Identification of Immunoreactive Antigens of Human Papillomavirus Type 6b by Using *Escherichia coli*-Expressed Fusion Proteins

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Human papillomaviruses (HPVs) infect the genital epithelium and are found in proliferative lesions ranging from benign condylomata to invasive carcinomas. The immunological response to these infections is poorly understood because of the lack of purified viral antigens. In this study, bacterially derived fusion proteins expressing segments of all the major open reading frames (ORFs) of HPV type 6b (HPV-6b) have been used in Western blot (immunoblot) assays to detect antibodies directed against HPV-encoded proteins. The most striking reactivities present in sera from patients with genital warts were to the HPV-6b L1 ORF protein and, to a lesser extent, to the HPV-6b L2 ORF protein. Two cases of reactivity to HPV-6b E2 ORF were observed, but no reactivities were seen with other HPV-6b constructs. Two sera reacted with the HPV-16 L2 fusion protein, and two sera reacted with the HPV-16 E4 protein. The antibodies directed against the HPV-6b fusion proteins showed no cross-reactivity with comparable regions of the HPV-16 ORFs. This assay provides a useful approach for further studies of HPV serology.

Human papillomaviruses (HPVs) are a heterogeneous group of double-stranded DNA viruses associated with a variety of epithelial lesions, including plantar warts, common skin warts, laryngeal papillomata, condylomata acuminata (common genital warts), and anogenital dysplasias and carcinomas (for a review, see reference 33). Although the overall genetic organization of human and animal papillomavirus genomes is conserved (9, 40), HPV isolates from histologically distinct lesions share only low levels of overall DNA sequence homology. Considerable variability is seen even among different HPV isolates from a single category of epithelial lesion. The viruses are classified into groupings called types; i.e., a single type includes all isolates which display greater than 50% DNA homology in a liquid hybridization assay (6). Approximately 50 types are currently recognized. This system of HPV genotyping is distinct from the serotyping systems commonly used to classify isolates in other viral groups, which group together antigenically related strains of virus.

Several studies, using whole virions extracted from human tissues as immunogens, suggest that HPVs isolated from different epithelial lesions may be antigenically distinct. Antisera generated in laboratory animals immunized with genotypically distinct HPVs isolated from skin warts do not cross-react (14, 29). Orth et al. prepared antisera in guinea pigs by using HPV virions isolated from two patients with epidermodysplasia verruciformis, the viral DNAs of which showed little sequence homology. The antibodies did not cross-react, nor did they react with whole HPV type 1 (HPV-1) or HPV-2 virions (30). Similarly, Pass et al. found that antisera prepared in rabbits immunized with HPV virions isolated from two siblings with epidermodysplasia verruciformis cross-reacted with each other but not with virions isolated from common skin warts of immunologically normal patients (32). On the other hand, some HPV isolates which are clearly genetically distinct may induce cross-reacting antibody responses (2, 28). Almeida et al. found that rabbit antisera prepared against skin wart-derived HPV

virions react both with skin wart virions and with HPV virions purified from condylomata acuminata (2).

Human antibodies induced by natural HPV infections have been detected by using HPV virions isolated from skin wart parings as the antigenic targets. Detection methods have included immune electron microscopy (1, 16), the agar-gel diffusion (Ouchterlony) method (1, 8, 16, 20, 24, 31, 34, 36, 38, 39), complement fixation (13, 17, 20, 26, 34, 36, 38, 39), radioimmunoassay (17), enzyme-linked immunosorbent assay (37), and hemagglutination inhibition (24). In the majority of these studies, antigen was prepared from pooled wart biopsies without the determination of the genetic characteristics of the HPV virions; two studies of anti-HPV antibody prevalence in skin wart patients have used type-specified HPV-1 virions as antigenic targets, but these virions were also derived from pooled wart biopsies (20, 37). In these studies, the reported prevalence of anti-HPV antibodies in patients with common skin warts varies from 20% (13) to 88% (24). The variation is related to the type of assay used, how long skin warts had been present, and whether the skin warts were persistent or resolving. Though the prevalence of anti-HPV antibodies has been examined in patients with epidermodysplasia verruciformis (37) and condylomata acuminata (36), the HPV antigen target used in these assays was prepared from pooled common skin warts rather than from the epithelial lesions characteristic of these conditions. The prevalence of antibodies among the study patients was less than that among a control group of patients with common skin warts. Pooled common skin warts have been used most commonly as a source of HPV virions because of the relatively high viral yield obtained from this source compared with other HPV-related lesions (3); the concentration of HPV virions in condylomata acuminata, for example, is estimated to be 10^{-4} less than that in skin warts (27, 36). The inability to propagate HPVs in tissue culture or to isolate large quantities of purified viral antigens from clinical tissues has hampered studies on the antigenic relatedness of HPVs and the thorough analysis of the immunological response to HPV infection.

We have initiated studies of the natural history of HPV

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infections of the human genital tract, specifically condylomata acuminata (most commonly associated with HPV-6 and HPV-11; 15) and uterine cervical dysplasia and carcinoma (most commonly associated with HPV-16, HPV-18, and HPV-33; 4, 7, 11). The use of HPV virions derived from these lesions as antigenic targets in serological assays was judged impractical because of the low virus yield from naturally infected human tissue sources. As an alternative source of HPV-encoded proteins, a series of plasmid constructs which express restriction enzyme fragments from the major open reading frames (ORFs) of HPV-6b and HPV-16 DNAs have been made (12). These HPV DNA fragments are expressed in *Escherichia coli* as fusion proteins linked to a portion of the *E. coli* tryptophan E synthetase (*trpE*) gene product through pATH vectors. The goals of the study presented here are (i) to determine whether serum antibodies recognize bacterially expressed HPV fusion proteins, (ii) to identify which HPV antigens are targets of the humoral immune response, and (iii) to verify and characterize any reactivities to the HPV fusion proteins that are observed. The decision to initiate the study by using serum from patients with genital condyloma was based on the assumption that this population would be most likely to have antibodies to HPV-6. The study was not designed to assess the prevalence of HPV antibodies in defined populations; rather, it represents a first step towards identifying the viral antigens which will be used as targets to screen populations.

MATERIALS AND METHODS

Plasmid constructs and synthesis of fusion proteins. Restriction enzyme fragments from ORFs of HPV-6b (40) and HPV-16 (41) were expressed in *E. coli* as tryptophan E synthetase (*trpE*)-HPV fusion proteins through the plasmid expression vectors pATH1, pATH2, pATH10, and pATH11 (gifts of T. J. Koerner) and have been described previously (12). Clone p6L1XP1 has not been described previously and was obtained by cleaving plasmid p6L1XX1 with *Pst*I and religating the large fragment. All constructs were sequenced by using a synthetic oligonucleotide primer homologous to sequences within the *trpE* gene to determine the nucleotide sequence through the polylinker and into at least the first 50 nucleotides of the DNA inserts to confirm the predicted reading frame. Transformed *E. coli* HB101 was grown overnight in M9 medium (23a) containing tryptophan (30 µg/ml) and ampicillin (50 µg/ml) at 37°C. The overnight culture was diluted 1:10 in M9 medium plus ampicillin (no tryptophan) and incubated for 1 h at 30°C. 3-Indoleacrylic acid was added to a final concentration of 5 µg/ml, and incubation was continued for 5 h at 30°C.

Constructs pHX1 and pHX2 (kindly provided by M. Churcher and A. C. Minson) contain restriction fragments from the L1 ORF of HPV-16 that are expressed in *E. coli* as λ *cro*- β -galactosidase-HPV fusions by the expression vector pEX (43). Cultures grown overnight at 30°C in 2 $\times$ yeast-tryptone medium containing ampicillin (50 µg/ml) were diluted 1:40 in the same medium and induced by growth at 42°C for 1 h.

Total bacterial lysates were prepared by pelleting 1.5 ml of bacterial culture, suspending the pellet in 250 µl of Laemmli sample buffer, sonicating at 50 W for 20 s, and heating to 100°C for 5 min. Fusion proteins were partially purified by means of their relative insolubility in nonionic detergents compared with native *E. coli* proteins. A 50-ml induced bacterial culture was pelleted, suspended in 10 ml of 50 mM Tris (pH 8.0)-5 mM EDTA, and then digested on ice for 90

min with lysozyme (2 mg/ml). NaCl (5.0 M) and 10% Nonidet P-40 were added to final concentrations of 0.3 M and 0.7%, respectively, and incubation on ice was continued for 30 min. The solution was passed three times through an 18-gauge needle to reduce viscosity, and incubation on ice was continued for 30 min. The insoluble fraction was pelleted at 16,000 $\times$ g at 4°C for 10 min, resuspended in 10 ml of 10 mM Tris (pH 8.0)-1.0 M NaCl, and incubated on ice for 10 min. The insoluble fraction was pelleted once again as described above, resuspended in 1.0 ml of Laemmli protein sample buffer and heated to 100°C for 5 min.

Polyacrylamide gel electrophoresis and Western blot (immunoblot) assay. Fusion protein preparations were subjected to electrophoresis through 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). Total bacterial lysates (50 µl) were loaded in 6-mm slots. The amount of fusion protein was determined by visual inspection of Coomassie blue-stained gels. The quantity of insoluble protein fraction was standardized to give a Coomassie blue staining intensity equivalent to 5 µg of bovine serum albumin per 6-mm slot. Proteins were transferred to nitrocellulose in 25 mM Tris-195 mM glycine (pH 8.5)-20% methanol (Western transfer buffer) at 250 mA for 8 h. Blots were soaked for 10 min in phosphate-buffered saline containing 10 mM N-ethylmaleimide, incubated for 2 h in 5% nonfat dry milk-0.9% NaCl-0.1% Antifoam-A (Sigma Chemical Co., St. Louis, Mo.)-0.1% sodium azide-1 mM potassium iodide (milk buffer) and then incubated for 1 h in milk buffer containing 10% fetal calf serum. Blocking agent was prepared from a 50-ml induced bacterial culture which was pelleted, resuspended in 3.6 ml of 50 mM Tris (pH 8.0)-5 mM EDTA, and sonicated at 50 W for 20 s; 0.4 ml of 20% SDS was added, and the lysate was heated to 100°C for 5 min. This mixture was diluted 1:20 in milk buffer, and Nonidet P-40 and sodium deoxycholate were each added to a final concentration of 0.1%. Human sera were diluted 1:50 in 2.5 ml of milk buffer plus appropriate blocking agent and preincubated at 4°C for 8 h. Nitrocellulose blots were then added, and incubation at 4°C was continued for 12 h. Blots were washed three times in 0.5% deoxycholate-0.1 M NaCl-0.5% Triton X-100-10 mM sodium phosphate (pH 7.5)-0.1 mM phenylmethylsulfonyl fluoride (wash buffer) for 30 min. The blots were reincubated in milk buffer for 1 h; ¹²⁵I-protein A (4 µCi/µg; New England Nuclear Corp., Boston, Mass.) was added to a final dilution of 1:1,000, and incubation was continued for 6 h at 4°C. Blots were washed three times for 30 min each in wash buffer, dried, and autoradiographed.

Immunospot assay. Insoluble fractions containing the *trpE*-HPV-6b fusion proteins were prepared from induced bacterial lysates as described above. The insoluble pellets were dissolved in Laemmli sample buffer to a final concentration of 50 µg of wet pellet weight per ml of sample buffer and heated to 100°C for 5 min. Of each fusion protein solution, 50 µl per well was filtered through nitrocellulose by using a dot-blotting device (Schleicher & Schuell, Inc., Keene, N.H.), and then the blot was rinsed with 500 µl of Western transfer buffer. Blots were preblocked in milk buffer and reacted with sera (1:50 dilution) preabsorbed with pATH10 blocking agent as described above. Antigen-antibody complexes were detected by incubating alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Promega Biotec, Madison, Wis.) at a dilution of 1:1,000 in milk buffer with the strips at 22°C for 2 h. Antibody complexes were visualized by allowing the strips to react for 30 min with 100 mM Tris (pH 9.5)-100 mM NaCl-5 mM MgCl₂ containing 0.33 mg of Nitro Blue Tetrazolium per ml and 0.165 mg of

TABLE 1. Identification of HPV fusion protein expression constructs

Name ^a	HPV type	ORF	Vector ^b	HPV restriction enzyme fragment ^c	Observed mol wt, 10 ³
p6L1XX1	6	L1	pATH10	<i>Xho</i> II (6013)– <i>Xho</i> II (7087)	75
p6L1(XX) ²	6	L1	pATH10	<i>Xho</i> II (6013)– <i>Xho</i> II (7087) twice	110
p6L1XP1	6	L1	pATH10	<i>Xho</i> II (6013)– <i>Pst</i> I (6490)	52
p16L1BX3	16	L1	pATH11	<i>Bam</i> HI (6150)– <i>Xho</i> II (6951)	68
pHX1 ^d	16	L1	pEX	<i>Hph</i> I (5564)– <i>Bam</i> HI (6150)	125
pHX2 ^d	16	L1	pEX	<i>Bam</i> HI (6150)– <i>Sph</i> I (7463)	150
p6L2NX1	6	L2	pATH11	<i>Nco</i> I (4421)– <i>Xho</i> II (6013)	120
p6L2NB4	6	L2	pATH11	<i>Nco</i> I (4421)– <i>Bam</i> HI (4722)	48
p6L2BP2	6	L2	pATH10	<i>Bam</i> HI (4722)– <i>Pst</i> I (5408)	75
p6L2BX4	6	L2	pATH10	<i>Bam</i> HI (4722)– <i>Xho</i> II (6013)	100
p16L2XX5	16	L2	pATH10	<i>Xho</i> II (4519)– <i>Xho</i> II (5071)	82
p16L2XB5	16	L2	pATH10	<i>Xho</i> II (5071)– <i>Bam</i> HI (6150)	70
p6E2SP1	6	E2	pATH10 ^e	<i>Sca</i> I (2789)– <i>Pst</i> I (3912)	53
p16E2BP2	16	E2	pATH11	<i>Bal</i> I (3398)– <i>Pst</i> I 3692)	50
p6E4BP37	6	E4	pATH11	<i>Bbv</i> I (3288)– <i>Pst</i> I (3912)	48
p16E4BP6	16	E4	pATH10	<i>Bal</i> I (3398)– <i>Pst</i> I (3692)	48
p6E1ES4	6	E1	pATH11	<i>Eco</i> RI (2188)– <i>Sca</i> I (2789)	52
p6E6NN11	6	E6	pATH11	<i>Nsi</i> I (171)– <i>Nsi</i> I (530)	51
p6E7NN1	6	E7	pATH11	<i>Nsi</i> I (530)– <i>Nsi</i> I (1640)	53
p16E1aSN2	16	E1a	pATH10	<i>Sau</i> 3A (871)– <i>Nar</i> I (1309)	65
p16E6DS2	16	E6	pATH10	<i>Dra</i> II (111)– <i>Sau</i> 3A (525)	58
p16E7NP1	16	E7	pATH11	<i>Nsi</i> I (562)– <i>Pst</i> I (875)	59

^a The plasmids have been described fully by Firzlaff et al. (12).

^b The pATH vectors were gifts from T. J. Koerner.

^c Numbers in parentheses indicate nucleotide positions.

^d pHX1 and pHX2 were gifts from M. Churcher and A. C. Minson.

^e pATH10 was cleaved with *Eco*RV and *Sma*I.

5-bromo-4-chloro-3-indolyl phosphate per ml. The color reaction was stopped by soaking the strips in 20 mM Tris (pH 8.0)–5 mM EDTA for 30 min.

Human and rabbit sera. Human sera were obtained from patients with condylomata acuminata lesions present for at least 6 months at their initial visit to a treatment study to test the efficacy of topical alpha interferon-nonoxynol 9 for the treatment of refractory genital warts. Sera were stored at –20°C. Rabbit antisera against *trpE*-HPV fusion proteins were prepared as previously described (12). Rabbit antiserum prepared against SDS-disrupted bovine papillomavirus type 1 (BPV-1) virions was obtained from a commercial source (Dako Rabbit anti-BPV-1; Dako Corp., Santa Barbara, Calif.). Rabbit antisera were diluted 1:500 when used in Western immunoblot assays.

RESULTS

Reactivity to the major capsid protein. The fusion protein constructs which express defined segments of the genomes of HPV-6b and HPV-16 are listed in Table 1. The observed molecular weights of the fusion proteins on polyacrylamide gels correlated closely with the predicted values. The fusion proteins have been used previously to generate polyclonal antibodies, and the antibodies have been shown to react with the appropriate fusion proteins and, in some cases, with authentic HPV proteins (12; our unpublished data). Those studies indicated that all the fusion proteins described in Table 1 are immunoreactive.

As an initial approach to screen for anti-HPV-6b antibodies, the salt-insoluble fraction of the largest *trpE*-HPV-6b fusion protein for each ORF was prepared, and an equivalent mass of each protein was spotted onto a strip of nitrocellulose. Individual strips were reacted with sera from 75 patients with condylomata acuminata. That patient population was chosen because of the likelihood of HPV-6 infection. To

eliminate antibodies directed against *E. coli*-encoded proteins (including the *trpE* portion of the fusion proteins), sera were preabsorbed against denatured lysates of *E. coli* which express vector (pATH) sequences. Figure 1 shows a sample of 12 strips demonstrating the range of reactivities. The predominant reactivity was to L1 ORF, with four patients (A, B, F, and I) strongly positive, five patients (C, D, E, K, and L) weakly positive, and three patients (G, H, and J) equivocal or negative. Clear reactivities to L2 (B, C, D, and

FIG. 1. Immunospot assay for antibodies to HPV-6b fusion proteins. The salt-insoluble fraction of the fusion proteins (listed on the left of strips) was filtered onto nitrocellulose by using a dot-blotting device (Schleicher & Schuell) and then rinsed with 500 μ l of Western transfer buffer. Filters were cut into strips, preblocked in milk buffer, and then incubated with human sera (from patients A to L) diluted 1:50 in 2.5 ml of milk buffer which had been preabsorbed with pATH10 blocking agent. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G and visualized by reacting the strips with Nitro Blue Tetrazolium.

FIG. 2. Characterization of antibody response to HPV-6b L1 fusion protein. A salt-insoluble fraction of the fusion protein of p6L1XX1 was subjected to electrophoresis through 10% polyacrylamide gels containing 0.1% SDS and transferred to nitrocellulose in Western transfer buffer. Human sera were used at a 1:50 dilution either unblocked (lanes 1) or preabsorbed with pATH10 blocking agent (lanes 2) or p6L1XX1 blocking agent (lanes 3). (A) Serum positive for antibodies to p6L1XX1; (B) negative serum. Numbers on the left are molecular masses in kilodaltons.

H), E2 (F and K), and HPV-16 E4 (L) were also seen. There were no detectable reactivities to the HPV-6 E1, E4, E5 (data not shown), E6, and E7 fusion proteins nor to the 37-kilodalton (kDa) pATH protein (results not shown here; see Fig. 4 to 6).

The reactivity to the HPV-6b L1 ORF was examined more fully by using Western blot analysis to confirm the specificity of the reaction. The plasmid construct p6L1XX1, which contains a 1,074-base-pair *Xho*II fragment (positions 6013 to 7087) of HPV-6b DNA, was used to prepare the target antigen. When induced to synthesize fusion protein, p6L1XX1-transfected *E. coli* produced a fusion protein with an apparent molecular mass of 75 kDa (the predicted molecular mass is 76.6 kDa, made up of 37 kDa from the *trpE* moiety plus 39.6 kDa from the *Xho*II insert-encoded moiety). In Western blot assays (Fig. 2), many of the sera contained antibodies which appeared to react with native *E. coli* proteins and which were eliminated or markedly reduced by preabsorbing the sera with lysates of bacteria expressing vector (pATH) sequences. The patterns of anti-*E. coli* reactivity for a given serum were similar when the serum was tested against bacterial lysates expressing different fusion proteins, both in the uninduced and induced states. In contrast, human sera with anti-p6L1XX1 antibodies showed a characteristic reactivity against the lysates of induced bacterial cultures expressing p6L1XX1. This reactivity was not seen when the serum was tested against lysates of p6L1XX1-transfected bacteria grown in the uninduced state, nor was it observed when the serum was tested against lysates of bacteria expressing other fusion proteins. The intensity of the anti-p6L1XX1 reactivity was not affected by preabsorbing the serum with pATH10-expressing bacterial lysates (Fig. 2, lanes 2) but was obliterated or markedly diminished by preabsorption with bacterial lysates expressing p6L1XX1 (Fig. 2, lanes 3). The characteristic anti-p6L1XX1 reactivity consisted of a prominent band at the mobility of the full-length fusion protein (75 kDa), as well

as a group of five minor bands in the 25- to 32-kDa range (Fig. 2A, lanes 2); some of the positive sera also showed a minor reactivity at 57 kDa. These smaller minor bands probably represent degradation products of the full-length fusion protein, which maintain the immunoreactive epitope(s).

To further demonstrate that the anti-p6L1XX1 reactivity was directed against the HPV-6b L1 fusion protein and not against a contaminating bacterial protein, a second *Xho*II fragment (6013 to 7087) was inserted 3' to the *Xho*II insert in p6L1XX1. The resulting plasmid p6L1(XX)² expressed two head-to-tail in-frame inserts, producing a fusion protein with an apparent molecular mass of 110 kDa. Four sera with anti-HPV-6b L1 reactivity were tested in Western blot assays against the p6L1(XX)² fusion protein; all showed reactivity at 110 kDa, which was unaffected by preabsorption with pATH lysates but was significantly reduced by preabsorption with p6L1XX1 lysates. Four sera with anti-*E. coli* reactivities which mimicked the anti-p6L1XX1 pattern failed to react with the 110-kDa p6L1(XX)² fusion protein. A positive serum and a negative serum are shown in Fig. 3. In this case, the positive serum was not completely blocked by a single cycle of preabsorption. Furthermore, an additional plasmid, p6L1XH1, expressing an *Xba*I (position 5903)-*Hpa*II (position 7862) fragment of the HPV-6b L1 ORF has been constructed, and the fusion protein was also recognized by the positive sera (data not shown). Taken together, the experiments indicate that the sera of many patients with condylomata acuminata contain antibodies which react with the HPV-6b-encoded portion of an L1 ORF bacterial fusion protein.

FIG. 3. Shift of reactivity to HPV-6b L1 to 110 kDa when a dimeric fusion protein is used as target. The insoluble protein fractions of p6L1XX1 and p6L1(XX)² were electrophoresed and transferred. The strips were reacted with sera shown to be positive for antibodies to p6L1XX1 (A) and to p6L1XX1-negative sera (B). The sera were preabsorbed with blocking agent as indicated. The blocking with p6L1XX1 in panel A was incomplete. Numbers on the left are molecular masses in kilodaltons.

It seems likely that the L1 ORF encodes the major capsid antigen. First, antisera prepared against SDS-denatured papillomaviruses from both human and animal sources are known to be widely cross-reactive (19), and comparative DNA sequence analysis has shown that the L1 ORF is the most highly conserved region of the genome (9, 40). Second, antisera raised against SDS-denatured BPV-1 virions react with an *E. coli*-expressed BPV-1 L1 fusion protein (35). It was therefore important to begin to assess the type specificity of the human antibody response and to compare it with the type-common response displayed by sera prepared against denatured papillomavirus virions.

Three HPV-16 L1 fusion proteins were used: p16L1BX3 expressed a 267-amino-acid segment from the middle of the L1 ORF in a pATH11 vector (68 kDa), pHX1 expressed the amino-terminal one-third of HPV-16 L1, and pHX2 expressed the carboxy-terminal two-thirds of the HPV-16 L1 ORF. The latter two were expressed as *cro*- β -galactosidase fusion proteins (125 and 150 kDa, respectively) from a pEX vector (43). In addition to the HPV-6b L1 fusion protein expressed by p6L1XX1 (75 kDa), a truncated protein, p6L1XP1 (52 kDa), which expressed only 159 amino acids of the HPV-6 L1 ORF, was constructed. The Coomassie blue-stained gel showed that all the fusion proteins were present in nearly equal amounts (Fig. 4). The human sera recognized only the p6L1XX1 fusion protein and not the smaller p6L1XP1 fusion protein. The very small amount of reactivity to p6L1XP1 in frame 2 (Fig. 4A) was not seen in frames 3 to 5 nor in other experiments. None of the human sera reacted with any of the HPV-16 L1 fusion proteins. Reactivity with a contaminating *E. coli* protein of 75 kDa was seen in the other lanes but not reactivity in the position of any of the fusion proteins.

Three explanations for this result were possible: (i) the antibody reactivity in human sera was specific for HPV-6b relative to HPV-16, (ii) the immunoreactive region of the capsid was located at residues which span the *Bam*HI site of HPV-16 and was disrupted in the HPV-16 L1 constructs but intact in the HPV-6b constructs, or (iii) the HPV-16 L1 fusion proteins were not immunogenic or recognizable by other antibodies. The immunoreactive region of the HPV-6b L1 fusion protein recognized by human sera was mapped to a region bounded by the *Pst*I site at position 6490 and the *Xho*II site at position 7087 by the failure of human sera to react with the p6L1XP1 fusion protein (Fig. 4A). Therefore, the immunoreactive region does not overlap the *Bam*HI site in HPV-16, ruling out the second explanation. Rabbit antiserum prepared against SDS-disrupted BPV-1 virions (Dako) reacted strongly with HPV-6b L1 fusion proteins p6L1XX1 and p6L1XP1 and less strongly with HPV-16 L1 fusion proteins p16L1BX3 and pHX2. Also, polyclonal rabbit sera raised against the L1 fusion proteins recognized the appropriate antigens, ruling out the third explanation (J. M. Firzlaff et al., Virology, in press). Therefore, we conclude that human sera obtained from patients with condyloma acuminata have antibodies to the major capsid protein which appear to be specific for HPV-6b relative to HPV-16.

The immunoreactive region of the rabbit anti-BPV-1 serum mapped to a region delimited by the *Bam*HI site (position 6150) in HPV-16 and the *Pst*I site (position 6490) in HPV-6b (Fig. 4B). The absence of reaction with pHX1 defined the leftward limit; the removal of the reactivity to p6L1XX1 by preabsorption with p6L1XP1 established the rightward limit. The reactivity of rabbit anti-BPV-1 serum to p16L1BX3 and pHX2 was abolished by preabsorption with the homologous antigen (data not shown). Because of the exten-

FIG. 4. Reactivities of human sera and rabbit antisera to SDS-disrupted BPV-1 virions and to HPV-6b and HPV-16 L1 fusion proteins. (A) Total bacterial lysates (50 μ l) of p6L1XX1, p6L1XP1, p16L1BX3, pHX1, pHX2 or insoluble fractions of pATH10, p6L1XP1, p6L1XX1 were electrophoresed and transferred. A Coomassie blue-stained gel (top left) indicates that an equivalent amount of fusion protein was present in each lane. The arrows indicate the positions of the fusion proteins. The filters were reacted with a p6L1XX1-positive human serum (diluted 1:50). (B) Identical filters reacted with rabbit anti-BPV-1 (Dako) serum (diluted 1:500). The sera were preabsorbed with blocking agent as indicated. The maps of the L1 ORFs and the restriction enzyme fragments present in the expression plasmids are shown below the filters.

sive colinearity of the L1 ORF proteins, we interpret our mapping data to indicate that the type-common epitope is located at a colinear position on the capsid antigens of HPV-6 and HPV-16 within the 64-amino-acid region delineated by the *Bam*HI site of HPV-16 and the *Pst*I site of HPV-6. Experiments to test this prediction directly are in progress. It appears that the type-common antibodies induced by immunization with denatured virions recognize a site on the major capsid antigen distinct from that recognized by the type-specific antibodies in human sera induced by infection with intact virions. Furthermore, our data indicate that a conserved antigenic sequence is encoded by the L1 ORFs of HPV-6b and HPV-16 and that the site is more highly conserved between BPV-1 and HPV-6b than between BPV-1 and HPV-16.

The experiments described above indicate unambiguously that some sera obtained from patients with condyloma react with an HPV-6b L1 fusion protein and that these sera do not react with the homologous region of the HPV-16 capsid antigen. The conclusion that the reactivity to the HPV-6b L1

fusion protein is authentic was based on the following: (i) the reactivity to p6L1XX1 was not affected by absorption with bacterial lysates expressing the vector or other fusion proteins but was abolished by absorption with bacteria expressing p6L1XX1; (ii) reactivity was seen with fusion proteins of different mobility if the recombinant plasmids expressed sequences between the HPV-6b *Pst*I (position 6490) and *Xho*II (position 7087) sites, e.g., p6L1XX1, p6L1(XX)², and p6L1XH1, and was not seen with other *trpE* fusion proteins; and (iii) although the majority of sera from patients with condyloma were reactive with the HPV-6 L1 fusion protein, many sera were negative, including those of some of the condyloma patients (used as the negative sera in these experiments) and those of 30 6-month-old babies (data not shown).

However, we could not determine whether the positive antibodies were induced by HPV-6b infection or by infection with an antigenically similar HPV type. The issue of the use of a control population for HPV infections is extremely complicated. Presently, no antigenic groupings of papillomaviruses exist, and therefore, it is unclear whether infections with other HPV types would induce antibodies which cross-react with the HPV-6b L1 fusion protein. This is likely to be true for some but not all HPV infections. Furthermore, many HPV infections are subclinical or go undiagnosed, and therefore, exposure to HPV cannot be assessed independent of serology in most cases. The challenge of categorizing HPV serotypes and further delineating the specificity of the antibody response to the HPV-6b L1 fusion protein are future goals.

Reactivity to other HPV proteins. The initial screen by immunospot revealed several sera with apparent reactivity to the HPV-6b L2 ORF expressed by p6L2NX1. In general, far fewer sera showed reactivity to L2 than to L1, and the most intensely L2-positive sera were less intense than many of the sera with reactivity to L1. To confirm the L2 reactivity, human sera were reacted to a filter containing the salt-insoluble fraction of p6L2NX1 which expressed a fusion protein with an apparent molecular mass of 120 kDa (of which 49.4 kDa is due to the HPV insert). Positive human sera reacted most strongly with the full-length fusion protein (Fig. 5A). There was also a prominent band seen at 29 kDa and a smear of reactivity in the 80- to 98-kDa range. Two human sera which reacted strongly with p6L2NX1 fusion protein were tested against two HPV-16 L2 fusion proteins, p16L2XX5 (82 kDa) and p16L2XB5 (70 kDa), which together encode approximately 85% of the HPV-16 L2 ORF. Neither HPV-16 L2 fusion protein was recognized by the anti-HPV-6b L2 reactive sera, nor did preabsorbing these sera with lysates derived from the HPV-16 L2 constructs affect the intensity of the anti-p6L2NX1 reactivity. Rabbit anti-BPV-1 serum (Dako) did not react with either the HPV-6b or HPV-16 L2 fusion protein (data not shown).

To map the immunoreactive regions within the HPV-6b L2 protein, positive human sera were reacted with the fusion proteins p6L2NB4 (48 kDa), p6L2BP2 (75 kDa), and p6L2BX4 (100 kDa) which express portions of the HPV-6b L2 ORF (Fig. 5). Positive human sera reacted with p6L2NX1 and p6L2BX4, and the reactivity was abolished by preabsorption with lysates of p6L2NX1. This placed the immunoreactive epitope in the carboxy-terminal one-third of the HPV-6b L2 ORF located between the *Pst*I (position 5408) and *Xho*II (position 6013) sites or in a region spanning the *Pst*I (position 5408) site.

Two sera (Fig. 5B) were detected which displayed antibodies to p16L2XX5 which expresses a 552-base-pair frag-

ment from the middle of the HPV-16 L2 ORF. With these sera, no reactivity was seen against p16L2XB5 which expresses a segment from the carboxy terminus of HPV-16 L2 ORF. Although these sera did not appear to cross-react with p6L2NX1, there was some decrease in their anti-p16L2XX5 reactivity when they were preabsorbed with p6L2NX1-expressing bacterial lysates.

Experiments with antisera to BPV-1 (35) and to HPV-1a (21) L2 ORF fusion proteins indicated that L2 is a minor capsid antigen. Our data indicated that some human sera have antibodies which recognize the minor capsid protein in a type-specific fashion. Furthermore, the observation that the reactive epitopes were not colinear, i.e., that the HPV-6b reactivity was to the carboxy-terminal end of L2 and that the reactivity to the HPV-16 L2 ORF was to the central region, is intriguing.

The initial screening by immunospot assay revealed the slight reactivity of two sera with the HPV-6b E2 protein expressed by p6E2SP1. The E2 ORF of BPV-1 and HPV have been shown to encode proteins which act as transcriptional activators and repressors (18, 22, 42) and are expressed at very low levels. The detection of antibodies directed against this protein was unexpected and was verified by Western blot analysis. The full-length fusion protein had an apparent molecular mass of 53 kDa (predicted molecular mass of 44.8 kDa, made up of a 38.8-kDa HPV insert linked to a 6-kDa truncated form of *trpE* which terminates at

FIG. 5. Reactivities of human sera to HPV-6b and HPV-16 L2 ORF fusion proteins. The insoluble fractions of pATH, p6L2NX1, p16L2XX5, and p16L2XB5 and total bacterial lysates (uninduced [u] and induced [i]) of p6L2NX1, p6L2NB4, p6L2BP2, and p6L2BX4 were electrophoresed and transferred. Sera were preabsorbed with blocking agent as indicated. (A) Filters reacted with a serum that showed anti-p6L2NX1 antibodies; (B) filters reacted with a serum that showed anti-p16L2XX5 antibodies. The maps of the L2 ORFs and the restriction enzyme fragments present in the expression plasmids are shown below the filters.

FIG. 6. Reactivity of human sera with p6E2SP1. Total bacterial lysates from both uninduced (u) and induced (i) cultures of pATH11, p6E2SP1, and p16E2BP2 were electrophoresed and transferred. A serum with anti-p6E2SP1 activity was reacted with the filters after preabsorption with blocking agent as indicated. The maps of the E2 ORFs and the restriction enzyme fragments present in the expression plasmids are shown below the filters.

an *EcoRV* site). Representative positive human sera reacted most strongly with the full-length fusion protein (Fig. 6); in addition, there was weaker activity to a pair of bands at 36 and 33 kDa. One serum containing anti-HPV-6b E2 antibody was tested against an HPV-16 E2 fusion protein encoded by p16E2BP2, which expressed a much smaller portion of that ORF than did its HPV-6b counterpart. No reactivity was seen against the HPV-16 fusion protein, nor was there any apparent decrement in anti-p6E2SP1 activity when the serum was preabsorbed with lysates expressing the HPV-16 fragment. Rabbit anti-BPV-1 antibody was not reactive with these E2 ORF fusion proteins (data not shown).

A function for the E4 ORF has not yet been discerned from a genetic analysis of BPV-1 or the HPVs. However, it has been shown that the E4 protein is extremely abundant in HPV-1-containing plantar warts (10) and that an mRNA encoding an E4 protein is the most abundantly expressed message in HPV-11 containing condylomata (5). Therefore, human sera were screened for reactivity to HPV-6 and HPV-16 E4 ORF fusion proteins. Of 75 sera from patients with condyloma acuminata, two showed reactivity to the HPV-16 E4 ORF expressed by p16E4BP6 and none showed reactivity to its HPV-6 counterpart expressed by p6E4BP37. The positive reactivity to the HPV-16 E4 ORF fusion protein was confirmed by Western blot analysis (Fig. 7) and was further analyzed by preabsorption to confirm its authenticity (data not shown). Both fusion proteins spanned nearly the entire E4 ORF; in fact, p6E4BP37 contained 12 amino acids more than p16E4BP6 did. At this point, it is impossible to determine whether the individual whose serum reacted with both the HPV-16 E4 and HPV-6 L1 fusion proteins was infected with both types or with another serotype. Although the E4 mRNA is abundantly expressed, the protein has not been convincingly identified in condylomata. Our data sug-

gest that the E4 protein is not a major target in eliciting an immune response.

The 75 sera analyzed by immunospot assay did not show any reactivity to the HPV-6b E1, E6, and E7 ORFs as defined by the fusion proteins encoded by p6E1ES4, p6E6NN11, and p6E7NN1, respectively. A limited set of sera were screened by reactivity to those fusion proteins and to the HPV-16 E1, E6, and E7 ORFs encoded by p16E1aSN2, p16E6DS2, and p16E7NP1, respectively, in Western blot assays, and no additional reactivities were detected (data not shown). The immunospot assay was useful in screening for the most prominent reactivities but was not sufficiently sensitive or specific to give accurate numbers for prevalence studies. Only a few sera were analyzed rigorously to confirm that the reactivity to the HPV fusion proteins was authentic.

DISCUSSION

This study shows that bacterially expressed fusion proteins can be used to define HPV antibody reactivities and to delineate regions of ORF-encoded proteins which contain immunoreactive epitopes. Although the problem of antibodies directed against *E. coli* proteins was formidable, it was overcome by extensive preabsorption of the sera with large amounts of bacterial lysates. Among all the sera screened, none reacted with the *trpE* protein alone, suggesting that this vector system may be particularly attractive if the fusion proteins are to be used as immunologic targets.

These data provide unambiguous identification of viral antigens which are recognized by patients with condyloma acuminata. The predominant reactivity in this population is to the major capsid antigen. L1 encodes the major structural component of the virion, and earlier electron microscopy studies (1, 16) had suggested that human antibodies react with the capsid. Furthermore, in a recent study (23), 18 of 30 sera from patients attending a colposcopy clinic reacted with a HPV-6b L1 fusion protein. The L1 ORF is highly conserved among HPV types; however, the immunoreactive region is not shared between HPV-6 and HPV-16. The L1 ORF of BPV-1 has been shown recently (25) to contain both type-common and type-specific epitopes. This finding may

FIG. 7. Reactivity of human serum to p16E4BP6. The insoluble fractions of the various fusion proteins were reacted with a serum preabsorbed with pATH10 blocking agent. The serum showed reactivity to p16E4BP6 and p6L1XX1. Numbers on the left are molecular masses in kilodaltons.

indicate that infections with some types will be cross-protective, and yet, other HPV types will not be recognized, thus leading to multiple infections. This is also the first report of other papillomavirus antigens (L2, E4, and E2) which are recognized by the immune system. The finding that a few patients reacted with HPV-16 E4 or L2 antigen but did not also react with the HPV-16 L1 antigen is unexplained. Either the HPV-16 major capsid antigen is not immunogenic, or the viruses infecting these people are more antigenically diverse than has been recognized by DNA typing. A Western blot assay primarily detects antibodies against linear epitopes. We cannot exclude the possibility that there are additional antibodies which recognize conformational epitopes, but no source of authentic viral antigen is available for those studies. Further, we cannot exclude the possibility of very weak antibody responses not detected in this assay.

At this stage, we have not attempted to comment on the prevalence of antibodies to HPV in a given population. The aim of the study was, first, to define which viral antigens serve as targets in one population (i.e., patients with genital warts), and, second, to develop an unambiguous method of identifying HPV antibodies. Although the immunospot method (Fig. 1) provides a rapid screen, it is less accurate than Western blot analysis with three strips per serum (Fig. 2). It is now possible to begin detailed prevalence studies in this population, to determine whether there is any correlation between antibody status and clinical disease, and to define those viral antigens which are recognized in other populations, e.g., patients with genital dysplasias or carcinomas.

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