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Cloning of Human Papilloma Virus Genomic DNAs and Analysis of Homologous Polynucleotide Sequences

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The complete DNA genomes of four distinct human papilloma viruses (human papilloma virus subtype 1a [HPV-1a], HPV-1b, HPV-2a, and HPV-4) were molecularly cloned in *Escherichia coli*, using the certified plasmid vector pBR322. The restriction endonuclease patterns of the cloned HPV-1a and HPV-1b DNAs were similar to those already published for uncloned DNAs. Physical maps were constructed for HPV-2a DNA and HPV-4 DNA, since these viral DNAs had not been previously mapped. By using the cloned DNAs, the genomes of HPV-1a, HPV-2a, and HPV-4 were analyzed for nucleotide sequence homology. Under standard hybridization conditions (T_m = −28°C), no homology was detectable among the genomes of these papilloma viruses, in agreement with previous reports. However, under less stringent conditions (i.e., T_m = −50°C), stable DNA hybrids could be detected between these viral DNAs, indicating homologous segments in the genomes with approximately 30% base mismatch. By using specific DNA fragments immobilized on nitrocellulose filters, these regions of homology were mapped. Hybridization experiments between radiolabeled bovine papilloma virus type 1 (BPV-1) DNA and the unlabeled HPV-1a, HPV-2a, or HPV-4 DNA restriction fragments under low-stringency conditions indicated that the regions of homology among the HPV DNAs are also conserved in the BPV-1 genome with approximately the same degree of base mismatch.

The papilloma viruses are members of the Papovaviridae family and are distinguished from the polyomaviruses, such as simian virus 40 (SV40) and polyoma virus, by a larger particle size (i.e., 55-nm versus 40-nm capsid diameter) and a larger double-stranded superhelical DNA genome (i.e., 5×10^6 versus 3.3×10^6 daltons). Papilloma viruses have been found in a large number of hosts including cottontail rabbits, domestic rabbits, horses, dogs, deer, sheep, chaffinches, cows, and humans.

A number of distinct papilloma viruses have been shown to infect humans. To date, at least five human papilloma viruses (HPVs) have been identified by restriction enzyme analysis and shown by DNA hybridization studies carried out under stringent conditions to share very little, if any, nucleic acid homology (3, 7, 22, 24). HPV type 1 (HPV-1) and HPV-4 are preferentially but not exclusively associated with deep plantar warts. HPV-2 is associated with common hand warts and mosaic plantar warts. HPV-3 has been isolated from flat warts of normal individuals as well as from flat warts of patients with epidermodysplasia verruciformis, a disease characterized by a generalized eruption of warty lesions (24). HPV-5 is associated with benign pityriasis-like lesions in patients with epidermodysplasia verruciformis. These lesions have the potential to undergo malignant transformation when located in areas of the body which receive considerable exposure to the sun (23, 24). Two additional distinct HPV types have also been described. The papilloma virus found associated with condylomata acuminata appears distinct from those previously described and as such is being classified as HPV-6 (24; L. Gissmann, personal communication). An additional HPV, HPV-7, has recently been isolated from the common hand warts of meat handlers (R. S. Ostrow, R. A. Kryzek, F. Pass, and A. J. Faras, manuscript submitted for publication).

Although the well-documented association of papilloma viruses with malignant lesions in their natural host has stimulated great interest in these infectious agents (14, 16, 24, 29), their biological and biochemical characterization has been seriously impeded by the lack of a tissue culture system for their in vitro propagation. To circumvent, in part, these limitations, we have constructed recombinant DNA molecules containing the complete genome of several HPVs (HPV-1, HPV-2, or HPV-4), using the certified plasmid vector pBR322 in *Escherichia coli* K-12. In this manuscript, we describe the cloning of these DNAs and present physical maps of two HPV DNAs which have not been previously mapped. By using these cloned DNAs, we have

examined the extent of genetic relatedness among these HPV DNAs by hybridization experiments carried out under varying degrees of stringency.

MATERIALS AND METHODS

Virus purification. Virus was purified from individual warts by grinding the tissue in sterile sand and phosphate-buffered saline, clarifying the supernatant by low-speed centrifugation (2,000 rpm for 10 min), and using two cycles of CsCl isopycnic density centrifugation ($\bar{\rho}$= 1.34 g/ml) at 35,000 rpm in a Beckman 50 Ti rotor for 16 h at 4°C.

Isolation of viral DNA. Viral DNA was purified directly from virions by disruption in 1% Sarkosyl containing 100 mM NaCl at 50°C for 30 min followed by two extractions with equal volumes of redistilled phenol equilibrated with 10 mM Tris-hydrochloride, pH 8. After dialysis against 10 mM Tris-hydrochloride buffer, pH 8, portions of the DNA were cleaved with restriction endonuclease *Eco*RI, *Bam*HI, or *Hin*dIII and analyzed by agarose gel electrophoresis.

Restriction endonuclease cleavage of viral DNA. Restriction endonucleases were purchased from New England Biolabs (Lowell, Mass.) and Bethesda Research Laboratories (Rockville, Md.). Analytical reaction mixtures (0.02 to 0.05 ml) contained 0.5 μg of DNA and 1 U of enzyme in the appropriate reaction buffer. The reaction mixtures were incubated at 37°C for 1 h, with the exception of *Tac*I and *Pst*I, which were incubated at 60 and 30°C, respectively, for 1 h.

Gel electrophoresis. Electrophoresis through 1.4% (wt/vol) agarose (Seakem) in vertical slab gels (17 by 17 by 0.3 cm) was carried out at 150 V for 3 h at 20°C in a Tris-acetate-EDTA buffer as previously described (11). After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) and the DNA was visualized on a shortwave UV light box and photographed, using orange-yellow filters and Polaroid type 57 film.

Low-molecular-weight DNA fragments generated by various restriction endonucleases were analyzed by electrophoresis in composite agarose-acrylamide gels as previously described (11). These gels consisted of 3, 4, or 5% acrylamide/bisacrylamide (19:1) and 0.5% agarose in a Tris-acetate-EDTA buffer. Electrophoresis in vertical slab gels was for 14 to 18 h at 50 V at 20°C.

Fragments generated by restriction endonuclease cleavage of adenovirus type 2 (Ad2) DNA and SV40 DNA were used as molecular weight standards to establish calibration curves for each gel. The error in size for DNA fragments less than 10^6 daltons is estimated to be $\pm 0.05 \times 10^6$ daltons, and for larger DNA fragments it may be as great as $\pm 0.1 \times 10^6$ daltons. Molecular weights were estimated from the electrophoretic mobility of the DNA fragments.

DNA blotting and hybridization. DNA fragments separated in agarose gels were blotted onto nitrocellulose filters as previously described (19), using the Southern blotting technique (15, 28). High-specific-activity in vitro-labeled viral DNA probes were prepared by using deoxynucleoside [α-^{32}P]triphosphates (Amersham Corp., Arlington Heights, Ill.) and *E. coli* DNA polymerase I (Boehringer Mannheim, Indianapolis, Ind.) (26).

In vitro ^{32}P-labeled viral DNA was denatured and annealed to unlabeled DNA immobilized on nitrocellulose filters under hybridization conditions as previously described (10). For comparison of conserved papilloma virus DNA sequences, 5-mm-wide strips cut from unlabeled DNA blots were preincubated at 60°C for 4 h in Denhardt solution (5) before hybridization. Radiolabeled viral DNA probes were annealed for 18 h to the unlabeled DNA immobilized on the filters at 34°C in 1 M NaCl, 0.01 M *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid (pH 7.4), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, sheared and denatured salmon sperm DNA (10 μg/ml), and the indicated formamide concentration. The nitrocellulose strips were washed extensively with 4× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) at temperatures equivalent to their respective hybridization conditions and then air dried. Identification of hybridized ^{32}P-labeled DNA was by exposure of the filters to Kodak RP Royal X-omat.

Cloning of HPV DNA in *E. coli*. Recombinant HPV/pBR322 DNA molecules were constructed between viral DNA purified from individual warts and pBR322 DNA under P2 physical containment in compliance with the current National Institutes of Health "Guidelines for Recombinant DNA Research," as previously described (13). After cleavage of viral and plasmid DNAs with the appropriate single-cut restriction endonuclease, the enzyme was inactivated at 68°C for 8 min and samples were precipitated with 2.5 volumes of 95% ethanol and washed in cold 80% ethanol. Equal molar quantities of linear plasmid and viral DNA (1 μg) were resuspended in 0.02 ml of 10 mM Tris-hydrochloride buffer, pH 8.0, containing 50 mM NaCl, 5 mM $MgCl_2$, 2 mM mercaptoethanol, 100 μM ATP, and 1.1 mg of bovine serum albumin per ml. Samples were chilled to 14°C, and 0.25 U of T4 DNA ligase (Miles Laboratories, Elkhart, Ind.) was added. Equal portions were taken at various times between 2 and 60 min and immediately frozen. The combined portions were diluted to a DNA concentration of 4 μg/ml and incubated at 14°C overnight after the addition of another 0.25 U of T4 DNA ligase.

A portion of this ligation mixture was then used to transform $CaCl_2$-treated *E. coli* K-12 (strain HB101) as previously described (30). Transformants were selected on agar plates containing either ampicillin (50 μg/ml) or tetracycline (20 μg/ml). These transformants were initially screened for the presence of recombinant plasmids by either replicate plating for altered antibiotic sensitivity (1) or by colony filter hybridization (9), using the appropriate ^{32}P-HPV DNA probe. After amplification by chloramphenicol in M9 medium (2), plasmid DNA was prepared from *E. coli* containing recombinant plasmids as previously described (30). Such recombinants invariably yielded only two DNA fragments after cleavage with the restriction enzyme used in their construction. These fragments corresponded in size to full-length linear plasmid and HPV DNAs.

Preparative amounts of linear viral DNA were isolated from recombinant DNA which had been cleaved

by one or several restriction endonucleases and fractionated on a 5 to 20% (wt/vol) sucrose velocity gradient (Beckman SW41 rotor at 27,000 rpm for 17 h at 10°C) or by electroelution of the electrophoretically separated viral DNA into dialysis bags.

RESULTS

Molecular cloning of HPV DNAs. HPV-1 (subtypes 1a and 1b), -2a, and -4 were isolated from deep plantar warts (types 1 and 4) or common hand warts (type 2). Viral DNAs isolated from purified virions were initially characterized by their sensitivities to the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII (3, 7, 8, 22). The DNA fragments obtained after cleavage of the different HPVs with these restriction endonucleases are summarized in Table 1. HPV-1a and HPV-4, each of which contains a single *Bam*HI restriction site, were cloned at the single *Bam*HI restriction site of the bacterial plasmid pBR322. Insertion of a DNA fragment at this location renders the plasmid incapable of encoding tetracycline resistance (1). Therefore, we identified ampicillin-resistant, tetracycline-sensitive bacterial colonies and screened for the presence of recombinant DNAs as described in Materials and Methods. Since HPV-1b was shown to contain a single *Pst*I restriction site, it was cloned at the *Pst*I restriction site in pBR322; cloning at the *Pst* site interrupts the β-lactamase gene, which confers ampicillin resistance. We therefore screened tetracycline-resistant, ampicillin-sensitive colonies for the presence of HPV-1b/pBR322 DNA. HPV-2a, containing one *Eco*RI restriction site, was similarly ligated to the plasmid vector at the single *Eco*RI restriction site. Since integration of the viral DNA at this site in the plasmid does not interrupt either of the antibiotic resistance genes, colonies containing HPV-2a recombinant DNA molecules were identified by colony hybridization, using in vitro ^{32}P-labeled HPV-2 DNA (9).

Bacterial colonies containing potential HPV/pBR322 hybrid DNAs were amplified for plasmid production with chloramphenicol (2). The supercoiled DNAs obtained from these cultures were then analyzed by agarose gel electrophoresis before and after restriction endonuclease digestion.

Characterization of HPV-4-containing recombinant DNA. Preparative quantities of recombinant DNA containing the HPV-4 genome (HPV-4/pBR322) were obtained from chloramphenicol-amplified bacterial cultures for use in the physical mapping studies. After isolation of the supercoiled recombinant DNA from CsCl-ethidium bromide density gradients (Fig. 1A, lane i), linear HPV-4 DNA was purified, either after cleavage with the restriction enzyme *Bam*HI alone (Fig. 1A, lane g) to generate full-length linear HPV-4 DNA (5×10^6 daltons) and full-length plasmid DNA (2.6×10^6 daltons), or with *Bam*HI and *Pvu*II (Fig. 1A, lane f) to generate full-length linear HPV-4 DNA and two plasmid fragments of molecular weight 1.5×10^6 and 1.1×10^6. The HPV-4 DNA was then separated from pBR322 DNA by sucrose velocity centrifugation (Fig. 1A, lane e) or electrophoresed and electroeluted, as described in Materials and Methods. Figure 1A, lanes d and c, shows the cleavage pattern obtained after digestion of HPV-1a/pBR322 DNA (lane d) and HPV-2a/pBR322 DNA (lane c) with the restriction endonucleases *Bam*HI and *Eco*RI, respectively. An identical cleavage pattern was obtained after digestion of HPV-1b/pBR322 DNA with *Pst*I (data not shown).

To determine the orientation of the linear HPV-4 DNA within the plasmid DNA, the recombinant DNA was cleaved with *Eco*RI. Three restriction fragments were generated with molecular weights 3.3×10^6, 2.6×10^6, and 1.7×10^6 (Fig. 1A, lane h). These fragment sizes indicate that the insertion of the HPV-4 DNA is in the orientation shown in Fig. 2.

To confirm the presence of HPV-4 DNA sequences in the anticipated restriction endonuclease fragments shown in Fig. 1A, and to evaluate the presence of any sequence homology between HPV-4 DNA and HPV-1a or HPV-2a DNAs under stringent hybridization conditions, the DNA fragments shown in Fig. 1A were denatured in situ, transferred to nitrocellulose filter paper (28), and hybridized to ^{32}P-labeled HPV-4 DNA, as described in Materials and Methods.

TABLE 1. *DNA fragments obtained after cleavage of HPV-1a, HPV-1b, HPV-2, HPV-2a, and HPV-4 DNAs with several restriction endonucleases*

Enzyme	Mol wt of fragments ($\times 10^6$)				
	HPV-1a	HPV-1b	HPV-2[a]	HPV-2a	HPV-4
*Bam*HI	4.8[b]	4.4[b]	4.00[c]	No cut	5.0
		0.45	0.98		
*Eco*RI	4.05[d]	4.1[b]	5.29[c]	5.0	2.7
	0.63	0.63			2.3
*Hin*dIII	3.75[d]	3.75[b]	2.7[c]	No cut	2.5
	0.9	0.85	1.7		1.6
	0.23	0.23	0.5		0.9
		0.07			

[a] In addition to the listed digest fragments, DNA molecules resistant to *Bam*HI, *Eco*RI, and *Hin*dIII were observed. Similar heterogeneity in viral DNA isolates from a single wart has been previously noted (23).

[b] From reference 8.

[c] From reference 20.

[d] From reference 6.

[e] From reference 23.

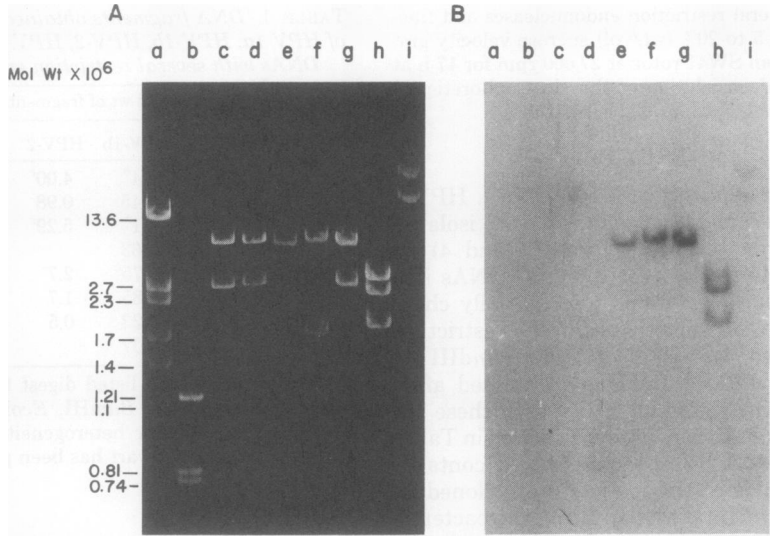

FIG. 1. *Electrophoretic separation in 1.4% agarose gel of restriction enzyme digests of recombinant plasmids containing pBR322 and HPV DNAs. (A) DNA fragments revealed by staining with ethidium bromide (0.5 μg/ml) and photographed with UV light. After photography, the DNA was blotted (28) and hybridized to ^{32}P-labeled HPV-4 DNA under standard stringent conditions at $T_m = -25°C$ as described in Materials and Methods. (B) Resulting autoradiograph. Lanes a and b, Ad2/EcoRI and SV40/HindIII DNA fragments, respectively. These were used as molecular weight standards. Lane c, BamHI digest of a recombinant plasmid containing HPV-1a inserted at the BamHI site. Lane d, EcoRI digest of a recombinant plasmid containing HPV-2a inserted at the EcoRI site. Lanes e, f, g, and i, Digests of the recombinant plasmid containing HPV-4 at various stages of purification; lane i after isolation on CsCl-ethidium bromide gradients, lane g after cleavage with BamHI, lane f after cleavage by BamHI and PvuII, and lane e the purified Bam HI/HPV-4 DNA after sucrose velocity centrifugation. Lane h, HPV-4 recombinant plasmid cleaved with EcoRI.*

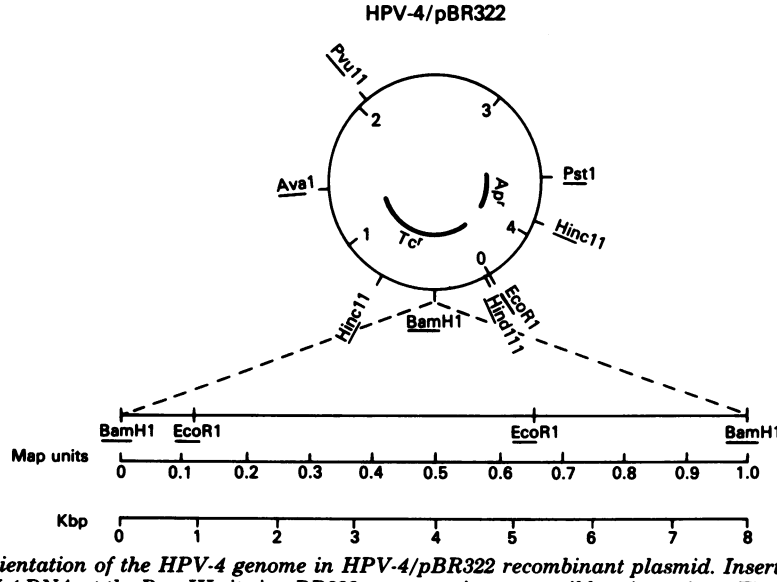

FIG. 2. *Orientation of the HPV-4 genome in HPV-4/pBR322 recombinant plasmid. Insertion of BamHI-cleaved HPV-4 DNA at the BamHI site in pBR322 can occur in two possible orientations. The HPV-4 genome is indicated at the bottom of the figure. The pBR322 genome is shown as a circle. Cleavage of the recombinant plasmid with EcoRI generates three fragments of molecular weights 3.3×10^6, 2.6×10^6, and 1.7×10^6, each containing sequences which hybridize to HPV-4 DNA (Fig. 1). The orientation of the HPV-4 DNA within the recombinant plasmid which corresponds to these EcoRI-generated DNA fragments is indicated. Tc^r represents the tetracycline-resistant region in pBR322; Ap^r represents the ampicillin-resistant region in pBR322. The size of pBR322 and HPV-4 in kilobase pairs (Kbp) is also indicated.*

^{32}P-labeled HPV-4 DNA hybridized only to those DNA fragments predicted to contain HPV-4 DNA sequences, including the HPV-4-containing recombinant plasmid (lane i) and the 5.0×10^6-dalton (full-length linear HPV-4) DNA fragment (lanes g, f, and e). In addition, each of the three fragments generated by *Eco*RI cleavage of the recombinant DNA molecule hybridized to the ^{32}P-labeled HPV-4 DNA probe to an extent proportional to the quantity of HPV-4 DNA present in each of the fragments (Fig. 1B, lane h). No homology was detected between HPV-4 and HPV-1a or HPV-2a DNAs under the stringent hybridization conditions used (Fig. 1B, lanes d and c), indicating that there are no significant regions of the genomes conserved at the level of one base mismatch in six (10). Further experiments under nonstringent hybridization conditions did, however, demonstrate extensive homology among these genomes (see below).

Fine-structure physical map of HPV-4 DNA. Linear HPV-4 DNA obtained from *Bam*HI-cleaved recombinant plasmid DNA (*Bam*HI/HPV-4 DNA) was used to construct the HPV-4 physical map. Several restriction endonucleases were initially screened to identify single-cut enzymes. Of 13 enzymes examined, 5 were identified which did not recognize any sites on the HPV-4 genome: *Ava*I, *Pvu*I, *Pvu*II, *Sal*I, and *Xho*I. Three enzymes, *Bam*HI, *Bgl*I, and *Hpa*I, each recognized one site. Five additional restriction endonucleases tested, *Tac*I, *Eco*RI, *Hin*dIII, *Hpa*II, and *Hinc*II, each recognized two, two, three, four, and five sites, respectively. A representative gel demonstrating the DNA fragments generated by cleavage of the linear *Bam*HI/HPV-4 DNA with various single-cut and multicut enzymes is shown in Fig. 3. The molecular weights of these fragments as calculated from their electrophoretic mobilities are summarized in Table 2.

By using the *Bam*HI restriction site as the zero coordinate, the two *Eco*RI sites were mapped. Digestion of supercoiled HPV-4 DNA by *Eco*RI resulted in two fragments (2.7×10^6 and 2.3×10^6 daltons), and cleavage of the *Bam*HI/HPV-4 DNA with *Eco*RI resulted in three fragments (2.7×10^6, 1.7×10^6, and 0.6×10^6 daltons), locating the *Eco*RI sites at approximately 0.12 and 0.66 map units (MU). A simple physical map of the HPV-4 genome was then constructed by using the other low-cut enzymes, *Bgl*I, *Hpa*I, and *Tac*I. Both single-cut enzymes, *Bgl*I and *Hpa*I, were found to cleave the 1.7×10^6-dalton *Eco*RI fragment of *Bam*HI/HPV-4 DNA, thus localizing each restriction site to this fragment. *Tac*I recognizes two sites on the HPV-4 genome and cleaves both the A and B fragments of *Eco*RI-cleaved *Bam*HI/HPV-4 DNA. The size of the fragments generated by these cleavages, as well as of the fragments generated by the sequential cleavages of *Bam*HI/HPV-4 with other combinations of low-cut restriction endonucleases, is summarized in Table 3. These results unambiguously locate the sites of these four low-cut restriction endonucleases on the HPV-4 genome relative to each other.

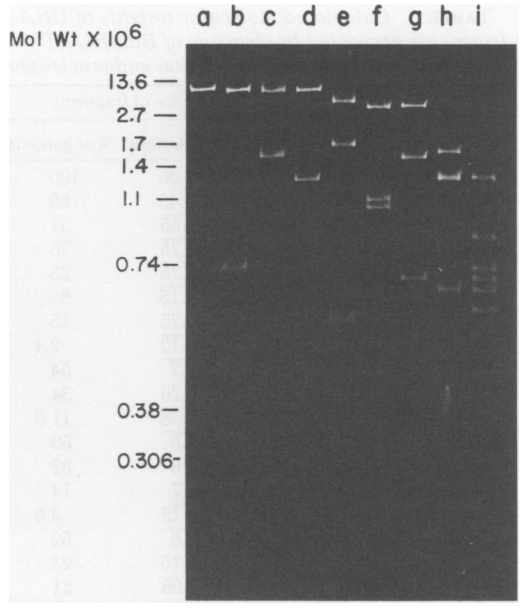

FIG. 3. *Electrophoretic separation in 3% acrylamide-0.5% agarose composite gels of restriction enzyme digests of BamHI/HPV-4 DNA. Full-length linear BamHI/HPV-4 DNA (lane a) was cleaved with a series of low-cut enzymes including TacI (lane b), BglI (lane c), HpaI (lane d), and EcoRI (lane e) and multicut enzymes including HpaII (lane f), HindIII (lane g), HincII (lane h), and HindII+III (lane i). The positions of the EcoRI fragments of Ad2 and the HindIII fragments of SV40 are included as molecular weight markers.*

By using the *Bgl*I, *Hpa*I, *Eco*RI, and *Tac*I sites on the *Bam*HI/HPV-4 DNA as reference points, the positions of the multicut enzymes, *Hin*dIII, *Hpa*II, and *Hinc*II, were next mapped. The size of each of the DNA fragments generated by cleavage with the multicut enzyme alone was compared with the size of the DNA fragments obtained after sequential digestion with the one- or two-cut enzymes (Table 4). By this method, it was possible to locate the sites of the multicut enzymes on the HPV-4 genome. For example, *Hin*dIII recognized three sites on the HPV-4 genome, thus giving four fragments when *Bam*HI/HPV-4 was cleaved with *Hin*dIII (2.5×10^6, 1.6×10^6, 0.7×10^6, and 0.2×10^6 daltons). Sequential cleavage of *Bam*HI/HPV-4 DNA

TABLE 2. *Calculated molecular weights of DNA fragments generated by cleavage of BamHI/HPV-4 linear DNA with several restriction endonucleases*

Enzyme	No. of fragments	Size of fragment	
		×10⁶ daltons[a]	% of genome[b]
*Bam*HI	1	5.00	100
*Bgl*I	2	3.45[c]	69
		1.55	31
*Hpa*I	2	3.75	75
		1.25	25
*Tac*I	3	4.15	83
		0.75	15
		0.12	2.4
*Eco*RI	3	2.7	54
		1.70	34
		0.58	11.6
*Hin*dIII	4	2.5	50
		1.6	32
		0.7	14
		0.18	3.6
*Hpa*II	5	2.6	52
		1.15	23
		1.05	21
		0.11	2.2
		0.10	2.0
*Hin*cII	6	1.6	32
		1.25	25
		1.25	25
		0.65	13
		0.165	3.3
		0.09	1.8
*Hin*dII + III	9	1.25	25
		0.9	18
		0.75	15
		0.7	14
		0.65	13
		0.55	11
		0.1	2
		0.09	1.8
		0.05	1.0

[a] Molecular weights of fragments were estimated from the electrophoretic mobilities of fragments in a composite 3.0% acrylamide–0.5% agarose gel and a 2.2% acrylamide–0.5% agarose gel. The six *Eco*RI fragments of Ad2 DNA and the six *Hin*dIII fragments of SV40 DNA were used as molecular weight standards.

[b] Percentage of genome for each fragment is equal to 100 × (molecular weight of fragment/sum of molecular weights of all fragments).

[c] Fragments which are underlined were determined to be joint fragments adjacent to the pBR322 genome in the HPV-4/pBR322 hybrid molecule by cleavage of the hybrid molecules with the indicated enzymes.

with *Hin*dIII and *Hpa*I or *Hin*dIII and *Bgl*I affected the mobility of the largest fragment. By comparing the sizes of the new fragments generated after cleavage of *Bam*HI/HPV-4 DNA with *Hin*dIII and *Hpa*I (2.0×10^6 and 0.55×10^6 daltons) and *Hin*dIII and *Bgl*I (1.7×10^6 and 0.85×10^6 daltons), the restriction sites delineating fragment A were located. Similarly, fragment B (1.6×10^6 daltons) was positioned between 0.04 and 0.31 MU after analysis of the new fragments generated by cleavage of *Bam*HI/HPV-4 DNA with *Hin*dIII and *Eco*RI as well as *Hin*dIII and *Tac*I. The mobility of fragment C_1 (0.7×10^6 daltons) was slightly altered after sequential digestion of *Bam*HI/HPV-4 DNA with *Hin*dIII and *Tac*I, suggesting that fragment C_1 was at the right-hand end of the *Bam*HI/HPV-4 genome and that the smallest fragment, fragment C_2, was at the left-hand end. The positions of the end fragments were confirmed by comparing the *Hin*dIII cleavage pattern of the recombinant plasmid (2.6×10^6, 2.5×10^6 1.6×10^6, and 0.9×10^6 daltons) with the pattern obtained after cleavage of *Bam*HI/HPV-4 DNA with *Hin*dIII (2.5×10^6, 1.6×10^6, 0.7×10^6, and 0.2×10^6 daltons). By this analysis, *Hin*dIII fragment C_1 of the *Bam*HI/HPV-4 DNA (0.7×10^6 daltons) was confirmed to be adjacent to the smaller fragment of pBR322 which is generated upon cleavage of the plasmid with *Bam*HI and *Hin*dIII (0.2×10^6 daltons), and fragment C_2 (0.2×10^6 daltons) of the *Hin*dIII-cleaved *Bam*HI/HPV-4 DNA was found to be adjacent to the larger fragment of *Bam*HI/*Hin*dIII-cleaved pBR322 (2.4×10^6 daltons).

TABLE 3. *Cleavage of BamHI/HPV-4 DNA by pairs of low-cut restriction endonucleases*

First enzyme	Second enzyme	No. of fragments	Mol wt of DNA fragment (×10⁶)[a]	Size of DNA fragment (% of HPV-4 genome)
*Bgl*I	*Hpa*I	3	3.45, 1.25, 0.3	69, 25, 6
*Bgl*I	*Tac*I	4	2.7, 1.45, 0.75, 0.12	54, 29, 15, 2.4
*Bgl*I	*Eco*RI	4	2.72, 1.55, 0.58, 0.15	54.4, 31, 11.6, 3
*Hpa*I	*Tac*I	4	3.0, 1.1, 0.75, 0.12	60, 22, 15, 2.4
*Hpa*I	*Eco*RI	4	2.72, 1.25, 0.58, 0.45	54.4, 25, 11.6, 9
*Tac*I	*Eco*RI	5	2.55, 1.6, 0.58	51, 32, 11.6
			0.17, 0.12	3.4, 2.4

[a] Molecular weights of fragments were determined from electrophoretic mobilities of fragments in a composite 3.0% acrylamide–0.5% agarose gel. The six *Eco*RI fragments of Ad2 DNA and the six *Hin*dIII fragments of SV40 DNA were used as molecular weight standards.

By similar analysis, the *Hin*cII and *Hpa*II sites were located on the *Bam*HI/HPV-4 genome. The detailed physical map is seen in Fig. 4.

Fine-structure physical map of HPV-2a DNA. The physical map of the HPV-2a genome was determined by an analysis similar to that used for HPV-4. The virus was purified from a single common wart and the viral DNA was prepared as described in Materials and Methods. The virion DNA was characterized by restriction endonuclease digestion as having a single *Eco*RI site and being resistant to *Bam*HI and *Hin*dIII. It was characterized as a subtype of HPV-2

TABLE 4. *Cleavage of BamHI/HPV-4 DNA by pairs of low-cut and multicut restriction endonucleases*

Enzyme	Fragment A		Fragment B		Fragment B_1		Fragment B_2		Fragment C		Fragment C_1		Fragment C_2		Fragment D		Fragment D_1		Fragment D_2		Fragment E	
	Mol wt ($\times 10^6$)[a]	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome	Mol wt ($\times 10^6$)	% of genome
*Hin*dIII	2.5	50	1.6	32							0.7	14	0.18	3.6								
*Hin*dIII + *Hpa*I	2.0, 0.55	40, 11	1.6	32							0.7	14	0.18	3.6								
*Hin*dIII + *Bgl*I	1.7, 0.85	33, 17	1.6	32							0.7	14	0.18	3.6								
*Hin*dIII + *Tac*I	2.5	50	1.05, 0.55	21, 11							0.58, 0.12	11.6, 2.4	0.18	3.6								
*Hin*dIII + *Eco*RI	1.5, 1.0	30, 20	1.2, 0.4	24, 8							0.7	14	0.18	3.6								
*Hpa*II	2.6	52	1.15	23					1.05	21							0.11	2.2	0.10	2.0		
*Hpa*II + *Hpa*I	2.6	52	1.05, 0.1	21, 2					1.05	21							0.11	2.2	0.10	2.0		
*Hpa*II + *Bgl*I	2.6	52	0.75, 0.4	15, 8					1.05	21							0.11	2.2	0.10	2.0		
*Hpa*II + *Tac*I	2.0, 0.6	40, 12	1.15	23					1.05	21							ND[b]	ND[b]	0.10	2.0		
*Hpa*II + *Eco*RI	2.1, 0.5	42, 10	0.6, 0.55	12, 11					1.05	21							0.11	2.2	0.10	2.0		
*Hinc*II	1.6	32			1.25	25	0.09	1.8	1.25	25					0.65	13					0.17	3.4
*Hinc*II + *Bgl*I	1.6	32			1.25	25	0.09	1.8	0.95, 0.3	19, 6					0.65	13					0.17	3.4
*Hinc*II + *Tac*I	1.6	32			1.15, 0.1	23, 2	0.09	1.8	1.25	25					0.5, 0.15	10, 3					0.17	3.4
*Hinc*II + *Eco*RI	1.6	32			1.25	25	0.09	1.8	0.8, 0.45	16, 9					0.35, 0.3	7, 6					0.17	3.4

[a] Molecular weights of fragments were determined from electrophoretic mobilities of fragments in a composite 3% acrylamide–0.5% agarose gel.

[b] ND, Not determined. The *Tac*I enzyme site located in the D_1 fragment of *Hpa*II-digested *Bam*HI/HPV-4 DNA was not localized because it was located very close to the *Hpa*II site.

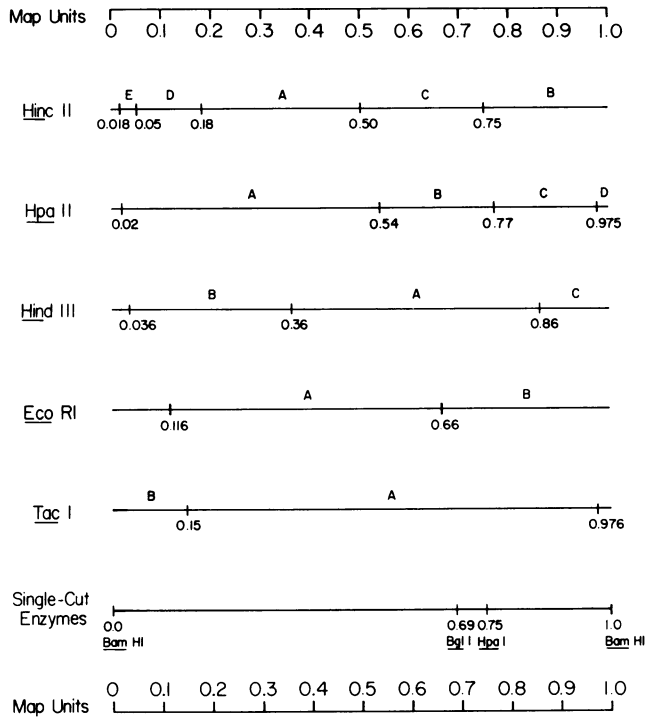

FIG. 4. *Restriction endonuclease cleavage map of HPV-4 DNA. Map units, expressed as a fraction of the circular HPV-4 genome commencing at the single BamHI cleavage site, were determined from the data presented in Tables 2 through 4.*

because it hybridized as efficiently to an HPV-2-specific probe under stringent conditions as did known HPV-2 DNA (data not shown). Orth and his collaborators have noted a heterogeneity among HPV-2 DNAs and have described molecules resistant to either *Eco*RI or *Bam*HI (22).

By using the single *Eco*RI site as the zero map position, a total of 11 additional restriction endonuclease sites were mapped. The mapping was performed by a series of single and multiple enzyme digestions of the HPV-2a/pBR322 hybrid DNA as well as of gel-purified *Eco*RI linear HPV-2a DNA as described above for HPV-4. The data obtained in constructing the physical map are presented in Table 5, and the physical map of HPV-2a is presented in Fig. 5.

Analysis of homology among the HPV DNAs. Previous studies from this laboratory have indicated that there are conserved sequences among the genomes of HPV-1, the bovine papilloma viruses BPV-1 and BPV-2, and the Shope papilloma virus (CRPV) which can be detected under nonstringent hybridization conditions (18). Thermally stable hybrids could be detected between these DNAs in 1 M NaCl at 37°C in 30% formamide (an effective temperature of T_m = −43°C for HPV-1 DNA). At higher temperatures, however, these hybrids melted out, indicating approximately 30% base mismatch in the regions of homology detected (18). Using the cloned HPV DNAs, we thus examined whether these conserved sequences were also present in the HPV-2a and HPV-4 genomes and estimated the degree of base mismatch within the homologous segments of these DNAs. In vitro ^{32}P-labeled DNA probes from each of the HPV DNAs as well as BPV-1 DNA were annealed under a range of hybridization conditions to restriction endonuclease-generated fragments of HPV-1a, HPV-2a, or HPV-4 DNA immobilized on nitrocellulose filters. *Bam*HI linear HPV-1a DNA purified from the recombinant plasmid was cleaved with *Hin*dIII and *Hpa*I to generate the following six fragments: A (0.285 to 0.62 MU); B (0.85 to 0.125 MU); C (0.67 to 0.85 MU); D (0.125 to 0.285 MU); E (0.62 to 0.66 MU); and F (0.66 to 0.67 MU) (6). Purified *Bam*HI linear HPV-4 DNA was cleaved with *Hin*dIII and *Hpa*I to generate five fragments: A (0.36 to 0.75 MU); B (0.036 to 0.36 MU); C (0.86 to 1.0 MU); D (0.75 to 0.86 MU); and E (0.0 to 0.036 MU) (Fig. 4). Purified *Eco*RI linear HPV-2a was similarly cleaved with *Xho*I and *Bgl*II to generate the following four fragments: A (0.22 to

TABLE 5. *Cleavage of EcoRI/HPV-2a DNA by restriction endonucleases*

Enzyme	No. of fragments	Mol wt of DNA fragments ($\times 10^6$)	Size of DNA fragments (% of HPV-2a genome)
*Hin*cII	3	3.0, 1.9, 0.12	60, 37.5, 2.5[a]
*Bgl*II	3	3.5, 1.25, 0.25	70, 25, 5
*Xho*I	2	3.9, 1.10	78, 22
*Pvu*II	7	1.6, 1.28, 0.70, 0.50, 0.45, 0.25, 0.22	32, 25.5, 14, 10, 9, 5, 4.5
*Hin*cII + *Xho*I	4	1.9, 1.88, 1.10, 0.12	38, 37.5, 22, 2.5
*Hin*cII + *Bgl*II	5	3.0, 1.25, 0.38, 0.25, 0.12	60, 25, 7.5, 5, 2.5
*Xho*I + *Bgl*II	4	2.4, 1.25, 1.10, 0.25	48, 25, 22, 5
*Pvu*II + *Xho*I	8	1.28, 1.10, 0.70, 0.50, 0.50, 0.45, 0.25, 0.22	25.5, 22, 14, 10, 10
*Pvu*II + *Hin*cII	9	1.6, 1.28, 0.70, 0.48, 0.45, 0.25, ND,[b] ND, ND	32, 25.5, 14, 9.5, 9, 5, (2.5), (2.0), (0.5)
*Pvu*II + *Bgl*II	9	1.6, 1.28, 0.70, 0.38, 0.25, 0.25, 0.22, 0.20, ND	32, 25.5, 14, 8, 5, 5, 4.5, 4, (2.0)

[a] Fragments which are underlined were determined to be joint fragments adjacent to the pBR322 genome in the HPV2a/pBR322 hybrid molecule by cleavage of the hybrid molecules with the indicated enzymes.

[b] ND, Fragments were not detected in the gel electrophoresis analysis of the digested DNA because of their size. Their size, estimated from other mapping data, is indicated in parentheses.

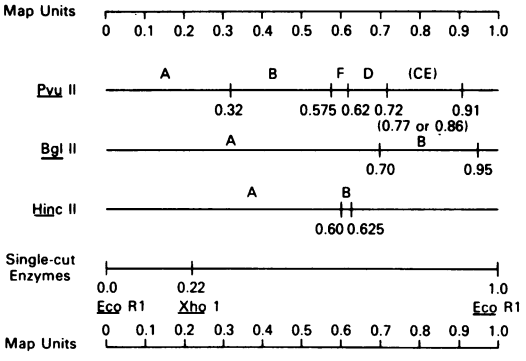

FIG. 5. *Restriction endonuclease cleavage map of HPV-2a DNA. Map units, expressed as a fraction of the circular HPV-2a genome commencing at the single EcoRI site, were determined from the data presented in Table 5. No-cut enzymes for HPV-2a include BamHI, HindIII, HpaI, and SstI.*

0.70 MU); B (0.70 to 0.95 MU); C (0.0 to 0.22 MU); and D (0.95 to 1.0 MU) (Fig. 5).

At 34°C in 50% formamide (T_m = −28°C), stringent hybridization conditions equivalent to those used by Lancaster and Olson (17) and our laboratory (18), no stable hybrids could be detected between these different HPV DNAs. At lower formamide concentrations, however, stable hybrids could be detected between certain regions of the HPV-1a, HPV-2a, and HPV-4 genomes, indicating that there are regions of significant homology among these DNAs with greater than 20% base mismatch. ^{32}P-labeled HPV-1a DNA formed a stable hybrid with the HPV-4 DNA fragment D when the hybridization reaction was carried out in 30% formamide (effective temperature of T_m = −43°C) (Fig. 6), indicating a region of homology in this fragment with a maximum of 30% base mismatch. Under even less stringent conditions (20% formamide, an effective temperature of T_m = −50°C), additional stable hybrids could be detected with HPV-4 DNA fragments A and B, indicating additional regions of homology with approximately 35% base mismatch. ^{32}P-labeled HPV-2a DNA also formed a stable hybrid with the HPV-4 DNA fragment D, but only under the least stringent hybridization conditions used (20% formamide with an effective temperature of T_m = −50°C). This same radiolabeled probe hybridized to a lesser degree with the HPV-4 DNA fragments A and B under these same conditions. No homology was detected between the HPV-1a or HPV-2a ^{32}P-labeled DNAs and HPV-4 DNA fragment C. The inefficient transfer of DNA fragments of less than 400 base pairs onto nitrocellulose filter paper prevented us from assessing with confidence the degree of homology between HPV-4 DNA fragment E and the heterologous DNAs. Interestingly, ^{32}P-labeled BPV-1 DNA also formed stable hybrids with the HPV-4 A, B, and D DNA fragments, and these hybrids melted at the same formamide concentrations as did the HPV-1a DNA probes.

The presence of related sequences among the HPV-1a, HPV-2a, and HPV-4 viral genomes was confirmed by reciprocal experiments using unlabeled HPV-1a or HPV-2a DNA fragments immobilized on nitrocellulose filters. No homology was detected between DNAs under the most stringent hybridization conditions used (T_m = −36°C and −28°C). However, stable hybrids were detected between HPV-1a DNA fragments A, B, and C and ^{32}P-labeled HPV-4 DNA when the hybridization reaction was carried out in 30 or 20% formamide. ^{32}P-labeled HPV-2a DNA also formed stable hybrids with HPV-1a DNA fragments A and B in 30 or 20% formamide and

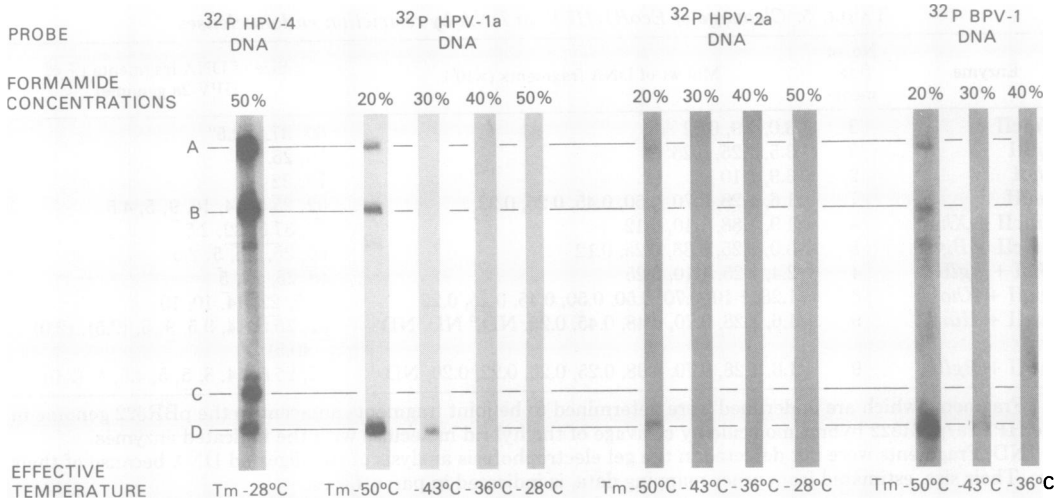

FIG. 6. *Hybridization of ^{32}P-labeled HPV-1a, HPV-2a, or BPV-1 DNA to the DNA fragments of the HPV-4 genome. Nitrocellulose strips containing the HindIII/HpaI fragments of the BamHI-cleaved HPV-4 genome (0.2 μg of DNA/strip) were incubated for 18 h in 1 ml of reaction mixture containing in vitro ^{32}P-labeled (5 × 10^5 cpm/reaction) and denatured HPV-1a DNA (specific activity, 6.4 × 10^7 cpm/μg of DNA), HPV-2a DNA (specific activity, 1.7 × 10^7 cpm/μg of DNA), or BPV-1 DNA (specific activity, 1.1 × 10^8 cpm/μg of DNA) at 34°C at the indicated formamide concentration. The strips were washed at a temperature equivalent to the T_m of the hybridization reaction as described in Materials and Methods. Filters examining homologous DNAs were exposed to RP Royal X-omat X-ray film for 6 h, and those examining heterologous DNAs were exposed for approximately 48 h. The effective temperature for hybridization in each case was determined by the melting temperature (T_m) of the HPV genome at each formamide concentration, as calculated from the equation T_m (°C) = 81.5 + 16.6 (log M) + 0.41 (%G+C) −0.72 (% formamide), where M is the molarity of the monovalent salt and %G+C is the percentage of guanine-plus-cytosine residues in the DNA (20, 27). The %G+C for HPV DNA used in this calculation was 41 (4, 21).*

with the HPV-1a DNA fragment C in 20% formamide. Again, hybridization with the HPV-2a DNA probe was not as extensive as the hybridization reaction between ^{32}P-labeled HPV-4 DNA and the HPV-1a DNA fragments (Fig. 7). Studies from this laboratory have previously shown that under these same conditions, BPV-1, BPV-2, and CRPV DNAs form stable hybrids with these same HPV-1a restriction fragments (18). Stable hybrids could be detected under the least stringent hybridization conditions (20% formamide with an effective temperature of T_m = −50°C) between ^{32}P-labeled HPV-1a DNA and HPV-2a DNA fragments A, B, and C and between ^{32}P-labeled HPV-4 DNA and HPV-2a DNA fragments A and B (data not shown). The degree of hybridization was not as extensive as that found between ^{32}P-labeled BPV-1 DNA and the HPV-2a DNA fragments. In this reaction, stable hybrids were formed between ^{32}P-labeled BPV-1 DNA and HPV-2a DNA fragment A in 30% formamide (an effective temperature of T_m = −43°C). Under less stringent conditions (20% formamide with an effective temperature of T_m = −50°C), additional stable hybrids formed between the radiolabeled BPV-1 DNA probe and HPV-2a DNA fragments B and C (data not shown).

These results confirm the results of others who have shown that under the standard hybridization conditions, nucleotide sequence homology cannot be demonstrated between the DNAs of the different HPV types (22, 23). However, under hybridization conditions which are more sensitive and detect segments of homologous DNA with up to 35% base mismatch, the genomes of HPV-1a, HPV-2a, and HPV-4 do contain homologous segments. Surprisingly, the sequences that appear most conserved among these HPV DNAs are the same sequences conserved in BPV-1 DNA. Additionally, the degree of base mismatch in the homologous segments between the human DNAs is approximately the same as the base mismatch between these homologous segments of each of the HPV DNAs and BPV-1 DNA, as measured by the effective temperatures at which the stable DNA hybrids melt. Thus, by this analysis, the different HPV types appear to have diverged as much from one another as they have from either BPV-1, BPV-2, or CRPV (18).

DISCUSSION

HPVs are capable of inducing a variety of epithelial proliferations in their natural host, humans, but have never been conclusively

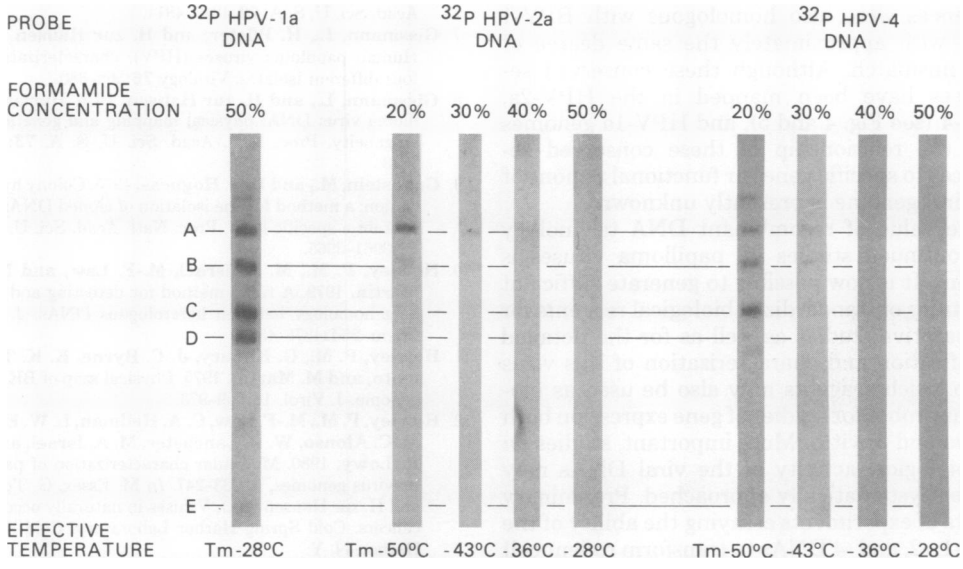

FIG. 7. *Hybridization of ^{32}P-labeled HPV-2a or ^{32}P-labeled HPV-4 DNA to the DNA fragments of the HPV-1a genome. Nitrocellulose strips containing the HindIII/HpaI fragments of the BamHI-cleaved HPV-2a genome (0.2 μg of DNA/strip) were incubated for 18 h in 1 ml of reaction mixture containing in vitro ^{32}P-labeled and denatured heterologous DNAs at 34°C at the indicated formamide concentration. The strips were processed as described in the legend of Fig. 6.*

shown to have any biological activity in heterologous hosts. Only during the past few years, by the analysis of virion DNA purified directly from individual warts, has the remarkable plurality of HPVs and their subtypes been revealed. The inability to propagate papilloma viruses in tissue culture has severely hampered attempts at biological and molecular characterization. As described here, the ability to molecularly clone HPV genomes by using recombinant DNA technology should circumvent this problem and provide the standardized biological reagents necessary to begin such studies. To date we have cloned the entire genomes of HPV-1a, HPV-1b, HPV-2a, and HPV-4 in pBR322. We are presently attempting to clone HPV-3 and HPV-5. The restriction endonuclease patterns generated for our cloned HPV-1a and HPV-1b DNAs were similar to those already published for viral DNA isolated from purified virions (7) and served as the basis for the assignment of the subtype. HPV-2 and HPV-4 DNAs have not been previously mapped, and we present here physical maps of these genomes.

The *Hin*dII+III cleavage pattern of our cloned HPV-4 DNA was in agreement with the cleavage pattern described by Orth and co-workers for HPV-4 (23). Slight differences were observed in the sizes of the *Hin*dIII fragments of our cloned HPV-4 DNA when compared with the HPV-4 pattern described by Pfister and coworkers (25); however, our cloned HPV-4 DNA hybridized extensively under stringent conditions to the HPV-4 DNA described by Pfister (H. Pfister, personal communication). Thus, there may be heterogeneity among individual isolates of HPV-4 with variation in the restriction endonuclease patterns.

There also appear to be several closely related subtypes of HPV-2 which share extensive nucleic acid homology under stringent hybridization conditions but which have distinct restriction endonuclease cleavage patterns (23). In the initial description of HPV-2, Orth and co-workers demonstrated that the HPV-2 DNA isolated from a single common wart was heterogeneous (22). In that description, the HPV-2 DNA was found to contain molecules which were resistant to *Bam*HI, *Eco*RI, and *Hin*dIII as well as molecules which contained two, one, and three cleavage sites for each enzyme, respectively. The HPV-2 DNA we have cloned contains a single *Eco*RI site, no *Bam*HI site, and no *Hin*dIII site. We have designated this isolate HPV-2a because of this variance in restriction endonuclease pattern from the HPV-2 DNA originally described (22).

By using the cloned HPV DNAs, the location and extent of nucleotide sequence homology among HPVs was examined. Under nonstringent hybridization conditions, conserved nucleotide sequences containing 25 to 35% base mismatch could be demonstrated among the genomes of HPV-1a, HPV-2a, and HPV-4, and these same

sequences were also homologous with BPV-1 DNA with approximately the same degree of base mismatch. Although these conserved sequences have been mapped in the HPV-2a, HPV-4 (see Fig. 4 and 5), and HPV-1a genomes (18), the relationship of these conserved sequences to specific genes or functional regions of the viral genome is presently unknown.

The value of recombinant DNA technology for continued studies on papilloma viruses is evident. It is now possible to generate sufficient quantities of standardized biological reagents for comparative studies as well as for the detailed classification and characterization of this virus group. Such reagents may also be used as molecular probes for studies of gene expression both in vivo and in vitro. More important, studies on the biological activity of the viral DNAs may now be systematically approached. Preliminary results of experiments assaying the ability of the HPV-1, -2, and -4 DNAs to transform rodent cell cultures are thus far negative. The oncogenic potential of these cloned HPV DNAs directly in animals is currently being examined. Nine months after injection of the cloned viral DNAs into newborn Syrian hamsters, no palpable tumors have been detected. These results contrast sharply with the transforming ability of molecularly cloned BPV-1 and -2 DNAs in NIH 3T3 cells and C127 cells (12, 19a).

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