JOURNAL OF VIROLOGY, Apr. 1992, p. 2008–2019
0022-538X/92/042008-12$02.00/0

Vol. 66, No. 4

Human Antibodies Recognize Multiple Distinct Type-Specific and Cross-Reactive Regions of the Minor Capsid Proteins of Human Papillomavirus Types 6 and 11

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Received 23 September 1991/Accepted 16 December 1991

Human serum samples derived from a case-control study of patients with cervical carcinoma (n = 174) or condyloma acuminatum (n = 25) were tested for the presence of immunoglobulin G antibodies to human papillomavirus type 6 (HPV6) L2 and HPV11 L2 recombinant proteins in a Western immunoblot assay. Thirty-six samples (18%) were positive for HPV6 L2 antibodies alone, 25 (13%) were positive for HPV11 L2 antibodies alone, and 34 (17%) were positive for both HPV6 L2 and HPV11 L2 antibodies. Thirty samples that were positive for both antibodies were tested for the presence of HPV6-HPV11 L2 cross-reactive antibodies. Fifteen (50%) serum samples contained HPV6-HPV11 L2 cross-reactive antibodies, and 15 (50%) contained independent, type-specific HPV6 L2 and HPV11 L2 antibodies. Altogether, 82% of the HPV6 L2 and HPV11 L2 antibody reactivities were type specific and 18% were HPV6-HPV11 cross-reactive. There was no significant difference in the prevalence of antibody reactivities between samples from patients with cervical carcinoma and those with condyloma acuminatum. Deletion mapping identified five HPV6 L2 regions that reacted with HPV6 type-specific antibodies: 6U1 (amino acids [aa] 152 to 173), 6U2 (aa 175 to 191), 6U3 (aa 187 to 199), 6U4 (aa 201 to 217), and 6U5 (aa 351 to 367). Five HPV11 L2 regions that reacted with HPV11 type-specific antibodies were identified: 11U1 (aa 49 to 84), 11U2 (aa 147 to 162), 11U3 (aa 179 to 188), 11U4 (aa 180 to 200), and 11U5 (aa 355 to 367). Two HPV6-HPV11 cross-reactive regions were identified: 6CR1 (HPV6 L2 aa 106 to 128)/11CR1 (HPV11 L2 aa 103 to 127) and 6CR2 (HPV6 L2 aa 187 to 199)/11CR2 (HPV11 L2 aa 180 to 200).

Human papillomavirus types 6 and 11 (HPV6 and HPV11) are closely related viruses that are associated with benign proliferative lesions of human epithelium. Both HPV6 and HPV11 are detected frequently in condylomata acuminata (benign genital warts) and in squamous laryngeal papillomas (4, 5, 12–14, 32, 40). HPV6 and HPV11 were classified as different HPV types because they had less than 50% overall DNA homology in a liquid hybridization assay (3). The prototype HPV6 genome, which was isolated from a genital wart (14), shared 25% overall homology with the prototype HPV11 genome, which was isolated from a laryngeal papilloma (12). The complete DNA sequences of the prototype genomes have subsequently been defined (4, 34). On the basis of nucleotide sequence comparison, HPV6 and HPV11 have 82% overall sequence similarity (4). The DNA sequences are particularly well conserved within the L1 open reading frames (ORFs) (92% amino acid similarity) and to a lesser extent within the L2 ORFs (85% amino acid similarity) (4). The L1 and L2 ORFs encode the major viral capsid protein and the minor viral capsid protein respectively (6, 36, 37).

HPV virions are spherical nonenveloped particles that are 55 nm in diameter (38). Virion capsids consist of two viral proteins, the major capsid protein (53 to 57 kDa) and the minor capsid protein (76 to 86 kDa) (8). The major capsid protein is estimated to compose approximately 90% of the capsid mass. HPV major capsid protein monomers are organized as 72 pentamers that are packed with icosahedral symmetry (23). The position of the L2 minor capsid proteins is less clear, but they may occupy positions at the apices of the L1 pentamers.

Although the L2 polypeptides make up a relatively small proportion of virion capsid mass, they appear to be important immunogens that elicit type-specific antibody reactivities. Immunization of mice with intact HPV1 virions (the HPV type commonly associated with plantar warts) generated a polyclonal antibody response that was directed against the L2 minor capsid protein and not against the L1 major capsid protein (39). These antibodies were specific for HPV1 L2 polypeptides and did not react with L2 proteins encoded by HPV type 6, 11, 16, or 18. Denatured HPV1 L2 and HPV6 L2 recombinant proteins elicit type-specific antibody reactivities (26, 36), whereas denatured L1 proteins elicit broadly cross-reactive antibodies that react with L1 proteins of other HPV types (20, 28, 37). Christensen et al. have shown that antibodies generated against cottontail rabbit papillomavirus L2 recombinant proteins neutralize the ability of cottontail rabbit papillomavirus virions to infect rabbit epithelium (2).

HPV-encoded recombinant proteins have been used as antigen targets in Western immunoblot assays to detect human serum antibody reactivities (16, 19, 21, 24, 25, 28, 35). Human antibody reactivities to L2 recombinant proteins encoded by HPV types 6, 16, and 18 are prevalent in various human populations, and the L2 antibodies are generally type specific (16, 18, 19, 24, 25). HPV16 and HPV18 are the HPV types most commonly associated with uterine cervical carcinomas and cervical dysplastic lesions (40). We have previously mapped the locations of human antibody-reactive regions of the HPV16 and HPV18 L2 polypeptides by using nested sets of serially deleted recombinant proteins (18).

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TABLE 1. HPV expression plasmids for exoIII- and nuclease S1-deleted clones

Direction of exoIII/S1 deletion	Plasmid	HPV DNA sequences (nt)	Restriction enzymes for cloning	Vector (cloning sites)	Restriction enzymes for exoIII/S1 deletion
HPV6b					
5′ to 3′	p6L2PCR	4421–5802	*Xba*I-*Hin*dIII	pATH10 (*Xba*I-*Hin*dIII)	*Sst*I + *Xba*I
3′ to 5′	p6L2NX1	4421–6013	*Nco*I-*Xho*II	pATH11 (*Eco*RI-*Bam*HI)	*Hin*dIII + *Sal*I
HPV11					
5′ to 3′	p11L2HH1	4557–6552	*Hin*dIII-*Hpa*II	pATH10 (*Hin*dIII-*Cla*I)	*Sst*I + *Hin*dIII
3′ to 5′	p11L2PCR	4417–5784	*Eco*RI-*Sal*I	pATH1 (*Eco*RI-*Sal*I)	*Nsi*I + *Bgl*II

Human antibody binding sites mapped to multiple regions that were closely clustered in a segment between amino acid (aa) positions 110 and 211.

As part of a population-based case-control study of risk factors involved in cervical carcinoma, we have tested serum samples for immunoglobulin G (IgG) antibody reactivities to recombinant proteins encoded by HPV types 6, 11, 16, and 18. The prevalence of HPV antibodies among cervical carcinoma (CC) cases compared with community-based, age-matched controls is reported in detail elsewhere (29). Twenty-five serum specimens from patients with condyloma acuminatum (CA) were also tested to compare the prevalence of HPV6 and HPV11 L2 antibodies and the locations of human antibody-reactive regions with that found in the CC case-control study. The study reported here was undertaken specifically to determine whether HPV6 and HPV11 L2 antibody reactivities were type specific and to determine the locations of human antibody-reactive regions.

MATERIALS AND METHODS

Selection of human subjects. Serum samples were obtained from two sources. One hundred seventy-four serum samples were obtained from adult female subjects who were participants in a case-control study of cervical cancer through the Cancer Surveillance System, a population-based tumor registry serving 13 counties in western Washington State. Sixty-nine of the subjects were women who had been diagnosed with invasive carcinoma of the uterine cervix and who had been recruited through the tumor registry; 105 subjects were population-based, age-matched, female control subjects who were recruited by random-digit telephone dialing. The characteristics of the population are described in detail elsewhere (29). Twenty-five serum samples were obtained from adult female subjects who were clinically diagnosed as having CA.

Expression plasmid constructs. DNA fragments that were derived from the HPV6b L2 ORF and from the HPV11 L2 ORF were expressed as *trpE*-HPV fusion proteins in *Escherichia coli* by using the plasmid expression vector pATH (9, 16). The HPV DNA fragments were derived from the prototype HPV6b DNA (5, 34) and HPV11 DNA (4, 12) that were contained in plasmids pHPV6b and pHPV11 (kindly provided by L. Gissmann and H. zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Germany). Expression vectors pATH1, pATH10, and pATH11 were gifts from T. J. Koerner (American Cancer Society, Atlanta, Ga.). HPV L2 ORF fragments were generated either by restriction endonuclease digestions of pHPV6b or pHPV11 DNAs (33) or by the synthesis of HPV DNA segments from HPV6b or HPV11 DNA templates by the polymerase chain reaction (PCR) technique (7). HPV DNA fragments were ligated to pATH DNA at restriction endonuclease sites within the pATH polylinker sequences (33; see reference 9 for a description of the vectors).

The plasmid p6L2NX1 contained an *Nco*I (nucleotide [nt] 4421)-*Xho*II (nt 6013) HPV6b DNA fragment that encodes the entire HPV6b L2 polypeptide (Table 1). This plasmid has been described previously (9). The HPV6b L2 expression plasmid p6L2PCR contains an HPV6b DNA fragment (nt 4421 to 5802) that was synthesized from a p6L2NX1 template by the PCR technique. DNA synthesis with *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.) was primed by the synthetic oligonucleotides 5′-CCTCTAGAGCCCATGGCACATAGTAGGGCCCGA-3′ (sense strand primer) and 5′-CGAAGCTTGCTAGGCCGCCACATCTGA-3′ (antisense strand primer). The underlined nucleotide segments represent HPV6b nt 4421 to 4442 and nt 5785 to 5802, respectively. The 5′ segments of the PCR primers contained restriction endonuclease cleavage sites that were used to prepare the PCR-generated DNA molecules for ligation to the pATH vector. The PCR-generated DNAs were digested with *Xba*I and *Hin*dIII and then ligated to pATH10 DNA (*Xba*I-*Hin*dIII digest).

The plasmid p11L2HH1 contained a *Hin*dIII (nt 4557)-*Hpa*II (nt 6552) HPV11 DNA fragment that was ligated to pATH10 DNA (*Hin*dIII-*Cla*I digest) (Table 1). This construct has been described previously (39). The plasmid p11L2PCR contained an HPV11 DNA fragment (nt 4417 to 5784) that encodes the entire HPV11 L2 polypeptide. This DNA fragment was synthesized from a pHPV11 DNA template by the PCR technique. DNA synthesis was primed by the synthetic oligonucleotides 5′-GGAATTCTCATGAAACCTAGGGCACGCAGA-3′ (sense strand primer) and 5′-GGTCGACATGCATAGATCTCTAGGCCGCCACATCTGTAAA-3′ (antisense strand primer). The underlined nucleotide segments represent HPV11 nt 4417 to 4437 and nt 5764 to 5784, respectively. The PCR-generated DNA molecules were digested with *Eco*RI and *Sal*I and then ligated to pATH1 DNA (*Eco*RI-*Sal*I digest).

ExoIII and nuclease S1 deletions. Nested sets of unidirectional 5′-to-3′ deletions and 3′-to-5′ deletions were made in the HPV DNA inserts of expression plasmids p6L2PCR, p6L2NX1, p11L2HH1, and p11L2PCR (Table 1) (17, 18). Unidirectional DNA deletions were achieved by digesting the plasmid DNAs with exonuclease III (exoIII) and nuclease S1 (S1) by the protocol of Henikoff (15). Prior to exoIII-S1 digestions, plasmid DNAs were cleaved at two unique restriction enzyme sites located either near the 5′ end (for 5′-to-3′ deletions) or near the 3′ end (for 3′-to-5′ deletions) of the HPV DNA insert. The DNA end that was distal to the HPV DNA insert was rendered resistant to exoIII digestion. This was achieved either by cleaving the DNA with a restriction endonuclease that generated a 4-base 3′

TABLE 2. Prevalence of antibodies against HPV6 L2 and HPV11 L2 in human sera

Specificity	No. (%) of samples		
	CC (n = 174)	CA (n = 25)	Total (n = 199)
HPV6 L2 only	32 (18)	4 (16)	36 (18)
HPV11 L2 only	21 (12)	4 (16)	25 (13)
HPV6 L2 and HPV11 L2[a]	27 (16)	7 (28)	34 (17)
Cross-reactive	13	2	15
Independent	13	2	15
Total positive	80 (46)	15 (60)	95 (48)

[a] Sera that were cross-reactive to both HPV6 L2 and HPV11 L2 and sera with independent reactivities to HPV6 L2 and HPV11 L2. Twenty-six of the 27 CC samples and four of the seven CA samples were tested for cross-reactivity.

overhanging end or by cleaving the DNA with a restriction endonuclease that generated a 4-base 5′ overhanging end and by making that end blunt by using Klenow polymerase in the presence of α-phosphorothioate deoxyribonucleoside triphosphates (dNTPs) (α-phosphorothioate-dNTP mix; Promega, Madison, Wis.) (15). The DNA end that was proximal to the HPV DNA insert was rendered susceptible to exoIII digestion by cleaving the DNA with a restriction endonuclease that generated a 4-base 5′ overhanging end (15).

For making unidirectional 5′-to-3′ deletions in the HPV6b L2 DNA insert of p6L2PCR, plasmid DNA was digested with *Sst*I and *Xba*I. Both restriction endonuclease sites were located 5′ to the HPV DNA insert within the pATH polylinker segment. ExoIII deletions proceeded from the *Xba*I site.

For making 3′-to-5′ deletions in the HPV6b L2 insert of p6L2NX1, plasmid DNA was digested with *Hin*dIII; the *Hin*dIII end was made blunt by using Klenow polymerase in the presence of α-phosphorothioate-dNTPs. The DNA then

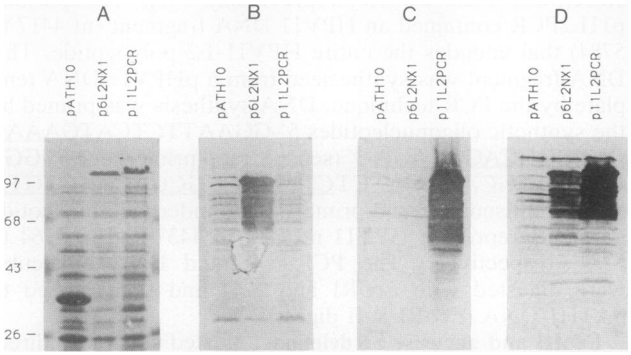

FIG. 1. Reactivities of human sera with HPV6 L2 and HPV11 L2 recombinant proteins. Lysates of p6L2NX1-, p11L2PCR-, and pATH10-containing bacteria were reacted with human serum samples in Western immunoblot assays. Antigen-antibody complexes were detected by using anti-human IgG antiserum. (A) Coomassie blue-stained SDS–12.5% polyacrylamide gel. Molecular size standards (in kilodaltons) are on the left. (B, C, and D) Three representative Western immunoblots. (B) Serum sample that contained IgG antibodies that reacted with the HPV6 L2 polypeptide p6L2NX1 and did not react with the HPV11 L2 polypeptide p11L2PCR. (C) Serum sample that reacted with the HPV11 L2 polypeptide p11L2PCR and did not react with the HPV6 L2 polypeptide p6L2NX1. (D) Serum that contained antibody reactivities to both the HPV6 and the HPV11 L2 polypeptides.

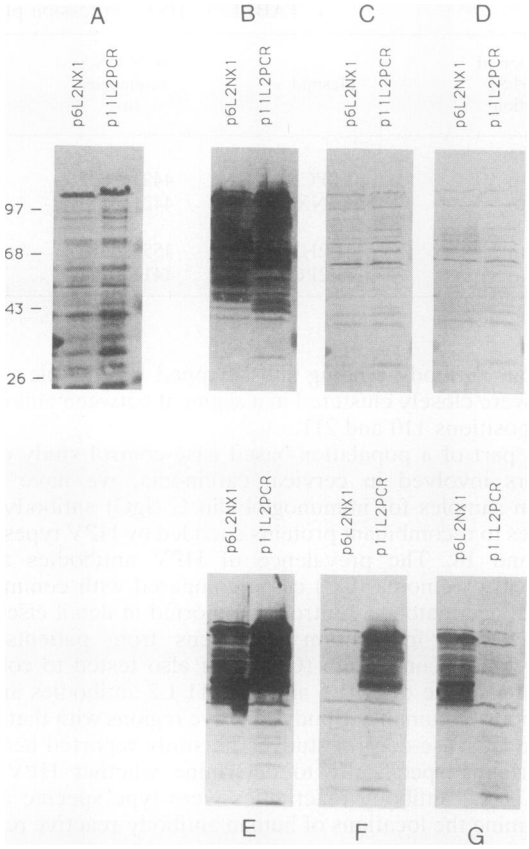

FIG. 2. Cross-reactivity versus type specificity of human serum samples containing antibodies that react with both HPV6 L2 and HPV11 L2 polypeptides. (A) Coomassie blue-stained SDS–12.5% polyacrylamide gel of lysates of bacteria containing plasmid p6L2NX1 or p11L2PCR. (B, C, and D) Replicate Western immunoblots that were reacted with a serum sample that contained an HPV6 L2-HPV11 L2 cross-reactive antibody specificity. (E, F, and G) Replicate Western immunoblots that were reacted with a serum sample that contains independent, type-specific HPV6 L2-reactive antibodies and HPV11 L2-reactive antibodies. (B and E) Serum samples were preabsorbed with lysates of bacteria that express vector (pATH) sequences. (C and F) Serum samples were preabsorbed with lysates of bacteria that express the p6L2NX1 fusion protein. (D and G) Serum samples were preabsorbed with lysates of bacteria that express the p11L2PCR fusion protein.

was digested with *Sal*I. Both restriction endonuclease sites were located 3′ to the HPV DNA insert within the pATH polylinker segment. ExoIII deletions proceeded from the *Sal*I site.

For making 5′-to-3′ deletions in the HPV11 L2 DNA insert of p11L2HH1, plasmid DNA was digested with *Sst*I and *Hin*dIII. Both restriction endonuclease sites are located 5′ to the HPV DNA insert within the pATH polylinker segment. ExoIII deletions proceeded from the *Hin*dIII site.

For making 3′-to-5′ deletions in the HPV11 L2 DNA insert of p11L2PCR, plasmid DNA was digested with *Nsi*I and *Bgl*II. Both restriction endonuclease sites are located 3′ to the HPV DNA segment within the antisense PCR primer sequence. ExoIII deletions proceeded from the *Bgl*II site.

Linearized plasmid DNAs were incubated with exoIII (New England BioLabs, Beverly, Mass.) at 25°C, and aliquots were removed from the reaction mix at 30-s intervals. Single-stranded portions of the DNAs were digested with

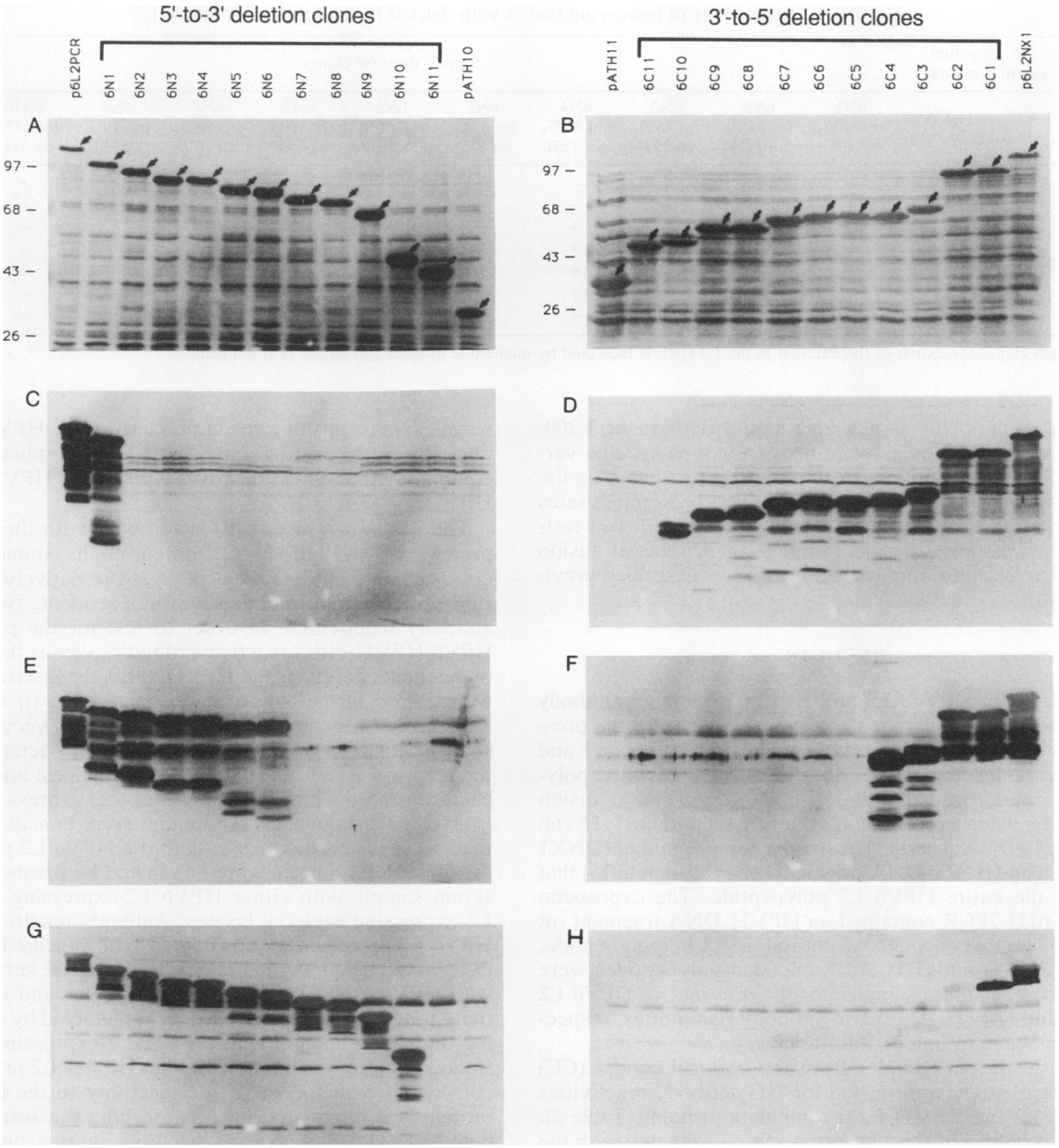

FIG. 3. Mapping of immunoreactive regions of the HPV6 L2 polypeptide. Nested sets of amino-to-carboxy terminus-deleted fusion proteins (A, C, E, and G) and carboxy-to-amino terminus-deleted fusion proteins (B, D, F, and H) that were used to map HPV6 L2 immunoreactive regions; the deleted fusion proteins were derived from nested sets of 5′-to-3′ and 3′-to-5′ deleted expression plasmids, respectively. (A and B) Coomassie blue-stained 12.5% polyacrylamide–SDS gels. The positions of the HPV6 L2-encoded fusion proteins are marked with arrows. The positions of molecular mass standards (in kilodaltons) are indicated at the left. (C and D) Replicate Western immunoblots that were reacted with a serum sample that contained an HPV6 L2-HPV11 L2 cross-reactive antibody specificity, whose binding site mapped to the 6CR1 region. (E and F) Replicate Western immunoblots that were reacted with a serum sample that contained an HPV6 L2 type-specific antibody reactivity, whose binding site mapped to the 6U3 region. (G and H) Replicate Western immunoblots that were reacted with a serum sample that contained HPV6 L2 type-specific antibodies, whose binding site mapped to the 6U5 region.

nuclease S1 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The digested DNA ends were made blunt with Klenow polymerase (Promega) and then ligated by using bacteriophage T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, Md.). The plasmid DNAs were used to transform *E. coli* HB101 bacteria. Selected DNAs were sequenced to evaluate the extent of the DNA deletions.

Synthesis of fusion proteins, SDS-polyacrylamide gel electrophoresis, and Western immunoblot assays. The expression of fusion proteins, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and Western immunoblot assays were performed as described previously (16). Human serum samples were screened for the presence of antibody reactivities to HPV6b and HPV11 L2-encoded polypeptides in

TABLE 3. Reactivities of human antibodies with deleted fusion proteins of HPV6 L2[a]

Region	No. of serum samples mapped		5′-to-3′ deletion clones										
	CC (n = 24)	CA (n = 4)	6N1 (nt 4738, aa 106)	6N2 (nt 4753, aa 111)	6N3 (nt 4874, aa 152)	6N4 (nt 4877, aa 153)	6N5 (nt 4943, aa 175)	6N6 (nt 4979, aa 187)	6N7 (nt 5003, aa 195)	6N8 (nt 5023, aa 201)	6N9 (nt 5133, aa 238)	6N10 (nt 5471, aa 351)	6N11 (nt 5498, aa 360)
6CR1	5	1	+	−	−	−	−	−	−	−	−	−	−
6U1	1		+	+	+	−	−	−	−	−	−	−	−
6U2	2	2	+	+	+	+	+	−	−	−	−	−	−
6CR2	1		+	+	+	+	+	+	−	−	−	−	−
6U3	11	1	+	+	+	+	+	+	−	−	−	−	−
6U4	1		+	+	+	+	+	+	+	+	−	−	−
6U5	3		+	+	+	+	+	+	+	+	+	+	−

[a] For each clone, the extent of the deletion in the L2 ORF is indicated by nucleotide number and amino acid number.

Western immunoblot assays at a serum dilution of 1:200. Human serum antibodies were tested for type-specific versus cross-reactive antibody reactivities by preabsorbing the serum samples in the liquid phase with bacterial lysates which express HPV-encoded fusion proteins and then testing the preabsorbed serum samples against target fusion proteins in Western immunoblot assays, as described previously (18).

RESULTS

Prevalence of HPV6b L2 and HPV11 L2 human antibody reactivities. Human serum samples were tested for the presence of IgG antibody reactivities to HPV6b-encoded and HPV11-encoded L2 polypeptides. The HPV-encoded polypeptides were produced in *E. coli* as *trpE*-HPV fusion proteins by using expression plasmids that contained HPV6b and HPV11 DNA inserts. The expression plasmid p6L2NX1 contained an HPV6b DNA fragment (nt 4421 to nt 6013) that encodes the entire HPV6 L2 polypeptide. The expression plasmid p11L2PCR contained an HPV11 DNA fragment (nt 4417 to 5784) that encodes the entire HPV11 L2 polypeptide. The p6L2NX1- and p11L2PCR-encoded polypeptides were used to screen serum samples for the presence of HPV6 L2 protein and HPV11 L2 protein antibody reactivities, respectively, in Western immunoblot assays.

First, the serum samples from the cervical cancer (CC) case-control study were tested for IgG antibody reactivities to HPV6 L2 and HPV11 L2 recombinant proteins (Table 2). Thirty-two (18%) of the 174 serum samples reacted with the HPV6 L2 protein and did not react with the HPV11 L2 protein (Fig. 1B). Twenty-one (12%) of the samples reacted with the HPV11 L2 protein and did not react with the HPV6 L2 protein (Fig. 1C). Twenty-seven (16%) serum samples contained antibodies that reacted with both the HPV6 L2 and HPV11 L2 proteins (Fig. 1D). Altogether, 46% of the serum samples from CC patients contained antibodies that reacted with the HPV6 L2 and/or HPV11 L2 protein. Twenty-five serum samples from CA patients were then similarly tested (Table 2). Four (16%) reacted with the HPV6 L2 protein and did not react with the HPV11 L2 protein. Four (16%) reacted with the HPV11 L2 protein and did not react with the HPV6 L2 protein. Seven (28%) reacted with both the HPV6 L2 and HPV11 L2 proteins. Altogether, 60% of the serum samples from CA patients contained antibodies that reacted with the HPV6 L2 and/or the HPV11 L2 protein. There was no statistically significant difference in the antibody reactivities between the CC and CA samples (χ^2 = 1.72, $P > 0.05$). As a whole, 18% of the human serum samples were positive for antibodies to the HPV6 L2 protein, 13% were positive for HPV11 L2 antibodies, and 17% contained antibodies reactive with both HPV6 L2 and HPV11 L2 (Table 2).

The serum samples that reacted with both the HPV6 L2 protein and the HPV11 L2 protein could contain a single cross-reactive antibody specificity. Alternatively, these serum samples could contain two independent, type-specific antibody reactivities. In order to test for the presence of HPV6-HPV11 cross-reactive antibodies versus the presence of independent HPV6 and HPV11 antibodies, serum samples were tested further under three conditions: (i) after being absorbed with bacterial lysates that express vector (pATH) sequences; (ii) after being absorbed with bacterial lysates that express HPV6 L2 (p6L2NX1) sequences; and (iii) after being absorbed with bacterial lysates that express HPV11 L2 (p11L2PCR) sequences. Antibodies were considered cross-reactive if the reactivities to both the HPV6 L2 protein and the HPV11 L2 protein were eliminated by preabsorbing the serum sample with either HPV6 L2-expressing or HPV11 L2-expressing bacterial lysates. Antibody reactivities to the HPV6 L2 protein were considered type specific if the reactivity could be eliminated by preabsorbing the serum sample with HPV6 L2-expressing bacterial lysates and if the reactivity to the HPV6 L2 protein was not affected by preabsorbing the serum sample with HPV11 L2-expressing bacterial lysates. Similarly, antibodies to the HPV11 L2 protein were considered type specific if the reactivity to the HPV11 L2 protein was eliminated by preabsorbing the serum sample with HPV11 L2-expressing bacterial lysates but was not affected by preabsorption with HPV6 L2-expressing bacterial lysates (Fig. 2).

Twenty-six of the 27 CC samples and four of the seven CA samples that reacted with both the HPV6 L2 protein and the HPV11 L2 protein were tested for the presence of cross-reactive versus type-specific antibody reactivities (Fig. 2 and Table 2). Thirteen of 26 CC samples and two of four CA samples contained HPV6 L2-HPV11 L2 cross-reactive antibodies (Fig. 2B, C, and D). The remaining 13 CC serum samples and two CA serum samples contained different IgG antibody reactivities that reacted independently with the HPV6 L2- and HPV11 L2-encoded polypeptides, respectively (Fig. 2E, F, and G).

The HPV6 L2-HPV11 L2 cross-reactive serum samples were tested further for the presence of HPV16 L2 and HPV18 L2 antibodies. This was done in order to determine whether the HPV6-HPV11 cross-reactivities were restricted to HPV6 and HPV11 or whether they were common to other HPV types that infect the genital tract epithelium. Two of

TABLE 3—*Continued*

3'-to-5' deletion clones										
6C11 (nt 4759, aa 112)	6C10 (nt 4806, aa 128)	6C9 (nt 4905, aa 161)	6C8 (nt 4941, aa 173)	6C7 (nt 4977, aa 185)	6C6 (nt 4993, aa 190)	6C5 (nt 4995, aa 191)	6C4 (nt 5019, aa 199)	6C3 (nt 5074, aa 217)	6C2 nt 5483, aa 353)	6C1 (nt 5523, aa 367)
−	+	+	+	+	+	+	+	+	+	+
−	−	−	+	+	+	+	+	+	+	+
−	−	−	−	−	−	+	+	+	+	+
−	−	−	−	−	−	−	+	+	+	+
−	−	−	−	−	−	−	+	+	+	+
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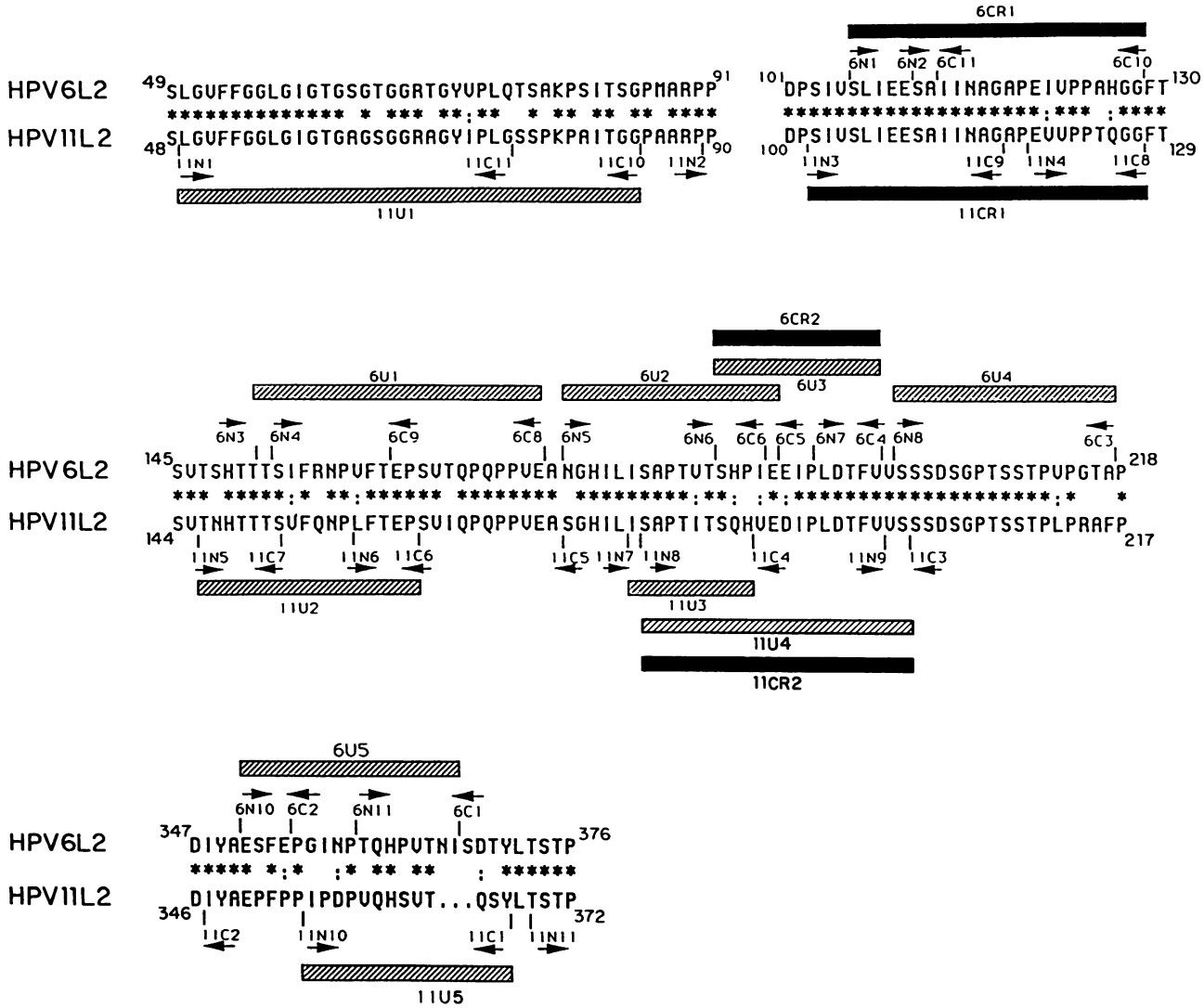

FIG. 4. Alignment of HPV6 L2 and HPV11 L2 amino acid sequences in the immunoreactive regions. Homologous amino acids that are identical are marked (*). Homologous amino acids with similar charge characteristics (conservative amino acid substitutions) are indicated (:). A blank space between the sequences indicates that the homologous amino acids are different and that the substitutions would not be considered conservative. The cross-hatched boxes indicate the locations of immunoreactive regions which react with type-specific antibodies. The black boxes indicate the positions of immunoreactive regions which react with HPV6 L2-HPV11 L2 cross-reactive antibodies. →, locations of the amino termini of amino-to-carboxy terminus-deleted fusion proteins. ←, locations of the carboxy termini of carboxy-to-amino terminus-deleted fusion proteins.

TABLE 4. Reactivities of human antibodies with deleted fusion proteins of HPV11 L2[a]

Region	No. of serum samples mapped		5′-to-3′ deletion clones										
	CC (n = 25)	CA (n = 4)	11N1 (nt 4559, aa 49)	11N2 (nt 4682, aa 90)	11N3 (nt 4718, aa 103)	11N4 (nt 4769, aa 119)	11N5 (nt 4853, aa 147)	11N6 (nt 4886, aa 158)	11N7 (nt 4952, aa 179)	11N8 (nt 4954, aa 180)	11N9 (nt 5003, aa 197)	11N10 (nt 5479, aa 355)	11N11 (nt 5422, aa 370)
11U1	4		+	−	−	−	−	−	−	−	−	−	−
11CR1	5	1	+	+	+	−	−	−	−	−	−	−	−
11U2	9	1	+	+	+	+	+	−	−	−	−	−	−
11U3	4	1	+	+	+	+	+	+	+	−	−	−	−
11CR2	1		+	+	+	+	+	+	+	+	−	−	−
11U4		1	+	+	+	+	+	+	+	+	−	−	−
11U5	2		+	+	+	+	+	+	+	+	+	+	−

[a] See Table 3, footnote *a*.

the HPV6 L2-HPV11 L2 cross-reactive serum samples contained antibodies that reacted strongly with the HPV18 L2 protein, and two of the cross-reactive serum samples contained antibodies that reacted strongly with both the HPV16 L2 and the HPV18 L2 proteins. However, the reactivities to the HPV6 L2 and HPV11 L2 proteins were not affected by preabsorbing the serum samples with HPV16 L2-expressing or HPV18 L2-expressing bacterial lysates (data not shown). Therefore, the HPV16 L2 antibodies and the HPV18 L2 antibodies were independent reactivities that were present in these serum samples and that were different from the HPV6-HPV11 L2 cross-reactive antibodies. Altogether, 82% of the HPV6 L2 and HPV11 L2 antibody reactivities were type specific and 18% were HPV6-HPV11 cross-reactive.

Mapping human antibody-reactive regions of the HPV6 L2 polypeptide. Human antibody-reactive regions of the HPV6 L2 polypeptide were mapped by using nested sets of serially deleted fusion proteins. The amino-terminal boundaries of the immunoreactive regions were determined by reacting antibodies with nested sets of HPV6 L2-encoded polypeptides that contained serial amino-to-carboxy terminus deletions. The deleted fusion proteins were expressed by plasmids that contained serial 5′-to-3′ deletions in the HPV6b L2 DNA insert of plasmid p6L2PCR. The carboxy-terminal boundaries were determined by using fusion proteins that contained carboxy-to-amino terminus deletions. The deleted fusion proteins were expressed by plasmids that contained serial 3′-to-5′ deletions in the HPV6b L2 insert of plasmid p6L2NX1. Both the p6L2PCR and the p6L2NX1 plasmids encode the entire HPV6 L2 polypeptide. Different expression plasmids were used for making the 5′-to-3′ and 3′-to-5′ deletion series because the plasmids contained different unique restriction endonuclease cleavage sites; these sites were used to prepare the plasmids for exoIII and S1 digestions (see Materials and Methods).

Immunoreactive regions recognized by 18 of the 32 CC samples that contained HPV6 L2-specific antibodies and by 6 of the 13 CC samples which contained HPV6 L2-HPV11 L2 cross-reactive antibodies were mapped.

Binding sites recognized by HPV6 L2-HPV11 L2 cross-reactive antibodies mapped to two distinct segments of the HPV6 L2 polypeptide. Five of six cross-reactive serum samples reacted with the 6N1 fusion protein (5′ end of the HPV6 DNA insert at nt 4738) and larger fusion proteins and did not react with the 6N2 fusion protein (5′ end at nt 4753) and smaller fusion proteins (Fig. 3C and Table 3). Therefore, the 5′ boundary of the immunoreactive region mapped to nt 4738, which encodes aa 106 of the HPV6 L2 polypeptide (first L2 methionine residue, aa 1). These serum samples also reacted with the 6C10 fusion protein (3′ end at nt 4806) and larger fusion proteins and did not react with the 6C11 fusion protein (3′ boundary at nt 4759) and smaller fusion proteins (Fig. 3D and Table 3). Therefore, the 3′ boundary of the immunoreactive region mapped to nt 4806, which encodes aa 128. The immunoreactive region of the HPV6 L2 polypeptide recognized by five of six cross-reactive serum samples was mapped to a segment between aa 106 and 128 (SLIEE SAIINAGAPEIVPPAHGG), encoded by nt 4738 through 4806; this region was designated 6CR1 (Fig. 4). The immunoreactive region of the HPV11 L2 polypeptide recognized by these same sera is described below.

Only one of the six cross-reactive serum samples reacted with a region that was distinct from 6CR1. This region was bounded by 6N6 (5′ end at nt 4979) and 6C4 (3′ end at nt 5019). Therefore, the region recognized by this cross-reactive serum sample was mapped to an amino acid segment between aa 187 and 199 (SHPIEEIPLDTFV); this region was designated 6CR2 (Table 3).

The antibody reactivities of 18 human CC samples that contained HPV6 L2-specific antibodies mapped to five distinct segments of the HPV6 polypeptide. These segments were designated 6U1 through 6U5; the immunoreactive regions were numbered in order from the most amino-terminal region to the most carboxy-terminal region (Table 3 and Fig. 4).

Eleven of the 18 CC samples contained antibodies that reacted with region 6U3, which includes aa 187 through 199 (SHPIEEIPLDTFV) and which is encoded by nt 4979 through 5019 (Table 3, Fig. 3E and F). The deletion clones that defined this region (6N6 and 6C4) were the same clones that defined the cross-reactive region 6CR2. Therefore, the 6U3-6CR2 immunoreactive region contains an epitope for HPV6 L2 type-specific antibodies as well as an epitope for HPV6 L2-HPV11 L2 cross-reactive antibodies. The amino-terminal half of the 6U3-6CR2 region is divergent between HPV6 L2 and HPV11 L2, whereas the carboxy-terminal half of this region is conserved (Fig. 4). Therefore, the HPV6 type-specific antibodies probably bind nearer to the amino-terminal end of this region, and the HPV6-HPV11 cross-reactive antibodies probably bind nearer to the carboxy-terminal end. The resolution of the deletion mapping was not sufficient to differentiate these specificities.

Two of the HPV6 L2 type-specific serum samples reacted with an amino acid segment between aa 175 and 191 (NG HILISAPTVTSHPIE), which is encoded between nt 4943 and 4995; this region was designated 6U2 (Table 3). Five amino acids at the carboxy-terminal end of 6U2 (SHPIE) overlapped with five amino acids at the amino-terminal end of region 6U3-6CR2 (Fig. 4). The glutamic acid residue at the

TABLE 4—*Continued*

3′-to-5′ deletion clones										
11C11 (nt 4639, aa 74)	11C10 (nt 4668, aa 84)	11C9 (nt 4765, aa 116)	11C8 (nt 4797, aa 127)	11C7 (nt 4872, aa 152)	11C6 (nt 4904, aa 162)	11C5 (nt 4940, aa 174)	11C4 (nt 4980, aa 188)	11C3 (nt 5018, aa 200)	11C2 (nt 5454, aa 346)	11C1 (nt 5518, aa 367)
−	+	+	+	+	+	+	+	+	+	+
−	−	−	+	+	+	+	+	+	+	+
−	−	−	−	−	+	+	+	+	+	+
−	−	−	−	−	−	−	+	+	+	+
−	−	−	−	−	−	−	−	+	+	+
−	−	−	−	−	−	−	−	+	+	+
−	−	−	−	−	−	−	−	−	−	+

carboxy-terminal end of 6U2 (aa191) was essential for antibody binding, because 6U2-reactive antibodies failed to react with deletion clone 6C6, which is missing this residue (Table 3 and Fig. 4). It is possible, therefore, that the 6U2, 6U3, and 6CR2 antibody binding sites are clustered together and have some of the same amino acids.

Three of the 18 HPV6 L2 type-specific serum samples reacted with an amino acid segment between aa 351 and 367 (ESFEPGINPTQHPVTNI), which is encoded by nt 5471 through 5523; this region was designated 6U5 (Table 3, Fig. 3G and H).

One HPV6 L2 type-specific serum sample reacted with an amino acid segment between aa 152 and 173 (TSIFRN PVFTEPSVTQPQPPVE), encoded by nt 4874 through 4941; this region was designated 6U1. The threonine residue at the amino-terminal end of this region appeared to be essential for antibody binding, because the serum failed to react with deletion clone 6N4, which lacked this residue (Table 3 and Fig. 4). Therefore, the epitope probably lies near the amino-terminal end of region 6U1.

One HPV6 L2 type-specific serum sample reacted with an amino acid segment between aa 201 and 217 (SSSDS GPTSSTPVPGTA), encoded by nt 5023 and 5074; this region was designated 6U4. The amino-terminal half of this region is well conserved between HPV6 and HPV11. Therefore, it is likely that HPV6 L2 type-specific antibodies bind nearer the carboxy-terminal end of the region, where the HPV6 and HPV11 amino acid sequences are more divergent (Table 3 and Fig. 4).

Three HPV6 L2 type-specific serum samples from CA patients were similarly mapped. Two of them reacted with 6U2 and one reacted with 6U3. One HPV6 L2-HPV11 L2 cross-reactive serum sample from a CA patient was mapped and reacted with 6CR1 (Table 3).

Mapping human antibody-reactive regions of the HPV11 L2 polypeptide. Human antibody-reactive regions of the HPV11 L2 polypeptide were mapped by using nested sets of serially deleted fusion proteins. The amino-terminal boundaries of the immunoreactive regions were defined by deletion clones derived from plasmid p11L2HH1; the carboxy-terminal boundaries were defined by deletion clones derived from plasmid p11L2PCR (see Materials and Methods).

The immunoreactive regions recognized by 19 of the 21 HPV11 L2 type-specific serum samples from CC patients and by 6 of the 13 HPV6 L2-HPV11 L2 cross-reactive serum samples from CC patients were mapped. The six HPV6 L2-HPV11 L2 cross-reactive serum samples were the same serum samples that were used to map the HPV6 L2 cross-reactive regions (see above).

The immunoreactive region of the HPV11 L2 polypeptide that was recognized by the five 6CR1-reactive serum samples mapped to a segment between aa 103 and 127 (SIVS LIEESAIINAGAPEVVPPTQGG), designated 11CR1 (Table 4 and Fig. 5C and D). Region 11CR1 is homologous to region 6CR1; their respective amino acid sequences are well conserved, particularly nearer their amino-terminal ends (Fig. 4). The HPV11 L2 region recognized by the 6CR2-reactive serum mapped to a segment between aa 180 and 200 (SAPTITSQHVEDIPLDTFVVS), designated 11CR2. Region 11CR2 is homologous to region 6CR2; their respective amino acid sequences are well conserved nearer their carboxy-terminal ends (Fig. 4).

Nine of the HPV11 L2 type-specific antibodies mapped to a region between aa 147 and 162 (TNHTTTSVFQNPLF TEP), encoded by nt 4853 through 4904; this region was designated 11U2 (Table 4 and Fig. 5E and F). 11U2 is homologous to the amino-terminal half of region 6U1, which is the half of the 6U1 region which probably contains the antibody binding site (see above).

Four of the 19 HPV11 L2 type-specific antibodies mapped to a region between aa 179 and 188 (ISAPTITSQH), encoded by nt 4952 through 4980, designated 11U3 (Table 4). The resolution of the deletion mapping was not sufficient to separate region 11U3 from region 11CR2, and these regions overlapped by nine amino acids (SAPTITSQH) (Fig. 4). Region 11U3 is homologous to segments of HPV6 L2 region 6U2.

Four of the HPV11 L2 type-specific antibodies mapped to a region between aa 49 and 84 (LGVFFGGLGIGTGAGSG GRAGYIPLGSSPKPAITGG), encoded between nt 4559 and 4668; this region was designated 11U1 (Table 4). No human antibody reactivities were mapped to the homologous region of the HPV6 L2 polypeptide.

Two of the HPV11 L2 type-specific antibodies mapped to a region between aa 355 and 367 (IPDPVQHSVTQSY), encoded by nt 5479 through 5518; this region was designated 11U5 (Table 4 and Fig. 5G and H). Region 11U5 is homologous to region 6U5. The HPV6 and HPV11 L2 amino acid sequences in these regions are highly divergent (Fig. 4).

Three HPV11 L2 type-specific serum samples from CA patients were similarly mapped. One reacted with 11U2, one with 11U3, and one with 11U4 (Table 4). Region 11U4 was defined with the same deletion clones that defined a cross-reactive region, 11CR2. Therefore, the 11U4-11CR2 immunoreactive region contains an epitope for HPV11 L2 type-specific antibodies as well as an epitope for HPV6 L2-HPV11 L2 cross-reactive antibodies (Table 4 and Fig. 4). Region 11U4 is homologous to 6U3.

One cross-reactive serum sample from a CA patient, which was the same serum sample that was used to map the

HPV6 L2 cross-reactive region, was mapped and reacted with 11CR1 (Table 4).

DISCUSSION

We have shown previously that the most prevalent human antibody reactivities to HPV6-encoded recombinant proteins are directed against the L1 polypeptide (16, 19). In Western immunoblot assays, human antibodies that reacted with the HPV6 L1 protein consistently cross-reacted with the HPV11 L1 protein but did not cross-react with L1 recombinant proteins encoded by HPV types 1, 2, 4, 5, 16, 18, 31, and 33 (17). Therefore, these L1 antibody reactivities were relatively specific for HPV types 6 and 11 compared with other HPV types that commonly infect the genital tract epithelium (HPV types 16, 18, 31, and 33). However, the HPV types 6 and 11 L1 antibodies could not differentiate between HPV6 and HPV11 L1-encoded proteins.

Prevalent human antibody reactivities have also been observed to L2 polypeptides encoded by HPV types 6, 16, and 18 (16, 19, 24, 25). The L2 polypeptide is well conserved between HPV6 and HPV11 (84% amino acid similarity), but it is less well conserved than the L1 polypeptide (92% amino acid similarity) (4). Therefore, it was possible that human antibody reactivities to the HPV6 L2 protein and to the HPV11 L2 protein would be type specific. In this study, we tested serum samples obtained from participants in a case-control study of CC and from CA patients for the presence of serum IgG antibody reactivities to HPV6 L2 and HPV11 L2 recombinant proteins. Because the prevalence of L2 antibodies was not different among cases than among controls, the results of HPV6 L2 and HPV11 L2 antibody type specificity assays and antibody mapping studies presented here are reported for the case and control populations as a whole. Similarly, no differences were observed between the prevalence of antibodies in the CA group compared with the case or control populations.

The majority of human serum antibody reactivities to the HPV6 L2 protein and to the HPV11 L2 protein were type specific. Eighteen percent of the serum samples contained antibodies that reacted with the HPV6 L2 protein and did not contain HPV11 L2-reactive antibodies. Thirteen percent of the serum samples contained antibodies that reacted with the HPV11 L2 protein and did not contain HPV6 L2-reactive antibodies. Seventeen percent of the serum samples contained antibody reactivities both to the HPV6 L2 protein and to the HPV11 L2 protein. In approximately half of these cases, the HPV6 L2 antibodies and the HPV11 L2 antibodies were two independent, type-specific reactivities that were both present in the same serum sample. In the remaining half of these cases, the HPV6-HPV11 L2 reactivity represented a single, cross-reactive antibody. Altogether, 48% of the serum samples contained antibodies that reacted with the HPV6 L2 protein and/or the HPV11 L2 protein, and the prevalence of L2 antibodies was not significantly different among CA and CC patient samples. Approximately 82% of these antibody reactivities were type specific, and 18% were HPV6-HPV11 cross-reactive.

The locations of human antibody-reactive regions were mapped by using nested sets of serially deleted recombinant proteins. Five HPV6 type-specific regions (6U1, 6U2, 6U3, 6U4, and 6U5), five HPV11 type-specific regions (11U1, 11U2, 11U3, 11U4, and 11U5), and two sets of HPV6-HPV11 cross-reactive regions (6CR1/11CR1 and 6CR2/11CR2) were identified. It is possible that additional rare epitopes would be found if more sera were screened. Human sera that reacted with either the HPV6 L2 protein or the HPV11 L2 protein recognized primarily only one of these regions. We have previously described human antibody-reactive regions of HPV16 L2 and HPV18 L2 polypeptides that were clustered in a segment between aa 110 and 211 (18). The majority of the human HPV6 L2 and HPV11 L2 antibodies mapped to regions of their respective polypeptides that were homologous to antibody-reactive regions of HPV16 L2 and HPV18 L2 polypeptides (Fig. 6). These findings may indicate that the segment of the minor capsid protein between aa 110 and 210 is immunodominant for the HPV types most commonly associated with genital tract lesions. The region of the HPV6 L2 protein with which human sera reacted most frequently (6U3) was not homologous to the region of the HPV11 L2 protein that was recognized most frequently by human sera (11U2) (Fig. 4). 6U3 was in fact homologous in location to the region of the HPV16 L2 protein with which human sera reacted most frequently (16REx), and 11U2 was homologous in location to the region of HPV18 L2 with which human sera reacted most frequently (18EEx) (18) (Fig. 6). Amino acid sequence divergence within the segment from aa 110 to 210 not only may account for the type specificity of L2 antibody reactivities, but it may also influence which of the antibody-reactive regions within the segment is immunodominant for a given HPV type.

The human antibody-reactive regions identified by deletion mapping represent linear segments of the L2 polypeptide that contain an antibody-binding site. The antibody-binding sites contained within the immunoreactive regions are by definition linear, or continuous, epitopes (1, 27). This technique can define a peptide segment that contains the antibody-binding site, but the resolution of the technique was not sufficient to define the minimal number of amino acids involved in the specific antibody binding. The resolution is limited by the spacing between the individual deletion clones. One antibody-reactive region defined by deletion mapping may contain more than one antibody-binding site. This must be the case for the 6U3-6CR2 region of the HPV6 L2 protein and the 11U4-11CR2 region of the HPV11 L2 protein, which contains antibody-binding sites for both HPV6 type-specific and HPV6-HPV11 cross-reactive antibodies and for both HPV11 type-specific and HPV6-HPV11 cross-reactive antibodies, respectively. In another case, two different regions recognized by HPV6 type-specific antibodies overlapped; five amino acids (SHPIE) of region 6U2 were shared by region 6U3. Because of the close spacing of the deletion clones in this instance, it was clear that the overlapping segment was necessary for maintaining antibody binding within both regions. Therefore, the unique antibody-binding sites within the 6U2 and 6U3 regions overlapped, but their antibody-binding specificities were distinct. In several cases, we have attempted to further localize human antibody-reactive binding sites by using overlapping sets of oligopeptides in an enzyme-linked immunoabsorbent assay (11). In our hands, human sera have generally displayed a very low signal-to-noise ratio in these assays and the data have been uninterpretable.

It is not known whether there is antigenic variability between different clinical isolates of a single HPV type. More than one serotype may exist within each HPV genotype. Multiple genetic subtypes of HPV6 have been identified that display polymorphisms of restriction endonuclease fragment sizes (5, 32); only one of these subtypes (HPV6b) has been fully sequenced (34). Genotypic variability within a type need not be associated with antigenic variability, how-

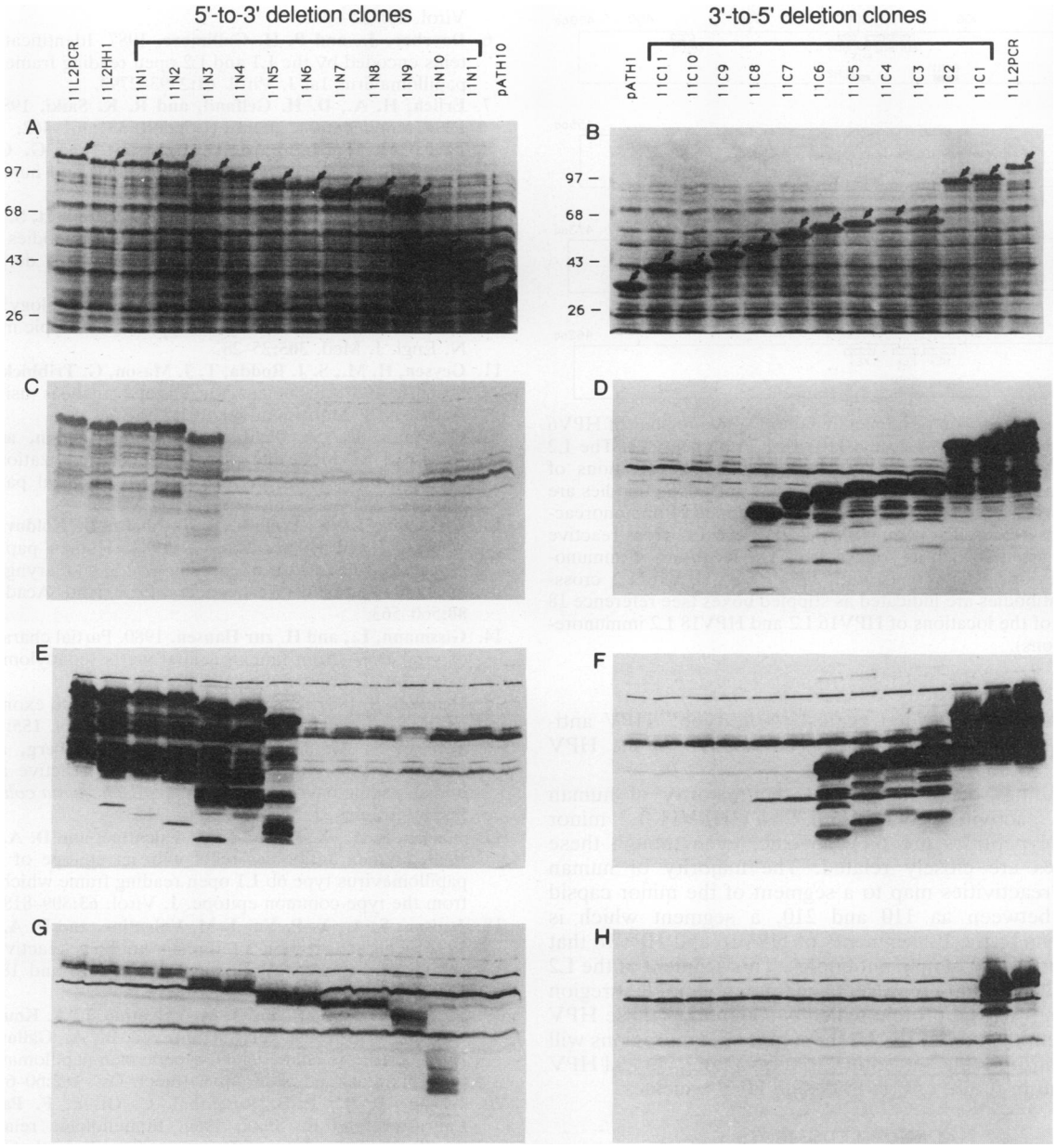

FIG. 5. Mapping of immunoreactive regions of the HPV11 L2 polypeptide. Coomassie blue-stained SDS–12.5% polyacrylamide gels and replicate Western immunoblots are arranged as in Fig. 3. (C and D) Western immunoblots that were reacted with the same serum sample shown in Fig. 3C and D, respectively. This serum sample contained an HPV6 L2-HPV11 L2 cross-reactive antibody specificity whose binding site mapped to region 11CR1. (E and F) Replicate Western immunoblots that were reacted with a serum sample that contained HPV11 L2 type-specific antibodies, whose binding site mapped to the 11U2 region. (G and H) Replicate Western immunoblots that were reacted with a human serum that contained HPV11 L2 type-specific antibodies, whose binding site mapped to the 11U5 region.

ever. Determining the locations of human antibody-reactive regions within the L2 polypeptides encoded by the prototype HPV6 and HPV11 DNAs will facilitate the comparison of these regions with homologous segments of clinical HPV isolates.

The fact that HPV L2-reactive antibodies from different humans recognize different L2 binding sites may be due to variability in individual human immune responses or variability in different HPV clinical isolates. It is possible that there is little or no antigenic variability between different clinical isolates of HPV6 or HPV11. In this case, differences in antibody recognition sites could be due to differences in individual host immune responses. This variability could stem from differences in human leukocyte antigen gene products, self-tolerance, specificity of helper T cells, antigen processing, idiotype networks, host structural antibody gene repertoire, and stochastic effects (1, 10, 22, 31). Alternatively, there may be marked antigenic variability between different clinical strains of both HPV6 and HPV11. Accordingly, there may be many serotypes of each HPV genotype. Because the prototype HPV6 and HPV11 DNAs were used to encode the antigen targets, we could potentially detect

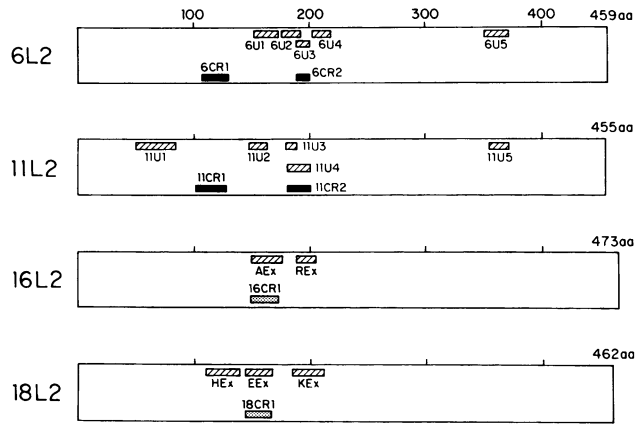

FIG. 6. Comparison of human immunoreactive regions of HPV6 L2, HPV11 L2, HPV16 L2, and HPV18 L2 polypeptides. The L2 polypeptides are represented as large blocks. The locations of immunoreactive regions that react with type-specific antibodies are indicated with cross-hatched boxes. The locations of immunoreactive regions that react with HPV6 L2-HPV11 L2 cross-reactive antibodies are indicated as solid boxes. The locations of immunoreactive regions which react with HPV16 L2-HPV18 L2 cross-reactive antibodies are indicated as stippled boxes (see reference 18 for details of the locations of HPV16 L2 and HPV18 L2 immunoreactive regions).

only antibodies generated against "wild-type" HPV antigenic sites that are well conserved relative to the HPV prototype strains.

Our findings demonstrate that the majority of human antibody reactivities to HPV6 L2 and HPV11 L2 minor capsid polypeptides are type specific, even though these viral types are closely related. The majority of human antibody reactivities map to a segment of the minor capsid proteins between aa 110 and 210, a segment which is homologous to the L2 segments of HPV16 and HPV18 that are recognized by human antibodies. This segment of the L2 polypeptide probably represents an immunodominant region of the minor capsid protein which is common to these HPV types. Identification of the L2 immunodominant regions will be useful in studying antigenic variation among clinical HPV strains and in future efforts to design HPV vaccines.

ACKNOWLEDGMENTS

We thank Janet R. Daling and Margaret T. Mandelson for providing human serum samples, Janette Valentine for valuable technical assistance, and Paul Su for artwork.

This study was supported by Public Health Service grants PO1-CA42792 and PO1-AI29363 to D.A.G. S.A.J. was supported by Physician Scientist Award K11-CA01391.

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