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Characterization of Human Antibody-Reactive Epitopes Encoded by Human Papillomavirus Types 16 and 18

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We have previously reported that the most common human serum immunoglobulin G antibody reactivities to human papillomavirus type 16 and type 18 (HPV16 and HPV18)-encoded proteins are directed against the minor capsid proteins (HPV16 L2 and HPV18 L2) and to the E7 protein of HPV16 (S. A. Jenison, X.-P. Yu, J. M. Valentine, L. A. Koutsky, A. E. Christiansen, A. M. Beckmann, and D. A. Galloway, J. Infect. Dis. 162:60–69, 1990). In this study, the antibody-reactive segments of the HPV16 E7, HPV16 L2, and HPV18 L2 polypeptides were mapped by using nested sets of deleted recombinant proteins. A single major immunoreactive region was identified in the HPV16 E7 polypeptide between amino acids (aa) 21 and 34 (DLYCYEQLNDSSEE). In contrast, three distinct immunoreactive regions of the HPV16 L2 polypeptide were present in the segment between aa149 and aa204, and three distinct immunoreactive regions of the HPV18 L2 polypeptide were present in the segment between aa110 and aa211. With the exception of one serum sample, serum immunoglobulin G antibodies which reacted with HPV16 L2 polypeptides or with HPV18 L2 polypeptides were not cross-reactive.

Human papillomavirus types 16 (HPV16) and 18 (HPV18) are the HPV types most frequently associated with cervical squamous cell carcinomas. These HPV types may play a role in the malignant transformation of cervical epithelial cells in vivo. This hypothesis is supported by the following evidence. (i) HPV16, HPV18, and related HPV types are present in the overwhelming majority of cervical squamous cell carcinomas and cervical carcinoma cell lines (3, 7, 43, 48, 49); (ii) these HPV types appear to be more prevalent in dysplastic and malignant cervical epithelium than in cervical epithelium which is cytologically normal (49); (iii) HPV16 and -18 immortalize primary human epithelial cells in vitro, but the HPV types commonly associated with benign genital tract lesions do not (23, 34); and (iv) the HPV open reading frames (ORFs) which mediate epithelial cell immortalization in vitro are generally intact and transcriptionally active in naturally occurring squamous cell carcinomas (2, 37, 39, 40–42; reviewed in references 13, 26, and 49). HPV type 6 (HPV6) is the HPV type most commonly associated with condylomata acuminata (benign genital warts) (5, 15, 16, 29) and is only infrequently associated with malignant lesions of the female genital tract (49).

The detection of human antibodies directed against HPV6, HPV16, and HPV18 has been problematic because neither virions nor viral proteins have been isolated from human tissues in sufficient quantities for use in routine serological assays (17). Humans are the only known animal hosts which support HPV infections, and HPV16 and -18 have not been propagated in vitro. As an alternative source of HPV-encoded proteins, we have expressed major ORF fragments of HPV6, -16, and -18 in *Escherichia coli* by means of the plasmid expression vector pATH (11, 20, 22). We have previously reported the prevalence of serum immunoglobulin G (IgG) antibodies to E2, E7, L1, and L2 recombinant proteins encoded by HPV6, -16, and -18 in women attending a sexually transmitted diseases (STD) clinic (n = 92) and in hospitalized children (n = 81) (22). Antibodies to late-gene products (L1 or L2 ORF) were more common than antibodies to early-gene products (E2 or E7) in both the adults and children. Different patterns of antibody reactivities to the HPV6, HPV16, and HPV18 proteins were observed. The most prevalent serum antibody reactivity to an HPV6-encoded recombinant protein was to the L1 protein, both among the STD clinic patients (66%) and among the children (57%). Antibodies to the L2 protein were the next most common reactivity detected among the HPV6 fusion proteins, both in the STD clinic patients (23%) and in the children (19%). In contrast to the high prevalence of reactivities to the HPV6 L1 protein, few serum samples reacted with homologous L1 proteins encoded by HPV16 and HPV18. Among the HPV16 and HPV18 fusion proteins that were tested, antibody reactivities were most commonly observed to the L2-encoded fusion proteins. Of the adult serum samples 47 and 25% reacted with the HPV16 L2 and HPV18 L2 fusion proteins, respectively; 30 and 26% of the children's serum samples reacted with the HPV16 L2 and HPV18 L2 proteins, respectively. Antibodies to the HPV16 E7 protein were observed in 13 and 12% of the STD clinic patients' and children's serum samples, respectively. In contrast, human antibody reactivities to the HPV18 E7 protein were rare, and were present in 3 and 1% of the STD clinic patients' and children's serum samples, respectively. The prevalence of antibody reactivities to HPV6 L1, HPV6 L2, HPV16 L2, HPV18 L2, and HPV16 E7 proteins in children was compared with the prevalence in adults. In each case, there was no significant difference in the prevalence between the children and the adults.

The identification of amino acid segments recognized by human serum antibodies may be useful both in designing more sophisticated serological assays and in designing HPV vaccines. We used nested sets of deleted recombinant proteins in Western immunoblot assays to localize linear amino acid (aa) segments recognized by HPV-reactive human serum antibodies. The human antibody-reactive epitope of the

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HPV6 L1 polypeptide has previously been mapped to a region near the carboxy terminus (21). In this report, we extend these studies to characterize human antibody-reactive regions of the HPV16 E7, HPV16 L2, and HPV18 L2 polypeptides.

MATERIALS AND METHODS

Selection of human subjects. Serum samples were obtained from three sources: (i) children hospitalized at Children's Hospital and Medical Center in Seattle, Wash.; (ii) patients attending an STD clinic at Harborview Medical Center in Seattle; and (iii) women with invasive cervical cancer who were identified through the Cancer Surveillance System, a population-based tumor registry serving 13 counties of western Washington State. Characteristics of the populations of hospitalized children and STD Clinic patients have been described previously (22). The cases of cervical carcinoma were diagnosed by histological examination of surgically excised tissues. Invasive carcinoma was defined as carcinoma which extended below the epithelial basement membrane.

Expression plasmid constructs. HPV DNA-encoded polypeptides were expressed in *E. coli* HB101 by means of the plasmid expression vector pATH (11, 20). pBR322-based bacterial plasmids which contain complete HPV16 (pHPV16) (7, 38) and HPV18 (pHPV18) (3, 4) DNA sequences were 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.). pATH vectors contain 5′ transcription control elements and a portion of the first structural gene (*trpE*) of the *E. coli* tryptophan synthetase operon. HPV DNA fragments, generated by restriction enzyme digestions, were inserted into pATH DNA at unique restriction enzyme sites within a polylinker segment located 3′ to the *trpE* gene (36; see reference 11 for a description of the vectors). HPV DNA-encoded polypeptides were expressed as fusion proteins linked to a 37,000-dalton polypeptide encoded by *trpE*. The identification of the expression constructs is shown in Table 1. Some of the constructions required multiple cloning steps. For p16L2OB1, pHPV16 DNA was digested with *Oxa*NI, the DNA ends were made blunt by using Klenow polymerase, and the DNA was digested with *Bam*HI. The *Oxa*NI (4335)-*Bam*HI (6151) fragment was ligated to vector pBS(−) (Stratagene, La Jolla, Calif.) DNA (*Sma*I-*Bam*HI digest); the pBS-HPV recombinant plasmid was digested with *Eco*RI and *Hin*dIII, and the HPV DNA-containing fragment was ligated to pATH1 DNA (*Eco*RI-*Hin*dIII digest). For p16L2AA1, p16L2XX5K DNA was digested with *Ase*I and the DNA ends were made blunt by using Klenow polymerase. The *Ase*I (4678)-*Ase*I (5061) fragment was ligated to a synthetic double-stranded DNA deoxyribonucleotide, dCGGAATTCCG (*Eco*RI linker; Promega Biotec, Madison, Wis.), was digested with *Eco*RI, and was ligated to pATH11 DNA (*Eco*RI digest). For p16L2PX1, p16L2XX5K DNA was digested with *Xba*I (located in the pATH polylinker 3′ to the HPV DNA insert), the DNA ends were made blunt by using Klenow polymerase, and the DNA was digested with *Pst*I. The HPV DNA-containing fragment was ligated to pATH11 DNA which had been prepared by digesting with *Cla*I, by making the DNA ends blunt by using Klenow polymerase, and by digesting with *Pst*I. The pATH11-HPV recombinant plasmid was digested with *Eco*RI and *Sal*I, the DNA ends were made blunt by using Klenow polymerase, and the DNA ends were religated. For p16L2XX1, p16L2XX5 DNA (described in reference 11) was digested with *Xmn*I (4815) and *Hin*dIII (located in the pATH polylinker 3′ to the HPV DNA insert) and the HPV DNA-containing fragment was ligated to pBS(−) DNA (*Sma*I-*Hin*dIII digest); the pBS-HPV recombinant plasmid was digested with *Eco*RI and *Hin*dIII, and the HPV-containing fragment was ligated to pATH1 DNA (*Eco*RI-*Hin*dIII digest). For p18L2OO1, pHPV18 was digested with *Oxa*NI, the DNA ends were made blunt by using Klenow polymerase, and the *Oxa*NI (4342 to 5800) fragment was ligated to pUC19 DNA (*Sma*I digest); the pUC19-HPV18 recombinant plasmid was digested with *Eco*RI and *Hin*dIII, and the HPV-containing fragment was ligated to pATH1 DNA (*Eco*RI-*Hin*dIII digest). Recombinant DNAs were sequenced across the pATH-HPV junction to confirm that the HPV DNA fragments were inserted in the desired reading frame orientations.

ExoIII and nuclease S1 deletions. Unidirectional 3′-to-5′ deletions were made in the HPV DNA inserts of expression plasmids p16E7NP1, p16L2XX5K, and p18L2OO1. Deletions were made by digesting linearized plasmid DNAs with exonuclease III (exoIII) and nuclease S1 according to the protocol of Henikoff (19). Plasmid DNAs were linearized by digestion at two unique restriction enzyme sites. The more 5′ restriction enzyme cleavage created a DNA end with a 4-base 5′ single-stranded segment, which was susceptible to exoIII digestion. The more 3′ restriction enzyme cleavage created a DNA end with a 4-base 3′ single-stranded segment, which was resistant to exoIII digestion (18). Plasmid p16E7NP1 DNA was cut at unique *Nco*I (863) and *Pst*I (875) sites within the HPV16 DNA insert, 3′ to the E7 translation termination codon (863). p16L2XX5K is a derivative of p16L2XX5 and contains the synthetic double-stranded deoxyribonucleotide dGGGTACCC (*Kpn*I linker; New England BioLabs, Inc., Beverly, Mass.) ligated into the *Hin*dIII site of the pATH polylinker segment. Plasmid p16L2XX5K DNA was digested with *Xba*I (the more 5′ site) and *Kpn*I (the more 3′ site), which cut within the pATH polylinker segment 3′ to the HPV16 DNA insert. Plasmid p18L2OO1 contains DNA sequences derived from the polylinker segment of plasmid vector pUC19, which were incorporated during an intermediate cloning step as described above. p18L2OO1 was digested with *Bam*HI (the more 5′ site) and *Sph*I (the more 3′ site), which cut within the pUC19 polylinker sequence 3′ to the HPV18 DNA insert. Linearized plasmid DNAs were incubated with exoIII (New England BioLabs) at 25°C, and aliquots were removed from the reaction mixture 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 by using Klenow polymerase (Promega), and the DNA ends were religated with T4 ligase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (19). The plasmid DNAs were used to transform *E. coli* HB101 bacteria. Selected DNAs were sequenced to evaluate the extent of the deletions. The identification of the deleted expression plasmids is shown in Table 1.

p16E7SEx664 was made by digesting p16E7NEx664 DNA with *Sau*3AI (622) and *Hin*dIII and by ligating the HPV DNA-containing fragment to pATH10 DNA (*Bam*HI-*Hin*dIII digest). p16L2SEx4675 was made by digesting p16L2XEx4675 DNA with *Spe*I (4570) and *Cla*I and by ligating the HPV-containing fragment to pATH11 DNA (*Xba*I-*Cla*I digest). p16L2AEx4770 was made by digesting p16L2XEx4770 DNA with *Ase*I (4678), by making the DNA

TABLE 1. HPV expression plasmids

HPV type	ORF	Construct name	Boundaries of HPV DNA insert		Vector (insertion sites)
			5′	3′	
16	E7	p16E7NP1	0562 (*Nsi*I)	0875 (*Pst*I)	pATH11 (*Pst*I-*Pst*I)
		p16E7NEx723	0562 (*Nsi*I)	0723[a]	—[a]
		p16E7NEx664	0562 (*Nsi*I)	0664[a]	—[a]
		p16E7NEx619	0562 (*Nsi*I)	0619[a]	—
		p16E7NEx615	0562 (*Nsi*I)	0615[a]	—
		p16E7SS1	0622 (*Sau*3AI)	0871 (*Sau*3AI)	pATH10 (*Bam*HI-*Bam*HI)
		p16E7PP1	0683 (*Pvu*II)	0875 (*Pst*I)	pATH11 (*Sma*I-*Pst*I)
		p16ESP1	0622 (*Sau*3AI)	0683 (*Pst*I)	pATH10 (*Bam*HI-*Pst*I)
		p16E7SEx664	0622 (*Sau*3AI)	0664[a]	—
16	L2	p16L2OB1	4335 (*Oxa*NI)	6151 (*Bam*HI)	pATH1 (*Eco*RI-*Hin*dIII)[b]
		p16L2XB5	5072 (*Xho*II)	6151 (*Bam*HI)	pATH10 (*Bam*HI-*Bam*HI)
		p16L2XX5K	4520 (*Xho*II)	5076 (*Xho*II)	pATH10 (*Bam*HI-*Bam*HI)
		p16L2XEx4866	4520 (*Xho*II)	4866[a]	—
		p16L2XEx4849	4520 (*Xho*II)	4849[a]	—
		p16L2XEx4804	4520 (*Xho*II)	4804[a]	—
		p16L2XEx4770	4520 (*Xho*II)	4770[a]	—
		p16L2XP1	4520 (*Xho*II)	4761 (*Pst*I)	pATH10 (*Bam*HI-*Pst*I)
		p16L2XEx4719	4520 (*Xho*II)	4719[a]	—
		p16L2XEx4675	4520 (*Xho*II)	4675[a]	—
		p16L2XEx4659	4520 (*Xho*II)	4659[a]	—
		p16L2SS1	4570 (*Spe*I)	4870 (*Spe*I)	pATH11 (*Xba*I-*Xba*I)
		p16L2AA1	4678 (*Ase*I)	5061 (*Ase*I)	pATH11 (*Eco*RI-*Eco*RI)[b]
		p16L2PX1	4756 (*Pst*I)	5076 (*Xho*II)	pATH11 (*Pst*I-*Cla*I)[b]
		p16L2RX1	4802 (*Rsa*I)	5076 (*Xho*II)	pATH10 (*Sma*I-*Bam*HI)
		p16L2XX1	4818 (*Xmn*I)	5076 (*Xho*II)	pATH1 (*Eco*RI-*Hin*dIII)[b]
		p16L2SX1	4866 (*Spe*I)	5076 (*Xho*II)	pATH11 (*Xba*I-*Bam*HI)
		p16L2SEx4675	4570 (*Spe*I)	4675	pATH11 (*Xba*I-*Cla*I)[b]
		p16L2AEx4770	4678 (*Ase*I)	4770	pATH11 (*Eco*RI-*Cla*I)[b]
		p16L2REx4849	4802 (*Rsa*I)	4849	pATH10 (*Sma*I-*Cla*I)
18	L2	p18L2OO1	4342 (*Oxa*NI)	5800 (*Oxa*NI)	pATH1 (*Eco*RI-*Hin*dIII)[b]
		p18L2OEx4945	4342 (*Oxa*NI)	4945[a]	—
		p18L2OEx4875	4342 (*Oxa*NI)	4875[a]	—
		p18L2OEx4823	4342 (*Oxa*NI)	4823[a]	—
		p18L2OEx4779	4342 (*Oxa*NI)	4779[a]	—
		p18L2OEx4746	4342 (*Oxa*NI)	4746[a]	—
		p18L2OEx4701	4342 (*Oxa*NI)	4701[a]	—
		p18L2OEx4661	4342 (*Oxa*NI)	4661[a]	—
		p18L2OEx4611	4342 (*Oxa*NI)	4611[a]	—
		p18L2HEx4661	4568 (*Hin*fI)	4661	pATH11 (*Sma*I-*Cla*I)[b]
		p18L2EEx4746	4669 (*Eco*RV)	4746	pATH10 (*Sma*I-*Cla*I)[b]
		p18L2KEx4875	4790 (*Kpn*I)	4875	pATH11 (*Eco*RI-*Cla*I)[b]

[a] —, These constructs were generated by digesting HPV DNA-containing plasmids with exoIII and nuclease S1 (see Materials and Methods).
[b] Detailed descriptions of plasmid constructions are provided in Materials and Methods.

ends blunt by using Klenow polymerase, and by digesting the DNA with *Cla*I. The HPV DNA-containing fragment was ligated to pATH11 DNA which had been prepared by digesting with *Eco*RI, by making the DNA ends blunt by using Klenow polymerase, and by digesting with *Cla*I. p16L2REx4849 was made by digesting p16L2XEx4849 DNA with *Rsa*I (4802) and *Cla*I and by ligating the HPV DNA-containing fragment to pATH10 DNA (*Sma*I-*Cla*I digest). p18L2HEx4661 was made by digesting p18L2OEx4661 DNA with *Hin*fI (4568), by making the DNA ends blunt by using Klenow polymerase, and by digesting the DNA with *Cla*I. The HPV DNA-containing fragment was ligated to pATH11 DNA (*Sma*I-*Cla*I digest). p18L2EEx4746 was made by digesting p18L2OEx4746 DNA with *Eco*RV (4669) and *Cla*I and by ligating the HPV DNA-containing fragment to pATH10 DNA (*Sma*I-*Cla*I digest). p18L2KEx4875 was made by digesting p18L2OEx4875 DNA with *Kpn*I, which cuts within the pUC19 polylinker 5′ to the HPV DNA insert (see description of p18L2OO1 above) and at HPV DNA coordinate 4790. The *Kpn*I-cut plasmid was religated, and the resulting plasmid DNA was digested with *Eco*RI and *Cla*I. The HPV DNA-containing fragment was ligated to pATH11 DNA (*Eco*RI-*Hin*dIII digest).

Synthesis of fusion proteins, SDS-polyacrylamide gel electrophoresis, and Western immunoblot assays. The expression of fusion proteins in *E. coli*, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and Western immunoblot assays were performed as described previously (20). In assays to measure antibody prevalence, the bacterial fusion proteins were partially purified from *E. coli* proteins as described previously (11). In epitope mapping studies, whole bacterial lysates were used as antigen targets. Human sera were tested at a 1:200 dilution in Western immunoblot assays. Antigen-antibody complexes were detected by using alkaline phosphatase-conjugated goat anti-human IgG (Boehringer Mannheim) at a 1:1,000 dilution. The type

specificity (HPV16 versus HPV18) of the antibody reactivities was tested by preabsorbing the sera with lysates of bacteria which expressed HPV16- or HPV18-encoded fusion proteins, as described previously (20, 21).

RESULTS

Mapping human antibody-reactive region of HPV16 E7 polypeptide. Human serum samples were tested for reactivity to HPV16 E7-encoded polypeptides by using the p16E7NP1 fusion protein. Expression plasmid p16E7NP1 contains an HPV16 DNA fragment (*Nsi*I [562]-*Pst*I [875]) which includes the entire E7 coding sequence (nucleotide [nt] 562 to nt855). Twenty-two serum samples were analyzed which contained IgG antibodies which reacted with the p16E7NP1 fusion protein. A region of the HPV16 E7 polypeptide recognized by human serum IgG antibodies was mapped by using nested sets of deleted expression plasmids (Fig. 1).

The carboxy-terminal boundary of the immunoreactive region was determined by reacting human antibodies with HPV16 E7-encoded polypeptides which contained serial carboxy-to-amino-terminus deletions. The serially deleted fusion proteins were expressed by plasmids which contained serial 3′-to-5′ deletions in the HPV16 E7 DNA insert. The results for one human serum sample are displayed in Fig. 1. The IgG antibodies reacted with the p16NEx664 (3′ end of HPV16 DNA insert at nt664) and larger fusion proteins and did not react with the p16NEx619 (3′ end at nt619) and smaller fusion proteins. Therefore, the 3′ boundary of the DNA sequence which encodes the immunoreactive epitope lies between nt619 and nt664.

The amino-terminal boundary of the immunoreactive region was mapped by using expression plasmids from which 5′ E7 ORF segments had been removed by restriction enzyme digestions. p16E7SS1 contains a *Sau*3AI (622 to 871) fragment, and p16E7PP1 contains a *Pvu*II (683)-*Pst*I (875) fragment; both plasmids include the E7 translation termination codon (nt856 to nt858). Human serum samples reacted with the p16E7SS1 fusion protein and did not react with the p16E7PP1 fusion protein (Fig. 1). Therefore, the 5′ boundary of the DNA sequence which encodes the immunoreactive epitope lies between nt622 and nt683.

The coding sequence for the human IgG-reactive epitope mapped to a segment between nt622 and nt664. This DNA segment was expressed in plasmid p16E7SEx664 (*Sau*3AI [622]-exoIII deletion site [664]). Twenty-one of 22 human serum samples which reacted with the p16E7NP1 fusion protein also reacted with the p16E7SEx664 fusion protein (Fig. 1). When the 21 p16E7SEx664-reactive serum samples were preabsorbed with lysates of bacteria which expressed the p16E7SEx664 fusion protein, the reactivities to both the p16E7SEx664 and p16E7NP1 fusion proteins in Western immunoblot assays were eliminated (data not shown). For the serum sample which reacted with the p16E7NP1 fusion protein but not the p16E7SEx664 fusion protein, the reactivity to the p16E7NP1 fusion protein was not affected by preabsorption with bacterial lysates which expressed the p16E7SEx664 fusion protein. Therefore, the predominant antibody binding site(s) recognized by the majority of human serum IgG antibodies in encoded between nt622 and nt664. This DNA segment encodes HPV16 E7 aa21 through aa34 (DLYCYEQLNDSSEE).

Mapping human antibody-reactive regions of HPV16 L2 polypeptide. The p16L2OB1 fusion protein was used to test human serum samples for the presence of HPV16 L2 anti-

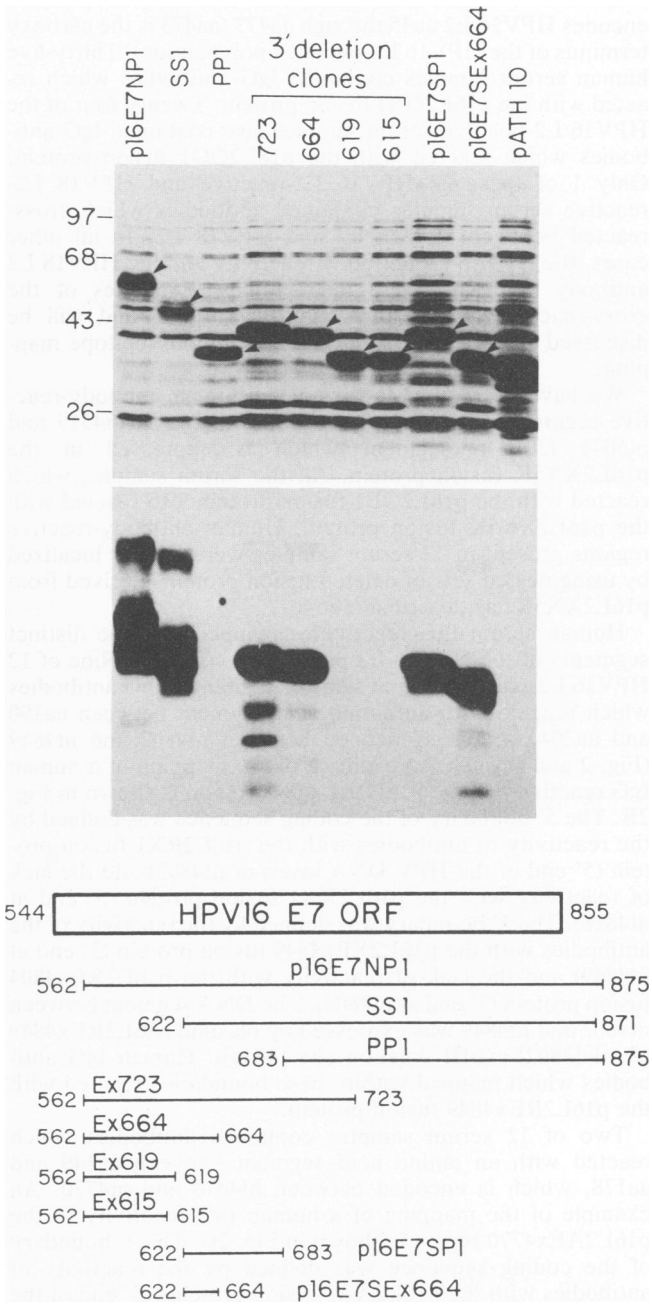

FIG. 1. Mapping of immunoreactive region of HPV16 E7 polypeptide. The immunoreactive region of the HPV16 E7 polypeptide was mapped by using nested sets of deleted fusion protein in Western immunoblot assays. Representative results for one serum sample are shown. The upper panel is a Coomassie blue-stained SDS-10% polyacrylamide gel. The positions of the full-length fusion proteins are indicated with arrows. The mobilities of protein standards in kilodaltons are shown along the left margin. The lower panel is a replicate Western immunoblot which was reacted with an HPV16 E7-positive serum sample. The results are described in the text. The map at the bottom of the figure displays the HPV DNA fragments present in the expression plasmids relative to the HPV16 E7 ORF. The nucleotide coordinates of the 5′ and 3′ ends of the DNA inserts are indicated at the left and right ends of the fragments, respectively.

body reactivities. The p16L2OB1 expression plasmid contains a DNA fragment (*Oxa*NI [4335]-*Bam*HI [6151]) which encodes HPV16 L2 aa35 through aa473 (aa473 is the carboxy terminus of the HPV16 L2-encoded polypeptide). Thirty-five human serum samples contained IgG antibodies which reacted with the p16L2OB1 fusion protein. Twenty-four of the HPV16 L2-positive serum samples also contained IgG antibodies which reacted with the p18L2OO1 fusion protein. Only 1 of these 24 HPV16 L2-reactive and HPV18 L2-reactive serum samples contained antibodies which cross-reacted between HPV16 L2 and HPV18 L2. In all other cases, the HPV16 L2 antibody reactivity and the HPV18 L2 antibody reactivity were independent. Examples of the cross-reactivity assays are shown in Fig. 5 and will be discussed later in the context of L2 antibody epitope mapping.

We have previously localized the human antibody-reactive region to a polypeptide encoded between nt4519 and nt5071 (20), a segment which is expressed in the p16L2XX5K fusion protein. All the serum samples which reacted with the p16L2OB1 fusion protein also reacted with the p16L2XX5K fusion protein. Human antibody-reactive regions present in 12 serum samples were further localized by using nested sets of deleted fusion proteins derived from p16L2XX5K, as described above.

Human serum IgG reactivities mapped to three distinct segments of the HPV16 L2 polypeptide (Fig. 2). Nine of 12 HPV16 L2-reactive serum samples contained IgG antibodies which reacted with an amino acid segment between aa190 and aa204, which is encoded between nt4802 and nt4849 (Fig. 2 and Fig. 3). An example of the mapping of a human IgG reactivity to the p16L2REx4849 region is shown in Fig. 2B. The 5′ boundary of the coding sequence was defined by the reactivity of antibodies with the p16L2RX1 fusion protein (5′ end of the HPV DNA insert at nt4802) and the lack of reactivity with the p16L2XX1 fusion protein (5′ end at nt4818). The 3′ boundary was defined by the reactivity of the antibodies with the p16L2XEx4849 fusion protein (3′ end at nt4849) and the lack of reactivity with the p16L2XEx4804 fusion protein (3′ end at nt4804). The DNA segment between nt4802 and nt4849 was expressed by plasmid p16L2REx4849 (*Rsa*I [4802]-exoIII deletion site [4849]). Human IgG antibodies which mapped within these boundaries reacted with the p16L2REx4849 fusion protein.

Two of 12 serum samples contained antibodies which reacted with an amino acid segment between aa149 and aa178, which is encoded between nt4678 and nt4770. An example of the mapping of a human IgG reactivity to the p16L2AEx4770 region is shown in Fig. 2C. The 5′ boundary of the coding sequence was defined by the reactivity of antibodies with the p16L2AA1 fusion proteins (5′ end of the HPV DNA insert at nt4678) and the lack of reactivity with the p16L2PX1 fusion protein (5′ end at nt4756). The 3′ boundary was defined by the reactivity of the antibodies with the p16L2XEx4770 fusion protein (3′ boundary at nt4770) and the lack of reactivity with the p16L2XP1 fusion protein (3′ boundary at nt4761). The DNA segment between nt4678 and nt4770 was expressed by plasmid p16L2AEx4770 (*Ase*I [4678]-exoIII deletion site [4770]). Human IgG antibodies which mapped within these boundaries reacted with the p16L2AEx4770 fusion protein.

Only 1 of the 24 HPV16 L2-reactive and HPV18 L2-reactive human serum samples contained an IgG antibody reactivity which cross-reacted between HPV16 L2- and HPV18 L2-encoded polypeptides. The 5′ boundary of this human IgG reactivity mapped to HPV16 nt4678, which is the 5′ boundary of the p16L2AEx4770 region. In contrast to type-specific antibodies which reacted with the p16L2AEx4770 region, the 3′ boundary of the cross-reactive region mapped to nt4761 (*Pst*I site in p16L2XP1) rather than to nt4770 (Fig. 2D). Therefore, the epitope recognized by the cross-reactive human antibody is different from the epitope recognized by HPV16 type-specific human antibodies which react within the p16L2AEx4770 region. However, the resolution of the deletion mapping did not allow for the complete dissection of these two epitopes. The region of the HPV16 L2 polypeptide which contains the cross-reactive epitope (aa149 to aa175) lies within the segment indicated with a cross-hatched bar in Fig. 4. The antibodies which reacted with this amino acid segment cross-reacted with the p18L2EEx fusion protein, which contains HPV18 L2 aa143 through aa167 (Fig. 4). Examples of HPV16 L2-reactive and HPV18 L2-reactive type-specific and cross-reactive human antibody reactivities are shown in Fig. 5.

Mapping human antibody-reactive regions of HPV18 L2 polypeptide. The p18L2OO1 fusion protein was used to test human serum samples for the presence of HPV18 L2 antibody reactivities. The p18L2OO1 expression plasmid contains a DNA fragment (*Oxa*NI [4342])-*Oxa*NI [5800]) which encodes HPV18 L2 aa34 through aa462 (aa462 is the carboxy terminus of the HPV18 L2-encoded polypeptide). Twenty-four serum samples were identified which reacted with the p18L2OO1 fusion protein. All these HPV18 L2-reactive serum samples also reacted with the HPV16 L2 fusion protein, but only one of these serum samples contained an antibody(s) which cross-reacted between the HPV16 L2 and HPV18 L2 polypeptides, as described above. The immunoreactive regions recognized by 12 HPV18 L2-reactive serum specimens were mapped by using sets of deleted fusion proteins. The carboxy-terminal boundaries of immunoreactive regions were mapped by using carboxy-to-amino-terminal-deleted fusion proteins as described above. For mapping the amino-terminal boundaries of the immunoreactive regions of the HPV18 L2 polypeptide, restriction enzyme sites were selected which were located immediately 5′ to the 3′ boundaries of the immunoreactive regions. These restriction sites were used to construct the expression plasmids p18L2HEx4661 (*Hin*fI [4568]-exoIII deletion site [4661]), p18L2EEx4746 [*Eco*RV [4669]-exoIII deletion site [4746]), and p18L2KEx4875 (*Kpn*I [4790]-exoIII deletion site [4875]), which express the immunoreactive regions in isolation.

The human antibody reactivities mapped to three distinct segments of the HPV18 L2 polypeptide. Two of 12 human serum specimens reacted with an amino acid segment encoded between nt4790 and nt4875 (Fig. 6B). The 3′ boundary was defined by the reactivity of the antibodies with the p18L2OEx4875 (3′ end of the HPV insert at nt4875) fusion protein and the lack of reactivity with the p18L2OEx4823 (3′ end at nt4823) fusion protein. These serum specimens also reacted with the p18L2KEx4875 (*Kpn*I [nt4790]-exoIII deletion site [nt4875]) fusion protein, which encodes HPV18 L2 aa184 to aa211 (Fig. 7).

Six of 12 serum specimens reacted with an amino acid segment encoded between nt4669 and nt4746. The 3′ boundary was defined by the reactivity of the antibodies with the p18L2OEx4746 (3′ end of the HPV DNA insert at nt4746) and the lack of reactivity with the p18L2OEx4701 (3′ end at nt4701) fusion protein. These serum specimens also reacted with the p18L2EEx4746 (*Eco*RV [nt4669]-exoIII deletion site [nt4746]), which encodes amino acids 143 to 167 (Fig. 7).

Four serum specimens reacted with p18L2OEx4661 (3′ end at nt 4661) and larger fusion proteins and did not react

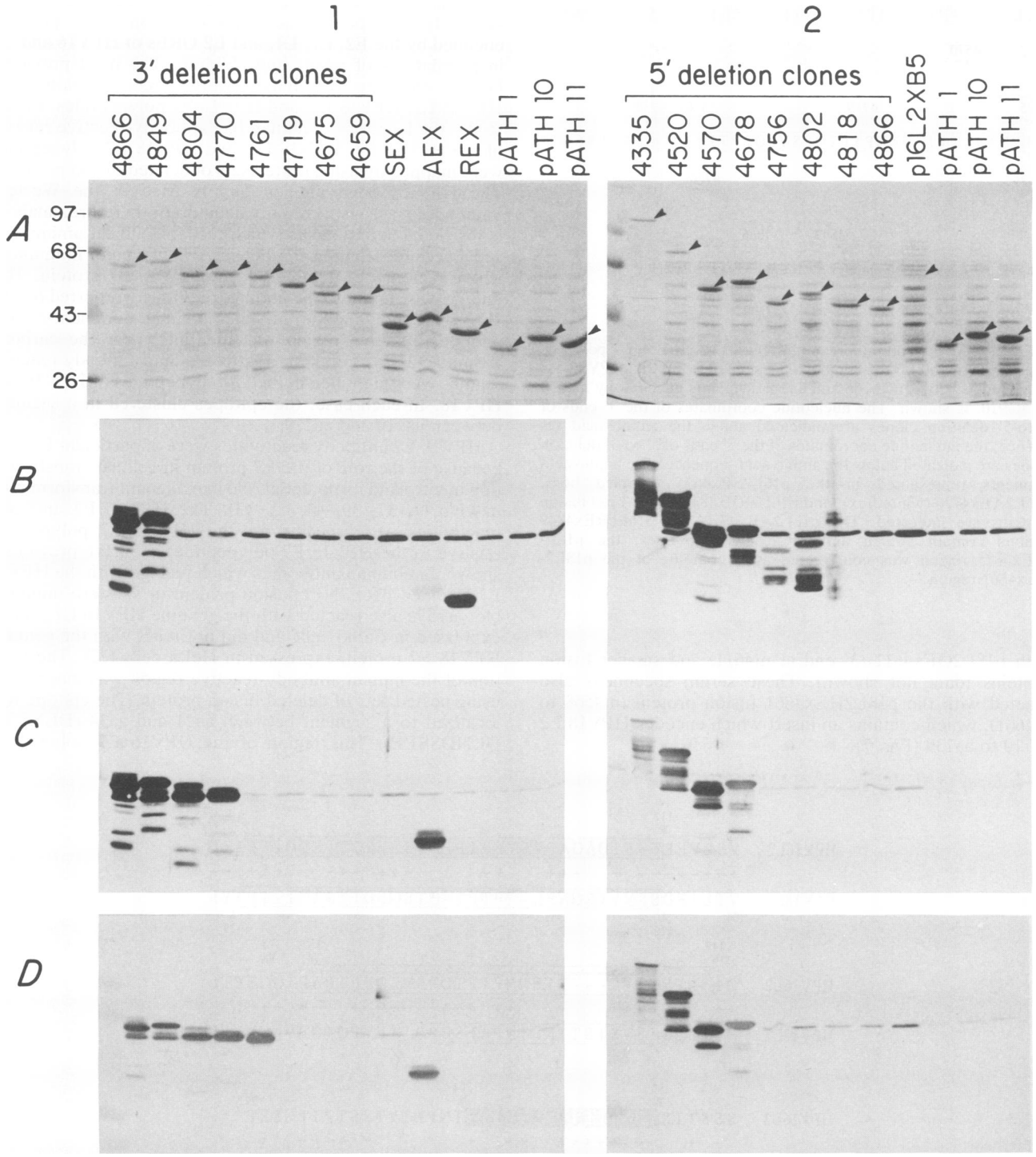

FIG. 2. Mapping of immunoreactive regions of HPV16 L2 polypeptide. Nested sets of deleted fusion proteins were used to define the 3′ and 5′ boundaries of the immunoreactive region coding sequences. A complete description of the expression plasmids is provided in Table 1. (A1 and A2) Coomassie blue-stained SDS-12.5% polyacrylamide gels which contain the deleted fusion proteins. Numbers on left are in kilodaltons. (B, C, and D) Replicate Western immunoblot assays which were reacted with three different serum samples. Panels B1 and B2 show an antibody reactivity which localized between nt4802 (reactive with 5′ deletion clone 4802 but not clone 4818 in panel B2) and nt4849 (reactive with 3′ deletion clone 4849 but not clone 4804 in panel B1). The antibodies also reacted with the p16L2REx4849 protein (REX in panel B1), which is encoded by nt4802 to nt4849. The results for panels C and D are described in the text.

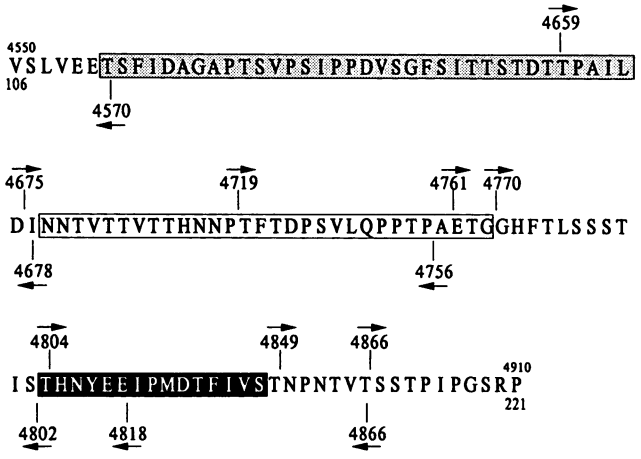

FIG. 3. Map of immunoreactive regions of HPV16 L2 polypeptide. A segment of the amino acid sequence of the HPV16 L2 polypeptide between aa106 and aa211, which is encoded by nt4550 to nt4910, is shown. The nucleotide coordinates of the 3′ ends of 3′-to-5′ deletion clones are indicated above the amino acid sequence; the nucleotide coordinates of the 5′ ends of 5′-to-3′ deletion clones are indicated below the amino acid sequence. The amino acid segments represented in the p16L2SEx4675 (stippled box), p16L2AEx4770 (white box), and p16L2REx4849 (black box) fusion proteins are indicated. The p16L2AEx4770 and p16L2REx4849 regions contain human antibody-reactive epitopes; the p16L-2SEx4675 region was constructed as a homolog of the p18L2-HEx4661 region.

with p18L2OEx4611 (3′ end at nt4611) and smaller fusion proteins (data not shown). These serum specimens also reacted with the p18L2HEx4661 fusion protein (nt4568 to nt4661), which contains an insert which encodes HPV18 L2 aa110 to aa139 (Fig. 7).

DISCUSSION

We have previously reported the prevalence of human serum IgG antibody reactivities to recombinant proteins encoded by the E2, E7, L1, and L2 ORFs of HPV16 and -18 in populations of adults and children. The most prevalent human antibody reactivities were directed against the HPV16 L2, HPV18 L2, and HPV16 E7 polypeptides. In this study, the locations of human IgG antibody-reactive regions of the HPV16 E7, HPV16 L2, and HPV18 L2 polypeptides were mapped by using nested sets of deleted fusion proteins. The HPV-encoded antigen targets used in the Western immunoblot assays were fully denatured; therefore, analysis is restricted to antibodies which recognize linear amino acid sequences (continuous epitopes). A single major immunoreactive region was identified in the HPV16 E7 protein. This finding was similar to the findings previously reported for the HPV6b L1 polypeptide, in which a single dominant human antibody reactive region was identified near the carboxy terminus (21). In contrast, multiple human antibody-reactive regions were identified in the L2 polypeptides of HPV16 and HPV18. In each case, the epitopes clustered in a segment between aa110 and aa210.

HPV16 E7 antibody reactivities were of particular interest because of the role of the E7 protein in cellular transformation in vitro and its potential role in malignant transformation in vivo (1, 37, 39, 40, 42, 43). The HPV16 E7 antibody reactivities were specific for the HPV16 E7 polypeptide relative to the HPV18 E7 polypeptide. We have previously shown that human antibodies which reacted with the HPV16 E7-encoded p16E7NP1 fusion protein in Western immunoblot assays also reacted with the genuine HPV16 E7 protein expressed in CaSki cells and did not react with the genuine HPV18 E7 protein expressed in HeLa cells (22). The location of the human antibody-reactive region was mapped by using nested sets of deleted fusion protein. The epitope was localized to a segment between aa21 and aa34 (DLYCYE QLNDSSEE). This region of the HPV16 E7 polypeptide

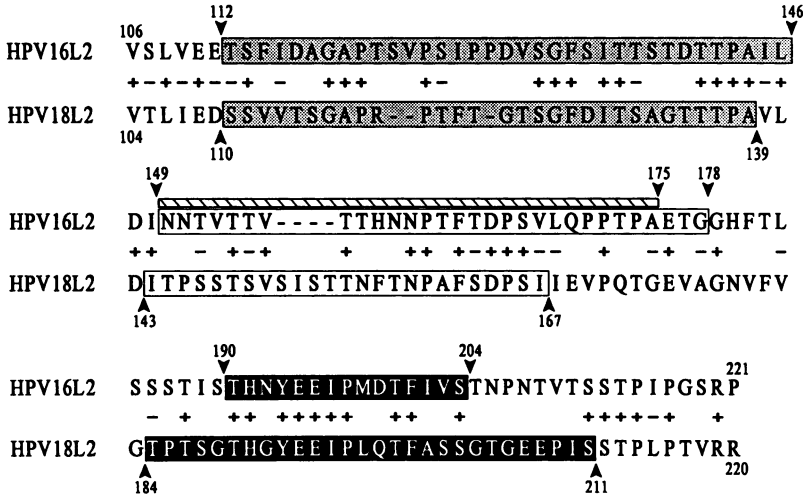

FIG. 4. Alignment of HPV16 L2 and HPV18 L2 amino acid sequences in the immunoreactive regions. A segment of the HPV16 L2 polypeptide (aa106 to aa221) is shown aligned with the homologous region of the HPV18 L2 polypeptide (aa104 to aa220). +, Homologous amino acids are identical. −, Homologous amino acids have similar charge characteristics (conserved amino acid substitutions). Blank spaces between the sequences indicate that the homologous amino acids are different and that the substitutions would not be considered to be conservative. The stippled, white, and black boxes represent the same regions indicated in Fig. 3 and 7. The amino acid boundaries of these regions are indicated. The cross-hatched bar above the p16L2AEx4770 region indicates the boundaries of the region of the HPV16 L2 polypeptide recognized by the HPV16 L2/HPV18 L2 cross-reactive antibodies (aa149 to aa175; see text).

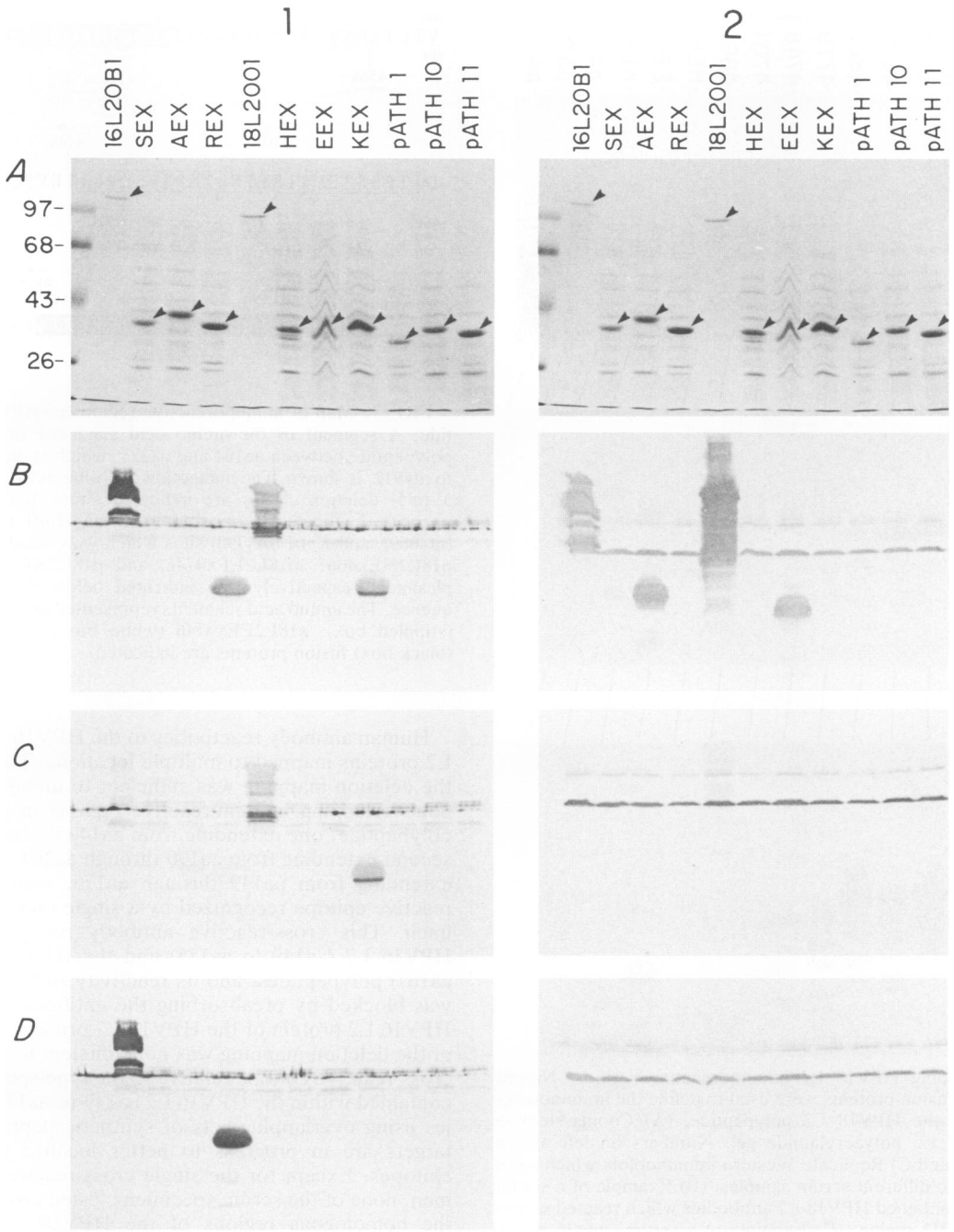

FIG. 5. Examples of HPV16 L2 and HPV18 L2 type-specific and cross-reactive antibody reactivities. (A1 and A2) Identical pictures of an SDS-12.5% polyacrylamide gel which contains the p16L2OB1 fusion protein and three fusion proteins which contain small regions of the HPV16 L2 polypeptide (SEX = p16L2SEx4675; AEX = p16L2AEx4770; REX = p16L2REx4849), and the p18L2OO1 fusion protein and three fusion proteins which contain small regions of the HPV18 L2 polypeptide (HEX = p18L2HEx4661; EEX = p18L2EEx4746; KEX = p18L2KEx4875). Numbers on the left show size in kilodaltons. (B, C, and D) Replicate Western immunoblots. The Western immunoblots in column 1 were all reacted with a single human serum sample (serum 1), and the immunoblots in column 2 were reacted with a second human serum sample (serum 2). Serum 1 reacted with both p16L2OB1 and p18L2OO1 and more specifically with the segments expressed in the p16L2REx4849 and p18L2KEx4875 fusion proteins (B1). When serum 1 was preabsorbed with lysates of bacteria which expressed the p16L2REx4849 fusion protein, the reactivities to the HPV16 L2 fusion proteins were blocked but the reactivities to the HPV18 L2 fusion proteins were unaffected (C1). When serum 1 was preabsorbed with lysates of bacteria which expressed the p18L2KEx4875 fusion protein, the reactivities to the HPV18 L2 fusion proteins were blocked, but the HPV16 L2 antibody reactivities were unaffected (D1). Serum 2 contains antibodies which cross-react with HPV16 L2 and HPV18 L2 proteins and which map to the p16L2AEx4770 and p18L2EEx4746 regions (B2). When serum 2 is preabsorbed with either p16L2Ex4770 lysates (C2) or p18L2EEx4746 lysates (D2), the reactivities to both the HPV16 L2 and HPV18 L2 fusion proteins are blocked.

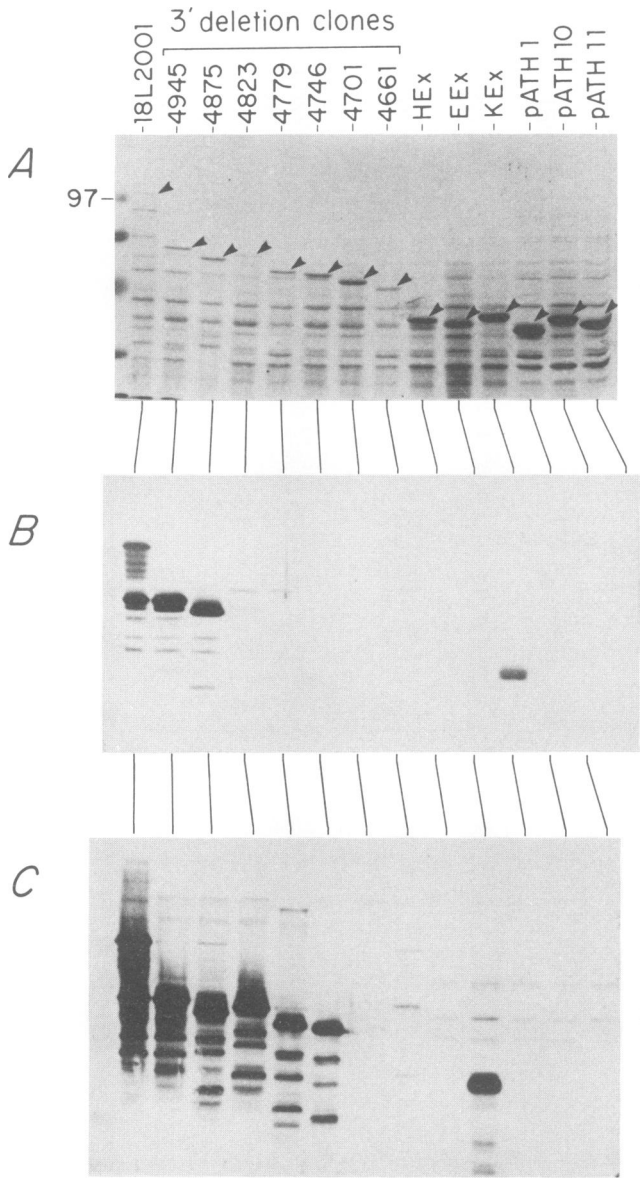

FIG. 6. Mapping HPV18 L2 immunoreactive regions. Nested sets of deleted fusion proteins were used to define the immunoreactive regions of the HPV18 L2 polypeptide. (A) Coomassie-blue stained SDS-12.5% polyacrylamide gel. Numbers on left are in kilodaltons. (B and C) Replicate Western immunoblots which were reacted with two different serum samples. (B) Example of a serum sample which contained HPV18 L2 antibodies which reacted within the aa184-to-aa211 region. (C) Example of a serum sample which contained HPV18 L2 antibodies which reacted within the aa143-to-aa167 region. The results are described in the text.

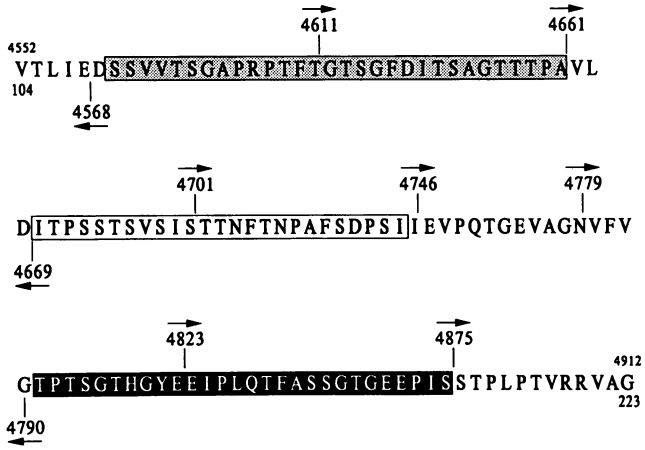

FIG. 7. Map of immunoreactive regions of HPV18 L2 polypeptide. A segment of the amino acid sequence of the HPV18 L2 polypeptide between aa104 and aa223, which is encoded by nt4552 to nt4912, is shown. The nucleotide coordinates of the 3' ends of the 3'-to-5' deletion clones are indicated above the amino acid sequence; the nucleotide positions of the *Hin*fI (nt4568), *Eco*RV (nt4669), and *Kpn*I (nt4790) sites which were used to construct the p18L2HEx4661, p18L2EEx4746, and p18L2KEx4875 expression plasmids, respectively, are indicated below the amino acid sequence. The amino acid segments represented in the p18L2HEx4661 (stippled box), p18L2EEx4746 (white box), and p18L2KEx4875 (black box) fusion proteins are indicated.

contains both a domain for binding the retinoblastoma gene produce (Rb) as well as a site for casein kinase II (CKII) phosphorylation (2, 8, 10, 33). Studies using overlapping synthetic peptides as antigen targets are in progress to further localize the human antibody-reactive epitope(s) within this region. The human antibody-reactive region was distinct from epitopes recognized by murine monoclonal antibodies generated against HPV16 E7 bacterial fusion proteins, results which were recently reported by Tindle et al. (44).

Human antibody reactivities to the HPV16 L2 and HPV18 L2 proteins mapped to multiple locations. The resolution of the deletion mapping was sufficient to identify two distinct nonoverlapping immunoreactive regions in the HPV16 L2 polypeptide, one extending from aa149 through aa178 and a second extending from aa190 through aa204. A third region, extending from aa149 through aa175, contained a cross-reactive epitope recognized by a single human serum specimen. This cross-reactive antibody recognized both the HPV16 L2 (aa149 to aa175) and the HPV18 L2 (aa143 to aa167) polypeptides, and its reactivity to both polypeptides was blocked by preabsorbing the antibody with either the HPV16 L2 protein or the HPV18 L2 protein. The resolution of the deletion mapping was not sufficient to define fully the cross-reactive epitope relative to the type-specific epitope(s) contained within the HPV16 L2 aa149-to-aa178 region. Studies using overlapping sets of synthetic peptides as antigen targets are in progress to better localize these different epitopes. Except for the single cross-reactive serum specimen, none of the serum specimens tested cross-reacted with the homologous regions of the HPV18 L2 polypeptide. HPV16 L2-reactive serum specimens which recognized one of the HPV16 L2 regions either reacted only minimally with the other HPV16 L2 regions or did not react at all with these other regions.

Human serum specimens which reacted with the HPV18 L2 protein recognized one of three distinct immunoreactive regions. Two of these regions, aa143 to aa167 and aa184 to aa211, were approximately homologous to the HPV16 L2 immunoreactive regions aa149 to aa178 and aa190 to aa204, respectively. A third region (aa110 to aa139), located more toward the amino terminus relative to the other immunoreactive regions, was located in a segment of the HPV18 L2 polypeptide where the homologous HPV16 L2 region contained no apparent antibody reactivities. As described

above, the aa143-to-aa167 region reacted with type-specific antibodies but also reacted with a cross-reactive antibody which recognized both HPV16 L2 and HPV18 L2. As for the HPV16 L2 protein, the resolution of the deletion mapping could not distinguish the cross-reactive epitope and the type-specific epitope(s) which are contained within this region. HPV18 L2-reactive sera which recognized one of the HPV18 L2 regions either reacted only minimally with the other HPV18 L2 regions or did not react at all with these other regions.

Papillomavirus virions are nonenveloped icosahedral particles which are 55 nm in diameter and which contain a single molecule of circular double-stranded DNA (24, 27). The L1 and L2 ORFs encode the major and minor capsid proteins of HPV virions, respectively (6, 9, 14, 25, 27, 28, 30–32, 35, 36). Our data suggest that the predominant IgG antibody responses induced by HPV16 and HPV18 infections are directed against the minor capsid proteins. It is not clear why prevalent antibody responses to the HPV16 L1-encoded and HPV18 L1-encoded recombinant proteins were not detected. The L1-encoded polypeptide is expressed in HPV16-associated dysplastic lesions of the uterine cervix in a distribution similar to that of the HPV16 L2-encoded polypeptide (12). It is possible that the HPV16 and HPV18 L1 proteins are not antigenic, that the L1 proteins are not accessible to antibodies due to steric interference imposed by the L2 polypeptide, or that the antibodies induced by the HPV16 and HPV18 L1 polypeptides recognize conformational epitopes and are not detected in Western immunoblot assays.

Different human serum specimens recognized different distinct epitopes which were clustered in the region between aa110 and aa210 of the HPV16 and HPV18 L2-encoded polypeptides. The observed difference in antibody recognition sites could be due to variation in HPV16 and HPV18 capsid proteins or due to variations in host responses to HPV infections. It is not known whether HPV16 and HPV18 virions in nature are relatively homogeneous or relatively divergent, because few HPV isolates have had their complete DNA sequences determined. It is possible that HPV16 and HPV18 infections induce antibody responses directed against multiple minor capsid epitopes in all human hosts but that the amino acid sequences at these locations vary among different viral isolates. Therefore, we could detect only those antibodies which were generated against epitopes which were conserved relative to the prototype HPV16 and HPV18 isolates (which were used to encode the target antigens in the Western immunoblot assays). Alternatively, minor capsid protein sequences might be well conserved among different viral isolates. In this case, variations in antibody recognition sites could be due to differences in immune responses between individuals. For example, HLA genotype variations could differentially affect the proliferation of HPV-specific B-lymphocyte clones so as to favor the proliferation of antibodies directed against one epitope versus antibodies directed against a different epitope, perhaps through affecting T-lymphocyte-mediated B-cell help. Further analysis of viral capsid protein sequences from different HPV16 and HPV18 isolates will help determine whether the observed variation in human antibody responses is due to variations in different HPV isolates. Determining whether certain HLA phenotypes are associated with the presence of antibodies directed against specific epitopes may help to determine whether the differences in antibody recognition sites are due to differences in host immune responses.

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