibody raised against SDS-disrupted BPV1 virions was mapped similarly (Fig. 3). Rabbit anti-BPV1 antiserum reacted strongly with p6L1XH1K and p6L1Ex12 fusion proteins but displayed a much weaker reactivity to fusion proteins expressed by deletion constructs p6L1Ex13 through p6L1Ex15. The antiserum also reacted strongly with the larger ExoIII deletion fusion proteins p6L1Ex0 through p6L1Ex6 (data not shown). The HPV DNA inserts of p6L1XP1, p6L1NP1, p6L1RP1, and p6L1HP1 share a common 3′ terminus at the *Pst*I site (6489) but are progressively smaller because of the location of their 5′ restriction enzyme cleavage sites. All of these fusion proteins retained reactivity with rabbit anti-BPV1. There was no detectable reactivity of rabbit anti-BPV1 with fusion protein expressed by the vector pATH. Therefore, sequences essential for coding the predominant rabbit anti-BPV1-reactive epitope mapped between HPV6b nucleotides 6377 and 6454.

The p6L1HX42 and p6L1HP1 fusion proteins appear to include the sole, or predominant, immunoreactive regions for human antibodies and for rabbit anti-BPV1 antiserum,

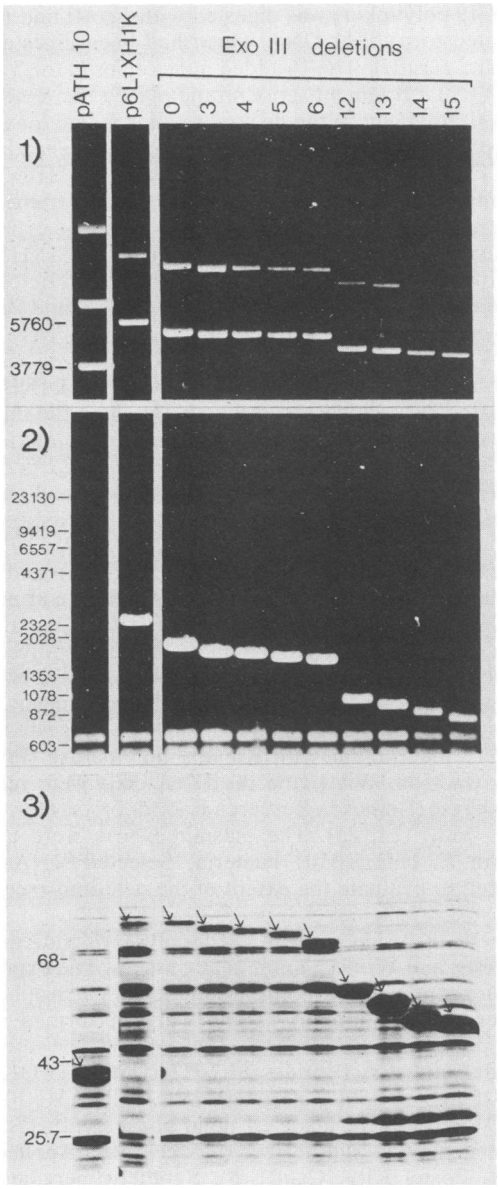

FIG. 1. ExoIII-deleted expression plasmids and fusion proteins. Selected ExoIII-deleted plasmid DNAs were subjected to electrophoresis through a 1.0% agarose gel and stained with ethidium bromide (panel 1). The numbers on the left indicate the positions of pATH10 (3779 nucleotide base pairs) and p6L1XH1K (5760 base pairs) supercoiled plasmid DNAs. DNA restriction fragments which contained the ExoIII-deleted segments were released from the plasmids by digestion with endonuclease *Hpa*II (panel 2). The positions of linear double-stranded DNA mobility references (in nucleotide base pairs) are indicated to the left. Proteins from *E. coli* which contained the expression plasmids were analyzed on a 10% SDS-polyacrylamide gel stained with Coomassie blue (30) (panel 3). The positions of the expression plasmid-encoded fusion proteins are marked with arrows. The numbers on the left are molecular masses in kilodaltons.

respectively. Human sera which had been shown previously to contain antibodies to the HPV6b L1 fusion protein and rabbit anti-BPV1 antiserum were reacted with p6L1XH1, p6L1HX42, p6L1HP1, and pATH fusion proteins under three conditions: (i) after preabsorbing the sera with bacterial lysates expressing pATH vectors sequences; (ii) after

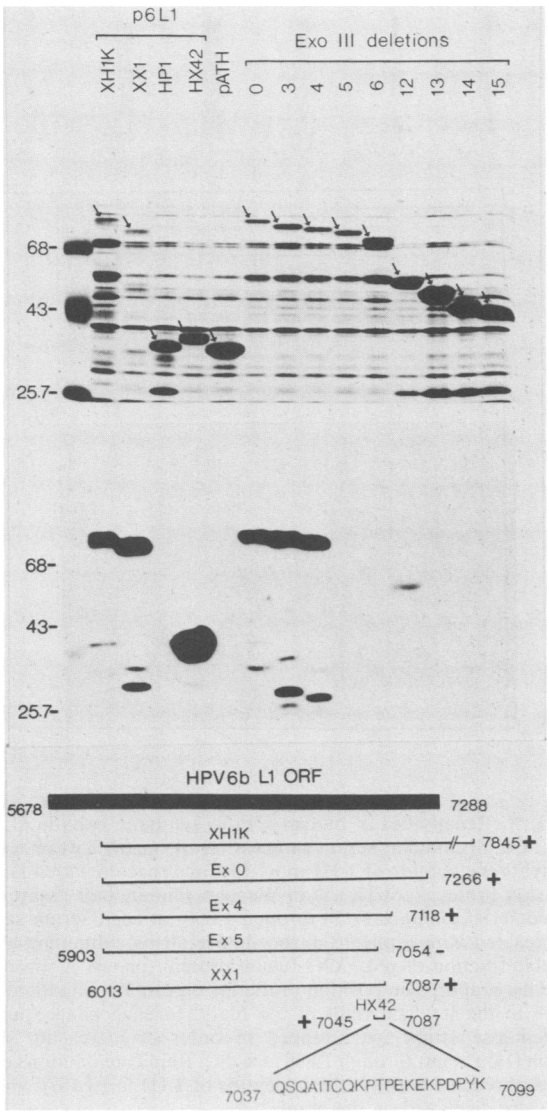

FIG. 2. Mapping of the human antibody-reactive region of the HPV6b L1 fusion protein. Bacterial fusion proteins were subjected to electrophoresis through a 10% SDS-polyacrylamide gel and stained with Coomassie blue (upper panel) (ExoIII deletions 0, p6L1Ex0; ExoIII deletions 3, p6L1Ex3; etc.). The positions of the fusion proteins are marked with arrows. The numbers on the left are molecular masses in kilodaltons. A replicate SDS-polyacrylamide gel was transferred to nitrocellulose and reacted with human serum in Western immunoblot assay (middle panel) (27, 52). Protein-antibody complexes were detected by using ^{125}I-labeled protein A and were visualized by autoradiography (27). The numbers on the left are molecular masses in kilodaltons. The maps of the HPV6b L1 ORF and the HPV6b DNA fragments contained within the expression plasmid are shown in the bottom panel. The + and − symbols indicate the reactivity of the human serum with the fusion protein encoded by that expression plasmid. The amino acid sequence of the synthetic peptide derived from the ExoIII mapping is shown at the bottom of the map.

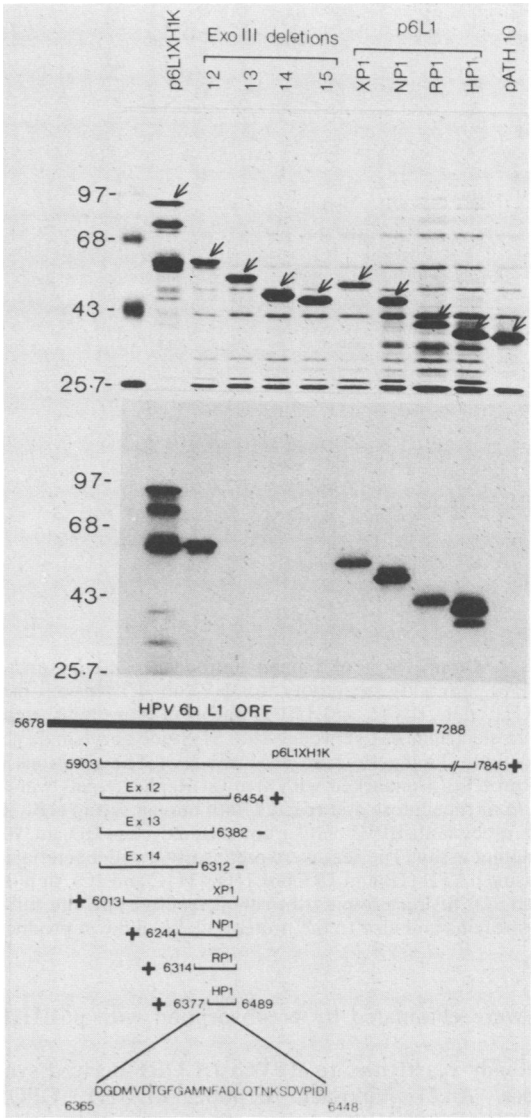

FIG. 3. Mapping of the dominant rabbit anti-BPV1 antibody-reactive region of the HPV6b L1 fusion protein. Bacterial fusion proteins were subjected to electrophoresis through a 12.5% SDS-polyacrylamide gel and stained with Coomassie blue (upper panel). The positions of the fusion proteins are marked with arrows. The numbers on the left are molecular masses in kilodaltons. A replicate SDS-polyacrylamide gel was transferred to nitrocellulose and reacted with rabbit anti-BPV1 antiserum in Western immunoblot assay (middle panel). The numbers on the left are molecular masses in kilodaltons. The maps of the HPV6b L1 ORF and the DNA fragments contained within the expression plasmids are shown in the bottom panels. The + and − signs indicate the reactivity of rabbit anti-BPV1 antiserum with the fusion protein encoded by that expression plasmid. The amino acid sequence of the synthetic peptide derived from the ExoIII mapping is shown at the bottom of the map.

preabsorption with p6L1HX42-containing bacterial lysates; and (iii) after preabsorption with p6L1HP1-containing bacterial lysates. Results are shown in Fig. 4. The positive human serum reacted with p6L1XH1 and p6L1HX42 but not with p6L1HP1 or with pATH fusion proteins. The human antibody reactivities were unaffected by preabsorption with p6L1HP1 lysates, but the reactivities to both p6L1XH1 and p6L1HX42 were markedly reduced by preabsorption with p6L1HX42 lysates. Rabbit anti-BPV1 antiserum reacted with p6L1XH1 and p6L1HP1 but did not react with p6L1HX42. The rabbit antibody reactivities were not reduced appreciably by preabsorption with p6L1HX42 lysates, but the reactivities to both p6L1XH1 and p6L1HP1 essen-

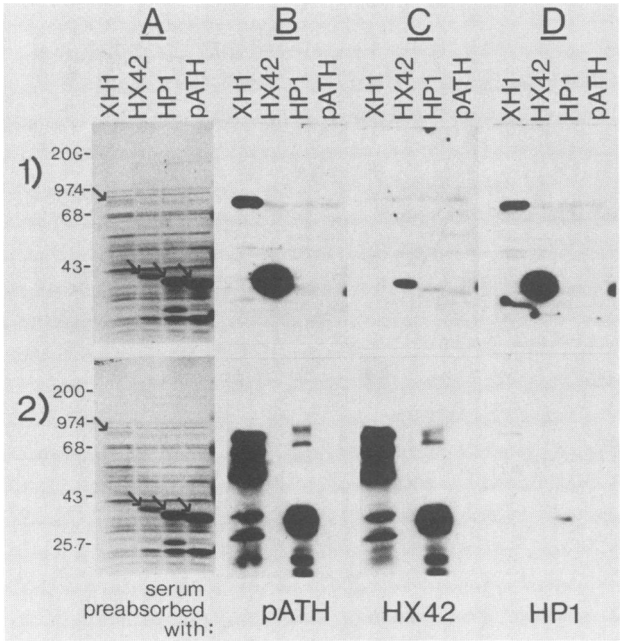

FIG. 4. Comparison of human antibody reactivity and rabbit anti-BPV1 antibody reactivity to HPV6b L1 fusion proteins. p6L1XH1, p6L1HX42, p6L1HP1, and pATH proteins were subjected to electrophoresis through 10% SDS-polyacrylamide gels and stained with Coomassie blue (1A and 2A). The positions of the fusion proteins are marked with arrows. Replicate gels were transferred to nitrocellulose and reacted with human serum (1B, 1C, and 1D) or rabbit anti-BPV1 antiserum (2B, 2C, and 2D) in Western immunoblot assay. The sera were preabsorbed with bacterial lysates expressing pATH (1B and 2B), p6L1HX42 (1C and 2C), or p6L1HP1 (1D and 2D) fusion proteins. In row 2 reactivity to the full-length fusion proteins and also to the proteolytic breakdown products was seen.

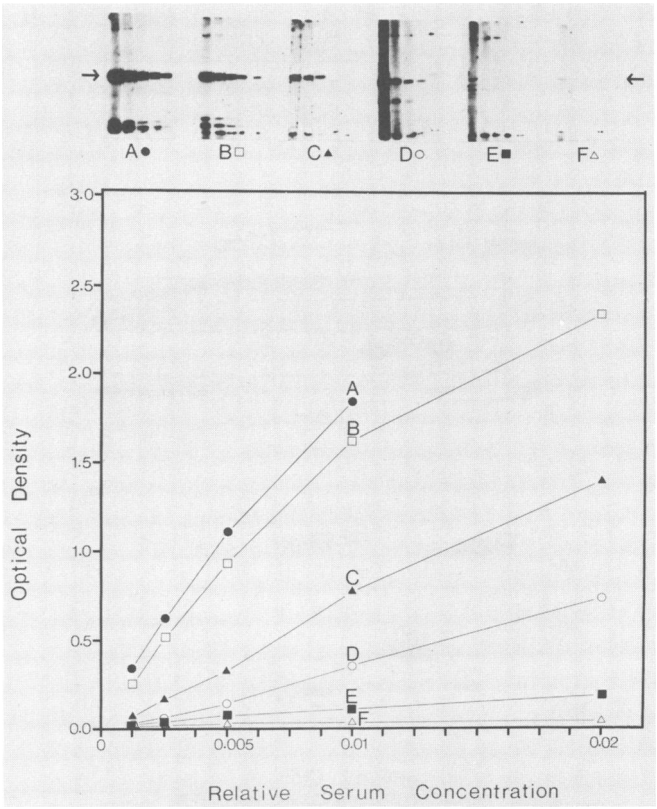

FIG. 5. Reactivities of human sera to synthetic peptide 6L1-H1 in ELISA. Six human serum samples (A through F) were reacted with synthetic peptide 6L1-H1 in ELISA and reacted with a HPV6b L1 fusion protein (p6L1XX1) in Western immunoblot assay. Five twofold serial dilutions (1:50 through 1:800) of each serum sample were reacted with replicate nitrocellulose strips containing a salt-insoluble fraction of p6L1XX1 fusion protein (panels A through F above the graph). The position of the fusion protein is marked with arrows to the left and right of the blots. In each panel, the five nitrocellulose strips are arranged in order of increasing serum dilution (1:50 at left through 1:800 at right). Replicate dilutions of the sera were reacted with synthetic peptide 6L1-H1 in ELISA, and the results are displayed on the graph.

tially were eliminated by preabsorption with p6L1HP1 lysates.

Antibody reactivities to HPV6b L1 ORF-derived synthetic peptides. A 21-amino-acid peptide, 6L1-H1 (QSQAITC QKPTPEKEKPDYPK), was synthesized on the basis of the human antibody epitope mapping and on considerations of the optimal-size peptide. It was reacted with human sera in ELISA. There was a marked correlation between the intensity of human antibody reactivity to the HPV6b-encoded fusion proteins in Western immunoblot assay and the reactivity of those sera to the synthetic peptide in ELISA. The results for six human serum samples are displayed in Fig. 5. Five twofold serial dilutions (1:50 through 1:800) of six human serum samples (A through F) were reacted in Western immunoblot assay with the p6L1XX1 fusion protein, which includes the coding sequence for the human antibody-reactive epitope (the position of the full-length fusion protein is marked with arrows). The results for replicate dilutions of the same sera reacted with the synthetic peptide in ELISA are displayed on the graph. As shown in Fig. 6A, the 6L1-H1 peptide also reacted strongly with a rabbit antiserum generated against the HPV6b L1 fusion protein p6L1XX1 but not with a rabbit antiserum generated against an HPV16 L1 fusion protein (p16L1BX3) or with rabbit antibodies to SDS-disrupted BPV1 virions. The HPV6b L1 construct p6L1XX1 includes the 6L1-H1 coding sequence, and the HPV16 L1 construct includes the homologous coding region of the HPV16 L1 ORF. Rabbit antibodies generated against the L2 fusion proteins of HPV6 (p6L2NX1) and HPV16 (p16 L2XX5) and nonimmune rabbit serum had no detectable reactivity with 6L1-H1 (results for the anti-L2 antisera are not shown).

A 28-amino-acid synthetic peptide, 6L1-X1 (DGDMVDT GFGAMNFADLQLTNKSDVPIDI), was made on the basis of ExoIII mapping of the rabbit anti-BPV1-reactive epitope and also on considering which residues were most highly conserved among the L1 ORFs of several papillomaviruses. This peptide reacted in ELISA with rabbit anti-BPV1, rabbit anti-p6L1XX1, and rabbit anti-p16L1BX3 sera (Fig. 6B). No detectable reactivity was seen with rabbit anti-p6L2NX1, rabbit anti-p16L2XX5, or nonimmune rabbit sera (results for the anti-L2 antisera are not shown). None of the human sera displayed a detectable level of reactivity to peptide 6L1-X1 (results not shown).

HPV type specificity of the antibody reactivities. Human sera which reacted with the HPV6b L1 ORF-encoded fusion proteins and rabbit anti-BPV1 were tested for reactivity to L1 fusion proteins encoded by HPV1a, HPV11, HPV16, and HPV18 DNAs. Characteristics of the fusion protein constructs are displayed in Table 1, and the Western immuno-

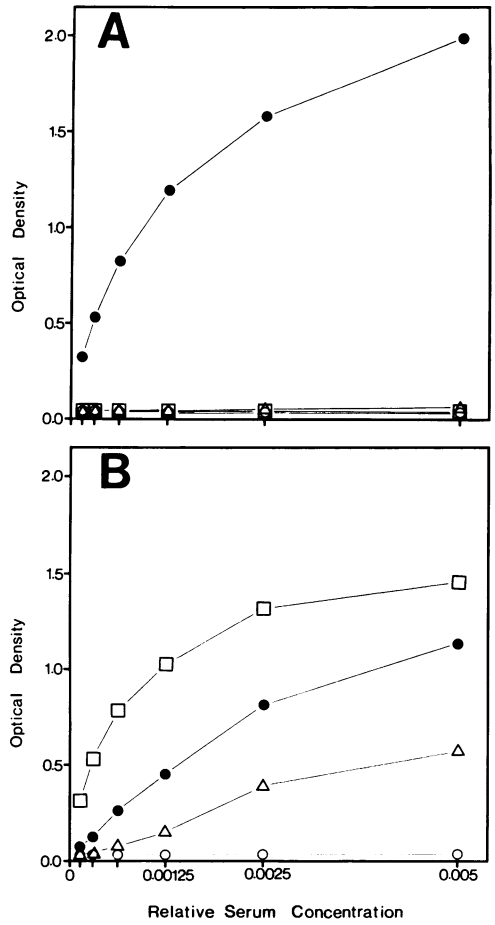

FIG. 6. Reactivities of rabbit antisera to synthetic peptides 6L1-H1 and 6L1-X1 in ELISA. Six twofold serial dilutions (1:200 through 1:6,400) of rabbit anti-p6L1XX1 (●), rabbit anti-p16L1BX3 (△), rabbit anti-BPV1 (□), and nonimmune rabbit serum (○) were reacted with synthetic peptides 6L1-H1 (graph A) and 6L1-X1 (graph B) in ELISA.

blot assays are shown in Fig. 7. Representative results for one human serum sample are displayed in the panels in row 1. The human serum reacted with the HPV6b and HPV11 L1-encoded proteins but did not react with L1 fusion proteins encoded by HPV1a, HPV16, or HPV18. After preabsorption of the human serum against bacterial lysates which express p6L1HX42, the reactivities to both p6L1XH1 and p11L1XN1 were markedly diminished. Thirty HPV6b L1-reactive human serum samples were tested similarly for reactivities with L1 fusion proteins encoded by HPV11, HPV16, and HPV18. Twenty-eight were positive for HPV11 L1 reactivity (the two HPV11 L1-nonreactive samples had very low levels of reactivity to the HPV6b L1 fusion protein), and all of the human samples were negative for reactivity to HPV16- and HPV18-encoded L1 fusion proteins.

Rabbit anti-BPV1 antiserum was reacted with the L1-encoded fusion proteins, and the results are displayed in the panels in row 2 of Fig. 7. The rabbit antiserum reacted to some extent with all of the L1 ORF-encoded fusion proteins (the 5′ construct, p1L1BB1, expresses the region of the HPV1a L1 ORF which is homologous to the rabbit anti-BPV1-reactive region of the HPV6b L1 ORF). After preabsorption of rabbit anti-BPV1 antiserum with bacterial lysates

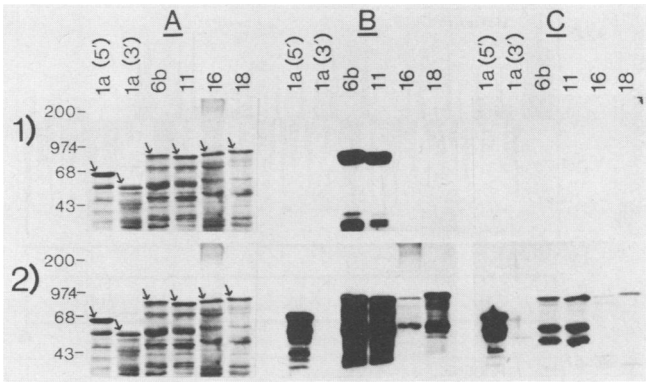

FIG. 7. HPV type specificity of the anti-HPV6b L1 reactivities of human serum and rabbit anti-BPV1 antiserum. Salt-insoluble fractions of fusion proteins (15, 27) were subjected to SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and stained with amido black (100 μg/ml in 45% methanol–10% acetic acid) (1A and 2A). The positions of the fusion proteins are marked with arrows. Replicate blots were reacted with human serum (1B and 1C) or with rabbit anti-BPV1 antiserum (2B and 2C). The human serum was preabsorbed with bacterial lysates expressing pATH (1B) or p6L1HX42 (1C) fusion proteins. Rabbit anti-BPV1 antiserum was preabsorbed with bacterial lysates expressing pATH (2B) or p6L1HP1 (2C) fusion proteins. 1a(5′), p1L1BB1; 1a(3′), p1L1BH1; 6b, p6L1XH1; 11, p11L1XN1; 16, p16L1BN1; 18, p18L1XX1. Reactivity to the full-length fusion protein and also to proteolytic breakdown products was seen.

which express p6L1HP1, the reactivities to the HPV6, HPV11, HPV16, and HPV18 fusion proteins were diminished. There was little effect upon the reactivity to the HPV1a-encoded L1 fusion protein.

DISCUSSION

We have presented a novel method for mapping linear (continuous) immunoreactive epitopes. The generation of a series of deleted plasmid molecules, each expressing a truncated fusion protein, provides a quick and inexpensive alternative to the more common use of a set of overlapping synthetic peptides. In the study presented here, a human antibody-reactive determinant encoded by the HPV6b L1 ORF was mapped by using ExoIII-deleted expression plasmids. The DNA sequence which encodes the epitope lies between HPV6b DNA coordinates 7045 and 7087. Expression plasmid p6L1HX42, which contains only the HPV6b L1 sequences from 7045 (*Hae*III site) to 7087 (*Xho*II site), encoded a fusion protein which retained immunoreactivity. The p6L1HX42 protein blocked the reactivity of human antibodies with the larger HPV6b L1 fusion protein p6L1XH1 [*Xba*I (5903) − L1 stop codon (7289)]; therefore, the 7045 to 7087 region appears to encode the sole (or predominant) human antibody-reactive linear epitope of the p6L1XH1 fusion protein. Human sera which reacted with HPV6b L1 fusion proteins in Western immunoblot assay also react with the 21-amino-acid synthetic peptide QSQAITCQKPTPEKEKPDPYK in ELISA. The synthetic peptide is equivalent to HPV6b L1 amino acids 417 through 437 (measured from the first L1 ORF-encoded methionine) derived from DNA coding sequences from coordinates 7037 through 7099. The human antibody epitope lies within a highly hydrophilic (29) segment near the carboxy terminus of the HPV6b L1-encoded polypeptide. The algorithm of Hopp and Woods (26) predicted the potential for an antibody-reactive site at this location.

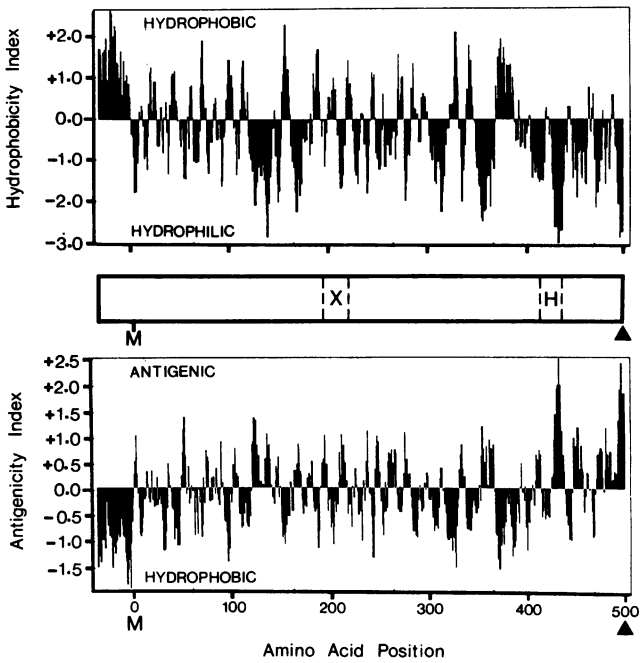

FIG. 8. Hydrophobicity and antigenicity plots of the HPV6b L1 ORF protein. Hydrophobicity (top graph) and antigenicity (bottom graph) profiles of the HPV6b L1 ORF-encoded polypeptide were generated by using the algorithms of Kyte et al. (29) and Hopp and Woods (26), respectively. The positions of the first L1 methionine codon (M) and the L1 stop codon (▲) are marked. The HPV6b L1 polypeptide is represented between the two plots. The positions of the cross-reactive (X) and human antibody-reactive (H) regions are indicated.

The human antibody-reactive epitope is distinct from the region of the HPV6b L1 fusion protein recognized by rabbit anti-BPV1 antiserum. The rabbit anti-BPV1-reactive epitope coding sequence mapped to the region between DNA coordinates 6377 (*Hin*cII site) and 6454 (3′ terminus of p6L1Ex12). Rabbit anti-BPV1 reacted with the synthetic peptide DGDMVDTGFGAMNFADLQTNKSDVPIDI in ELISA. This peptide is equivalent to HPV6b L1 amino acids 193 through 220 derived from the DNA coding sequence from coordinates 6365 through 6448. This region lies within an amphipathic segment of the HPV6b L1 polypeptide (29) which does not rank highly as a potential antigenic determinant according to the algorithm of Hopp and Woods. Computer-generated profiles of the hydrophilicity (29) and antigenicity (26) of the HPV6b L1 ORF are shown in Fig. 8.

Human sera which reacted with the HPV6b L1 fusion protein cross-reacted with an L1 fusion protein encoded by the closely related HPV11. The human sera did not cross-react, however, with L1 proteins encoded by HPV1a, HPV16, or HPV18. In contrast, rabbit anti-BPV1 reacted with L1 fusion proteins encoded by all of these HPV types. Anti-BPV1 antisera generated against SDS-disrupted BPV1 virions are known to contain both BPV type-specific antibodies as well as antibodies which broadly cross-react with capsid proteins of other papillomavirus types (8, 36). Conversely, rabbit anti-BPV1, rabbit anti-p6L1XX1 (HPV6b L1), and rabbit anti-p16L1BX3 (HPV16 L1) reacted with the 6L1-X1 synthetic peptide derived from mapping the rabbit anti-BPV1-reactive region of the HPV6b L1 ORF. But only rabbit anti-p6L1XX1 and not rabbit anti-BPV1 or rabbit anti-p16L1BX3 recognized the 6L1-H1 synthetic peptide derived from mapping the human antibody-reactive epitope of the HPV6b L1 ORF. The amino acid sequences of HPV1a, HPV6b, HPV11, HPV16, HPV18, and BPV1 are compared in the rabbit anti-BPV1-reactive (X1) and human antibody-reactive (H1) regions in Fig. 9. There is a striking conservation of amino acid sequences among the HPV types within the X1 (cross-reactive) region but considerably less conservation within the region which includes the human antibody-reactive epitope of HPV6b.

We conclude, therefore, that human antibodies are reacting with a specific linear (continuous) epitope near the carboxy terminus of the HPV6b L1 polypeptide, which is highly hydrophilic. We speculate that the epitope is located on the external surface of the viral capsid in the intact virion

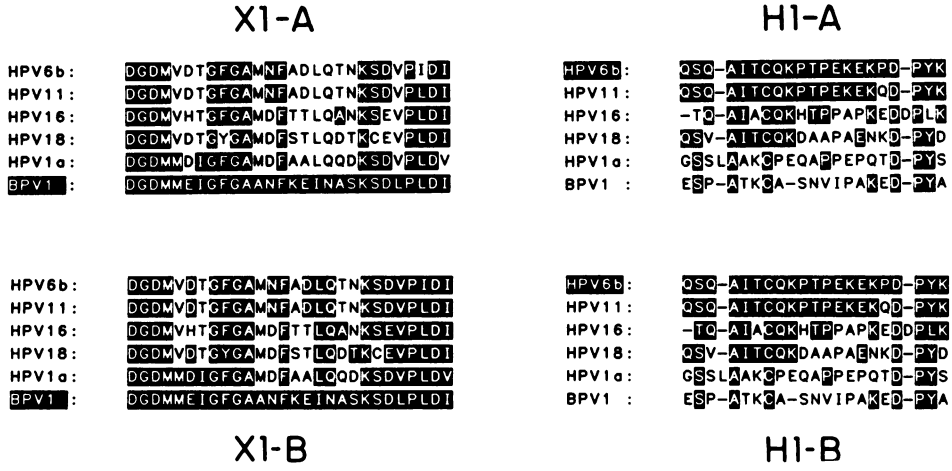

FIG. 9. Comparison of papillomavirus amino acid sequences in the HPV6b L1 cross-reactive and human antibody-reactive regions. The HPV6b peptide sequences 6L1-X1 (X1) and 6L1-H1 (H1) are compared to homologous regions of selected papillomavirus types. The reference sequence for the cross-reactive (X1) region is the BPV1 sequence, and the reference sequence for the human antibody-reactive (H1) region is the HPV6b sequence. In the upper panels (X1-A and H1-A), amino acids which are identical to the reference sequence are highlighted. In the lower panels (X1-B and H1-B), identical amino acids and conservative amino acid changes are highlighted. The papillomavirus amino acid sequences are derived from the published DNA sequences (4, 5, 7, 9–11, 48, 49).

and is recognized during a natural infection. The human antibodies cross-react with L1 proteins encoded by closely related HPV6b and HPV11, both of which are associated with condylomata acuminata and squamous laryngeal papillomas, but there is no detectable reactivity with the HPV types most commonly associated with uterine cervical carcinomas (HPV16 and HPV18) or plantar warts (HPV1). The human antibodies are not reacting with the broadly cross-reactive epitope recognized by rabbit anti-BPV1 antiserum.

The human antibody reactivities to the HPV6b L1 fusion proteins probably represent humoral immune responses induced by HPV6, HPV11, or closely related papillomavirus infections associated with genital warts and squamous laryngeal papillomas. But confirming this relationship is particularly problematic in the case of HPVs. HPVs are ubiquitous, and over 50 different genotypes have been described. Some proportion of the human antibody reactivities conceivably could have been induced by antigenically similar HPVs which cause epithelial lesions distinct from condylomata acuminata or squamous laryngeal papillomas. The lack of cross-reactivity with the HPV1, HPV16, and HPV18 L1 proteins demonstrates at least a degree of L1 antigenic specificity. We are in the process of expressing L1 ORF fragments from HPV2, HPV4, HPV5, and HPV33 to determine whether the HPV6b and HPV11 L1-reactive sera cross-react with any of these proteins.

The finding that none of the 30 serum samples from patients with condylomata acuminata reacted with the L1 proteins of HPV16 or HPV18 was surprising in view of the fact that HPV16 infection is frequent in patients with sexually transmitted disease. We have begun using bacterially expressed fusion proteins encoding all of the ORFs of HPV16 in Western blot assays with sera from patients attending a sexually transmitted disease clinic. Only one serum sample reacted with L1, whereas the most common reactivity was to the L2 ORF (our unpublished results). At this point it is unclear whether the HPV16 L1 protein is not immunogenic or whether the epitopes are discontinuous and not detected by our assays.

The association between the HPV6b L1 antibody reactivities and HPV6b-related infections would also be strengthened by comparing seroprevalence rates in people with HPV6b exposure with seroprevalence rates in people without HPV6b exposure. However, exposure to HPVs is difficult to define. The majority of papillomavirus infections are probably asymptomatic and establish prolonged periods of low-grade pathological changes or latency. The development of HPV-associated lesions may not be the result of recent infection only but also may be the manifestation of other factors which have reactivated latent virus. Therefore, the presence or absence of HPV-associated lesions may not be appropriate criteria for selecting HPV-exposed versus HPV-nonexposed individuals. In future studies, we plan to use the 6L1-H1 synthetic peptide and the p6L1HX42 fusion protein to screen larger populations for HPV6b and HPV11 L1 antibody reactivities. By determining HPV seroprevalence rates in populations which differ with regard to age, level of sexual activity, and the presence or absence of HPV lesions, we hope to better define patterns of HPV transmission and the relationships between HPV exposure and HPV disease.

ACKNOWLEDGMENTS

We thank Daniel Hansen and Reynaldo Verduzco for assisting in the construction of expression plasmids, Ernest Tolentino for synthesizing the peptides, Juliane Firzlaff for providing rabbit antisera, and Andria Langenberg, Lawrence Corey, and King Holmes for kindly providing human sera.

This study was supported by Public Health Service grants PO1-CA42792 and RO1-CA35568 from the National Cancer Institute to D.A.G. S.A.J. was a Fellow of the American Social Health Association.

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