Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4437–4441, July 1982
Medical Sciences

Isolation of a human papillomavirus from a patient with epidermodysplasia verruciformis: Presence of related viral DNA genomes in human urogenital tumors

(urogenital cancer/vulva carcinoma/hybridization)

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Communicated by Robert J. Huebner, April 22, 1982

ABSTRACT **The DNA genome of a human papillomavirus (HPV), tentatively designated HPV-EV, was molecularly cloned from hand and leg lesions of a patient with epidermodysplasia verruciformis, a chronic skin disease associated with a 30% risk of developing cancer. Using stringent hybridization conditions, we observed <5% homology between HPV-EV and the cloned genomes of HPV-1, HPV-4, HPV-5, and HPV-5a. HPV-EV DNA showed ≈6% homology with HPV-2 and 36% homology with HPV-3. These data suggest that HPV-EV is partially related to HPV-3. Using ^{32}P-labeled cloned HPV-EV as probe in Southern blot hybridization experiments, we detected HPV-EV-related DNA in the carcinoma *in situ* (Bowenoid lesion) of the vulva of the patient from which HPV-EV was isolated. HPV-EV-related DNA was detected in 2 of 10 vulva carcinomas and in 2 of 31 cervical carcinomas. Related DNA sequences were found in papillomas from each of two patients with condyloma acuminata (anogenital warts), which is of interest considering that condylomas have been reported to convert occasionally to carcinomas. The positive vulva DNAs were also probed with other cloned HPV DNAs: HPV-1-, HPV-4-, and HPV-5a-related sequences were not detected; HPV-3 and HPV-2 DNA probes detected strong and weak DNA bands, respectively, of the same size as found with HPV-EV. The HPV DNA sequences were present in the positive tumors mainly as free viral DNA molecules; no evidence for integration into cellular DNA was found. The emerging biological picture with papillomaviruses is that cells transformed by these viruses are maintained in a transformed state by free episomal genomes. Thus, our findings are consistent with the idea, but by no means establish, that HPVs play a role in human cancer by a similar mechanism.**

Very little is known about the human papillomaviruses (HPVs) because they do not grow readily in cell culture. There are five recognized human papillomaviruses, HPV-1 to HPV-5, whose circular superhelical DNA genomes (M_r, ≈5.0×10^6) differ considerably, as shown by molecular hybridization (1). HPV-1 and HPV-4 are associated with plantar warts, HPV-2 is associated with common warts, HPV-3 is associated with multiple flat warts, and HPV-3 and HPV-5 are associated with cutaneous lesions from patients with epidermodysplasia verruciformis (EV). Several additional HPVs have been associated with condyloma acuminata (anogenital warts) (2, 3) and with cutaneous warts of meathandlers (4).

HPVs are excellent candidates as etiological agents of cancer in man for several reasons (3, 5). First, HPVs are ubiquitous and epitheliotrophic, which is important considering that most human cancers are malignant growths made up of epithelial cells—i.e., carcinomas. Second, HPVs are the only viruses proven to induce tumors (papillomas or warts) naturally in man and papillomas have been reported to progress occasionally to malignancy. Most interesting is the occurrence of carcinomas in about one-third of patients with EV (6). Third, animal papillomaviruses are natural causes of cancer. Shope papillomavirus induces skin warts in cottontail rabbits that transform into squamous cell carcinomas in ≈25% of animals (7). Bovine papillomaviruses induce upper alimentary tract papillomas that convert to carcinomas in cattle fed with bracken fern (8). Bovine papillomaviruses also appear to be involved in the etiology of equine connective tissue tumors (9).

In a previous study, we analyzed ≈150 human tumors for the presence of HPV-1 and HPV-2 DNA sequences by molecular hybridization using as probes viral DNAs isolated from patients with plantar warts and common warts, respectively (10). No HPV-1- or HPV-2-specific DNA sequences were detected. The cancer types analyzed represented ≈50% of the cancer incidence in the United States. Our data are strong evidence that none of the cancers examined were induced by HPV-1 or HPV-2. However, these results are not relevant to the possible role of other HPVs in the etiology of human cancers—for example, HPV-3, HPV-4, HPV-5, and the as-yet incompletely characterized HPVs.

In the present report, we describe the isolation of a HPV (HPV-EV) from the skin lesions of a patient with EV. We detected HPV-EV DNA sequences in the carcinoma *in situ* of the vulva of the same patient. Using HPV-EV DNA as probe, we detected related DNA sequences in cancers of the urogenital tract of four patients. Since most of the HPV DNA sequences appeared to be present in a free state (i.e., not integrated into the cellular DNA of the tumors), it seems possible that the HPV sequences represent passenger viruses. On the other hand, considering the ability of bovine papillomaviruses to maintain the phenotype of the transformed cell while replicating as an episome (for example, see ref. 11), it is tantalizing to consider the possibility that human papillomaviruses may induce cancer by a "nonintegrative" mechanism.

MATERIALS AND METHODS

Isolation and Cloning of HPV-EV DNA. Skin shavings from multiple lesions of the hand and leg of patient B.S., who was afflicted with EV, were separately homogenized as 10% suspensions in TE buffer (10 mM Tris·HCl, pH 8.1/1 mM EDTA),

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Abbreviations: HPV, human papillomavirus; EV, epidermodysplasia verruciformis.

and the homogenate was clarified by brief centrifugation and then centrifuged to equilibrium in CsCl solution [density (ρ), $\approx$1.34] using a Beckman SW 50.1 rotor at 40,000 rpm for 24 hr at 4°C. The upper ($\rho \approx 1.33$) and lower ($\rho \approx 1.37$) virus-containing bands were collected and diluted 1:10 with TE buffer, and the virus was pelleted by centrifugation at 40,000 rpm in a Beckman SW 50.1 for 90 min and resuspended in TE buffer. HPV DNA was isolated as follows. To each virus suspension (200 μl) was added 10 μg of yeast tRNA (Sigma; type XI) and $NaDodSO_4$ to 0.5%, and these suspensions were incubated at 37°C for 60 min with proteinase K at 500 μg/ml. The digests were extracted twice with phenol and twice with water-saturated ether, and viral DNA was precipitated with 2 vol of ethanol at −20°C. The DNA precipitate was centrifuged for 5 min in an Eppendorf centrifuge and dissolved in 50 μl of TE buffer. Five-microliter aliquots of each preparation were subjected to electrophoresis in 0.8% agarose. Staining with ethidium bromide revealed bands of $\approx$20 ng of form I and form II HPV DNA from the upper CsCl bands isolated from the hand and leg lesions; one-half to one-fifth as much viral DNA was obtained from the lower bands.

To clone HPV-EV DNA, $\approx$30 ng of each HPV DNA was mixed with 2.3 ng of pBR322 DNA and digested with 4 units of *Bam*HI endonuclease for 30 min at 37°C, and the DNAs were ethanol precipitated and ligated with 0.25 units of T4 ligase at 15°C for 15 hr. The ligation mixture was used to transform *Escherichia coli* HB-101. Ampicillin-resistant tetracycline-sensitive colonies were isolated and shown to contain an HPV-EV DNA insert of M_r 3.0×10^6 or 2.0×10^6.

***In Vitro* Labeling of HPV DNAs.** Cloned HPV DNAs were labeled *in vitro* by nick-translation using [^{32}P]dCTP as labeled substrate to specific activities of 1–3 $\times$ 10^8 cpm/μg (12). HPV-1, HPV-2, HPV-3, and HPV-4 DNAs were cloned in our laboratory (unpublished). Cloned HPV-5a and HPV-5 DNAs were kindly provided by G. Orth and A. J. Faras, respectively. As shown by DNA hybridization measurements (unpublished), our cloned HPV-1, HPV-2, and HPV-4 DNAs are closely related (>80% homology) to the cloned HPV-1, HPV-2, and HPV-4 DNA isolates, respectively, recently described by Heilman *et al.* (13) and kindly provided to us by P. Howley. Our cloned HPV-3 is closely related to the cloned HPV-3 DNA isolate kindly provided to us by A. J. Faras. HPV DNAs were excised from recombinant preparations by digestion with *Bam*HI (pBR322 with HPV-1, HPV-3, HPV-4, HPV-5, HPV-5a, and HPV-EV) or *Eco*RI (pBR325 with HPV-2) and then separated by electrophoresis on agarose gels from linearized plasmid and eluted from the gels as described (14).

Hybridization Analysis of HPV DNAs. For hydroxylapatite analysis, 1,000 cpm of labeled HPV DNAs excised from the cloned pBR/HPV recombinant (HPV-1 to HPV-5, HPV-5a, and HPV-EV) was hybridized with 250 ng each of unlabeled cloned HPV-EV DNA fragments (M_r 3.0×10^6 and 2.0×10^6) in 20 μl of 0.45 M NaCl/0.045 M Na citrate/40% formamide for 4 hr at 37°C; the extent of hybridization was analyzed by chromatography as described (15). For nuclease S1 analysis, 1,000 cpm of labeled HPV DNA probe was hybridized with 100 ng of unlabeled HPV DNA in 20 μl of 750 mM NaCl/10 mM Pipes, pH 6.7/1 mM EDTA/0.05% $NaDodSO_4$ containing 2 μg of sonicated denatured calf thymus DNA for 2 hr at 68°C (15).

Isolation of Human Tumor DNA. Human tumors were provided by the Department of Obstetrics and Gynecology of the St. Louis University Medical Center and by J. Gruber of the Office of Program Resources and Logistics, National Cancer Institute, and were maintained in liquid nitrogen. High molecular weight DNA was extracted from the tumors as described (12).

Southern Blot Analysis of Tumor DNA for HPV-Specific DNA Sequences. Ten-microgram samples of tumor DNA were digested with appropriate restriction enzymes, and the digests were subjected to electrophoresis on 0.8% agarose gels, transferred to nitrocellulose, and hybridized with labeled HPV DNA probes by a modification of the method of Southern (16, 17).

RESULTS

Isolation of HPV-EV DNA from Hand and Leg Lesions of a Patient with EV. Patient B.S. is a 34-yr-old female with a history of recalcitrant verrucous lesions of her bilateral lower extremities since the age of 6 and a 2-yr history of verrucous lesions on the dorsal aspects of both hands. Her clinical presentation is consistent with epidermodysplasia verruciformis of Lewandowsky and Lutz (18). In 1972, she underwent a hysterectomy for carcinoma *in situ* of the cervix. Bowenoid lesions (carcinoma *in situ*) were first noted on the vulva in 1977; these were treated by surgical excision, efudex cream, and a laser. A small portion of the vulva tumor was available for DNA extraction and Southern blot analysis.

Virus was isolated from the hand and leg lesions of patient B.S. and viral DNA was extracted. As shown by agarose gel electrophoresis, distinct bands representing typical form I (superhelix) and form II (nicked circle) DNA molecules of the appropriate size for human papillomavirus genomes (M_r, $\approx 5.0 \times 10^6$) were isolated from both the hand and leg lesions (Fig. 1).

Molecular Cloning of HPV-EV DNA. HPV-EV DNA preparations isolated from the hand and leg lesions were digested with *Bam*HI and cloned into the *Bam*HI site of pBR322. We expected that recombinants would contain an intact HPV DNA insert of $M_r \approx 5.0 \times 10^6$ because the prototype strains of HPV-

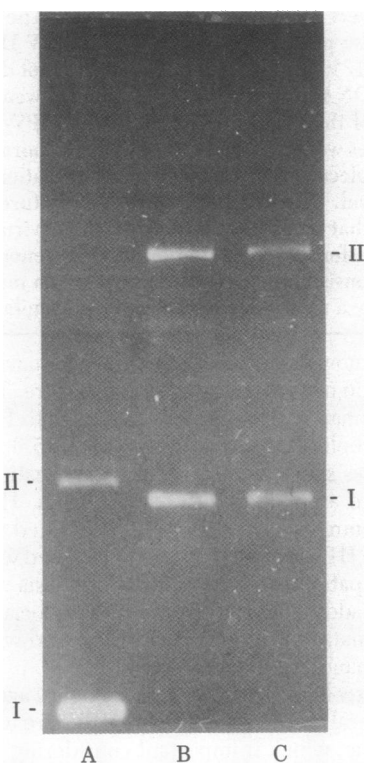

FIG. 1. Agarose gel electrophoresis of HPV-EV DNAs isolated from patient B.S. Viral DNAs were isolated from hand (lane C) and leg (lane B) lesions and subjected to electrophoresis on agarose gels. Lane A: pBR322 DNA. I, form I DNA; II, form II DNA.

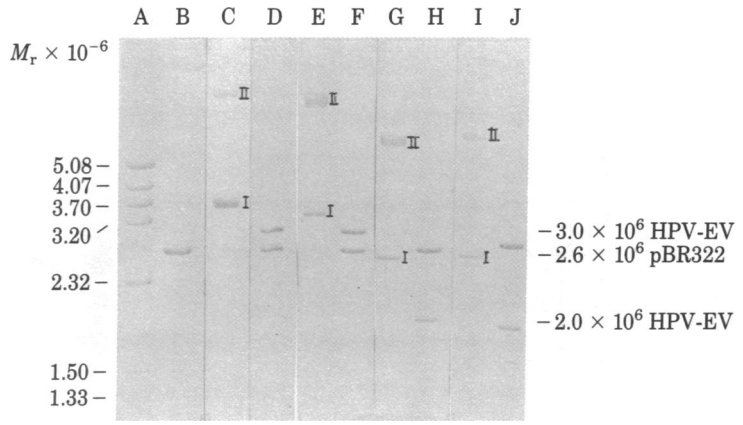

FIG. 2. Agarose gel electrophoresis of pBR322/HPV-EV DNA cloned from viral DNAs isolated from hand (lanes C, D, G, and H) and leg (lanes E, F, I, and J) lesions of patient B.S. Representative clones of pBR322/HPV-EV containing HPV-EV inserts of M_r 3.0 × 10^6 (lanes C–F) or M_r 2.0 × 10^6 (lanes G–J) were subjected to electrophoresis without digestion (lanes C, E, G, and I) or after digestion with *Bam*HI (lanes D, F, H, and J). An adenovirus serotype 2 DNA *Kpn* I digest (lane A) and *Bam*HI linearized pBR322 (lane B) were included as M_r markers. I, form I DNA; II, form II DNA.

3 and HPV-5, which are associated with EV, usually have a single *Bam*HI site. Instead, we found that each clone contained an insert of M_r 2.0 × 10^6 or 3.0 × 10^6. Agarose gel electrophoretograms of typical pBR322/HPV-EV recombinants are shown in Fig. 2. When cleaved with *Bam*HI, form I and form II molecules yielded linear pBR322 and HPV-EV DNA of M_r 3.0 × 10^6 or 2.0 × 10^6. We conclude that HPV-EV has two *Bam*HI sites and thus differs from the HPV-3 and HPV-5 prototypes (6).

Relationship of HPV-EV to Other HPVs. Liquid hybridization analyses were carried out under stringent hybridization conditions (melting temperature −28°C) to establish the relationship between HPV-EV and cloned HPV-1, HPV-2, HPV-3, HPV-4, HPV-5, and HPV-5a DNAs. As shown in Table 1, no significant hybridization (<5%) was detected between the HPV-EV M_r 2.0 × 10^6 and 3.0 × 10^6 fragments and HPV-1, HPV-4, HPV-5, and HPV-5a. HPV-2 DNA hybridized ≈3% to the HPV-EV M_r 2.0 × 10^6 and 3.0 × 10^6 fragments. HPV-3 DNA hybridized 23% to the M_r 3.0 × 10^6 fragment and 13% to the M_r 2.0 × 10^6 fragment using stringent nuclease S1 analysis. Thus, HPV-EV shows ≈36% (23% plus 13%) homology to HPV-3.

Southern Blot Analysis of Human Tumor DNAs by Using Cloned HPV-EV M_r 3.0 × 10^6 and 2.0 × 10^6 Fragments as Probes. A mixture of ^{32}P-labeled HPV-EV M_r 3.0 × 10^6 and 2.0 × 10^6 pBR322 recombinant plasmids was used initially as probe to analyze DNA extracted from the vulva tumor of patient B.S. and from other female urogenital tumors. As shown in Fig. 3, 0.2 and 1.0 copy equivalents of HPV-EV DNA per cell were detected in reconstruction experiments, ensuring excellent sensitivity for cancer analysis. Furthermore, DNA bands of M_r 5.0 × 10^6 were detected readily in two anogenital warts (condyloma acuminata) (samples K150D and K150E; Fig. 3); this suggests that a virus related to HPV-EV but containing only a single *Bam*HI site is present in these papillomas.

Table 1. Hybridization of ^{32}P-labeled HPV-1, HPV-2, HPV-3, HPV-4, HPV-5, and HPV-5a DNAs with the M_r 3.0 × 10^6 and 2.0 × 10^6 DNA fragments of HPV-EV

HPV-EV fragment	% hybridization with labeled DNA probe						
	HPV-1	HPV-2	HPV-3	HPV-3*	HPV-4	HPV-5*	HPV-5a
M_r 3.0 × 10^6	0	2.6	30.3	22.8	0	0	0
M_r 2.0 × 10^6	0	3.5	14.8	12.8	0	1	1

Results are averages of two experiments analyzed by hydroxylapatite chromatography and are corrected for background hybridization (2–4%) and normalized to maximum hybridization with homologous DNA (80–95%).

* These data were obtained by more stringent nuclease S1 analysis and are corrected for background hybridization (3–6%) and normalized to maximum hybridization with homologous DNA (75–95%).

The vulva tumor removed from patient B.S. contained the HPV-EV M_r 2.0 × 10^6 DNA fragment (Fig. 3) but not the M_r 3.0 × 10^6 DNA fragment that would be expected if free HPV-EV DNA were present and cleaved twice by *Bam*HI. It is possible that the sequences comprising the M_r 3.0 × 10^6 fragment were integrated into cellular DNA and that these were masked in the high background smear at the top of the blot. The analysis was repeated using a new blot with identical results. Further analyses were not possible because of the limited quantity of tumor DNA.

To evaluate whether other urogenital cancers might contain viral sequences related to HPV-EV, we analyzed DNAs extracted from 5 cancers of the endometrium, 31 cancers of the cervix, and 10 cancers of the vulva. Two of 31 cervical tumor DNAs contained bands of M_r 5.0 × 10^6 that hybridized with the HPV-EV probe; one of the positive (H110K) and two of the negative samples are shown in Fig. 3. Two of the 10 vulva tumors had hybridization bands of M_r 5.0 × 10^6 (samples H115S and J123H). Blots of the two positive vulva DNAs, two negative vulva DNAs, and a second extraction of the same tumor used for sample H115S (K150A) are shown in Fig. 3. The detection of a M_r 5.0 × 10^6 DNA fragment in each case suggests that free unintegrated HPV DNA is present in each tumor. This HPV DNA appears to have a single *Bam*HI restriction site whereas HPV-EV has two *Bam*HI sites. No HPV-EV-related DNA sequences were found in the endometrial tumors (Fig. 3).

Southern Blot Hybridization Analysis of the Two Positive Vulva Carcinoma DNAs with DNAs of Different HPVs. To obtain evidence as to which HPV type was present in the tumors, blots of the two positive vulva tumor DNAs (samples H115S and J123H in Fig. 3) were probed with ^{32}P-labeled DNAs of cloned HPV-1, HPV-2, HPV-3, HPV-4, and HPV-5a, as well as with the individual M_r 3.0 × 10^6 and 2.0 × 10^6 fragments of HPV-EV. The results of Southern blot analysis with the different HPV DNA probes is shown in Fig. 4. Both vulva tumor DNAs yielded a prominent hybridization band of M_r 5.0

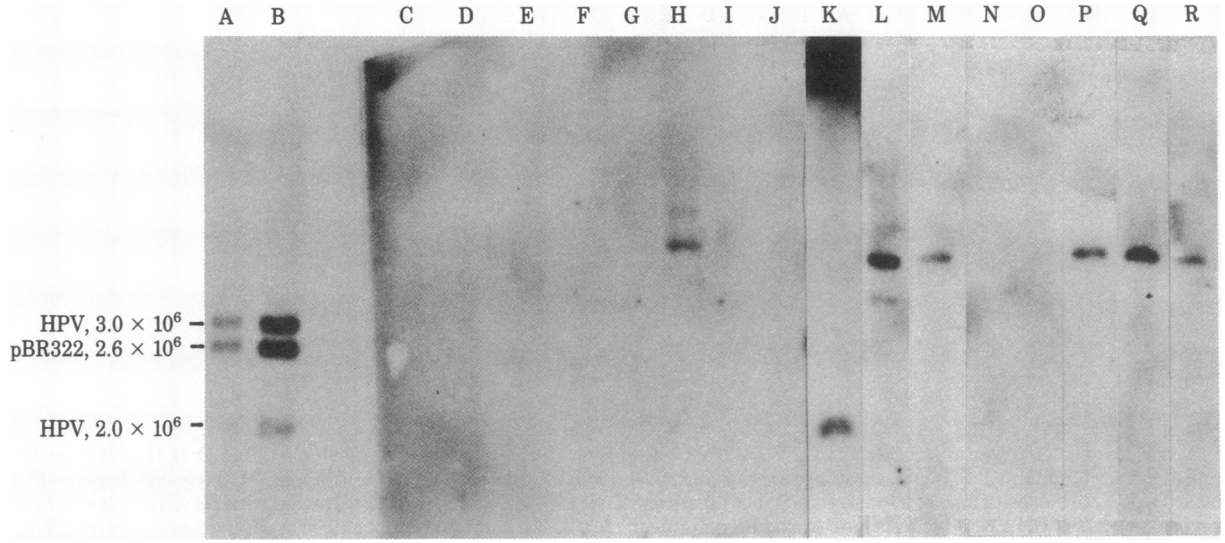

FIG. 3. Detection of HPV-EV-related DNA sequences in human urogenital carcinomas and benign condylomas. Total high M_r DNAs extracted from benign condylomas, urogenital cancers, and the carcinoma *in situ* of patient B.S. were digested with *Bam*HI, subjected to electrophoresis on agarose gels, transferred to nitrocellulose paper, and hybridized to an equimolar mixture of ^{32}P-labeled pBR322/HPV-EV DNAs containing the M_r 3.0 × 10^6 and 2.0 × 10^6 fragments. The HPV copy control (lane A, 0.2 equivalents; lane B, 1.0 equivalents) consisted of an equimolar mixture of *Bam*HI-digested pBR322/HPV-EV DNAs containing the M_r 3.0 × 10^6 and 2.0 × 10^6 fragments. Lanes: C–G, endometrial tumor DNAs J125M, J120D, J126M, J126N, and J133T, respectively; H–J, cervical tumor DNAs H110K, H110J, and H110L, respectively; K, patient B.S. vulva tumor DNA; M–P, vulva tumor DNAs H115S, K150B, K150C, and J123H, respectively; Q and R, condyloma acuminata DNAs K150D and K150E, respectively.

× 10^6 when analyzed with the HPV-EV M_r 3.0 × 10^6 or 2.0 × 10^6 probes (the copy controls show the expected M_r 3.0 × 10^6 or 2.0 × 10^6 bands, as well as a M_r 2.6 × 10^6 pBR322 DNA band, since *Bam*HI-digested pBR322/HPV-EV was used as both probe and copy control). Of interest, the M_r 2.0 × 10^6 probe consistently detected in sample H115S (in addition to the M_r 5.0 × 10^6 band) a M_r ≈4 × 10^6 DNA fragment. We do not know the origin of this fragment. Conceivably, it could represent a subgenomic fragment of HPV DNA integrated into cellular DNA. No hybridization bands were detected by using HPV-1, HPV-4, or HPV-5a DNAs as probe. However, the major M_r 5.0 × 10^6 band was detected in both tumor DNAs by using HPV-3 DNA as probe; the M_r ≈4 × 10^6 band was also detected in H115S. In addition, faint but distinct bands of M_r 5.0 × 10^6 were detected in both tumor DNAs by using HPV-2 as probe. These data are consistent with the cross-hybridization studies in Table 1, which show partial homology of HPV-EV with HPV-3 and lesser homology with HPV-2.

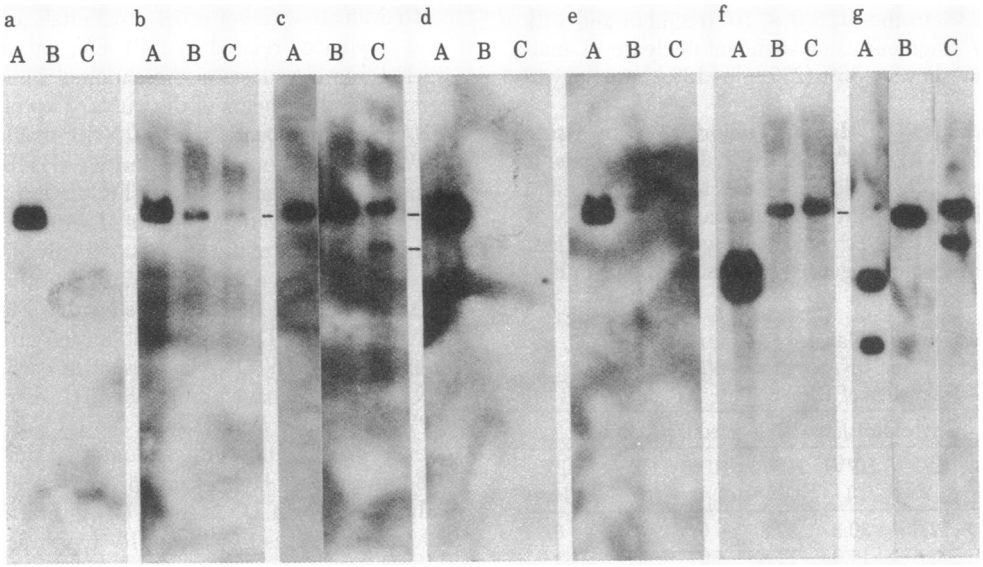

FIG. 4. Hybridization analyses of the DNAs of two positive vulva carcinomas with ^{32}P-labeled cloned DNAs of HPV-1 (*a*), HPV-2 (*b*), HPV-3 (*c*), HPV-4 (*d*), HPV-5a (*e*), and the M_r 3.0 × 10^6 (*f*) and 2.0 × 10^6 (*g*) DNA fragments of cloned HPV-EV. All of the radioactive DNA probes and copy controls except HPV-EV DNA consisted of HPV DNAs excised from the pBR/HPV recombinant and purified; thus, each copy control for HPV-EV contains the pBR322 M_r 2.6 × 10^6 band in addition to the HPV-EV band of M_r 3.0 × 10^6 or 2.0 × 10^6. Lanes: A, copy controls containing the equivalent of 5 copies per cell of linearized viral DNA excised from the pBR322/HPV recombinant with *Bam*HI (HPV-1, HPV-3, HPV-4, HPV-5a, and HPV-EV) or *Eco*RI (HPV-2); B, vulva tumor DNA sample J123H; C, vulva tumor DNA sample H115S. Southern blot analyses were performed using *Bam*HI-digested tumor DNA as described in Fig. 3.

DISCUSSION

In this communication, we describe the isolation and molecular cloning of a HPV genome, tentatively called HPV-EV, from a patient with EV. Cross-hybridization analysis, using stringent hybridization conditions, with cloned DNAs of prototype HPV-1 to HPV-5 and HPV-5a established that HPV-EV is a new virus that is partially related to HPV-3. Using labeled HPV-EV DNA as probe, we showed that HPV-EV related DNA sequences were present in a carcinoma *in situ* of the vulva that arose at age 31 *de novo* in the patient from whom HPV-EV was isolated; the patient first developed EV at age 6. Subsequently, we showed that HPV-EV related DNA sequences were present in two vulva carcinomas and two cervical carcinomas. In addition, we found HPV-EV related sequences in each of two condylomas (anogenital warts) that we analyzed.

The viral DNA genomes that we detected in the vulva and cervical carcinomas and in the benign condylomas do not appear to be that of HPV-EV, since these DNAs possess only one *Bam*HI cleavage site whereas HPV-EV has two *Bam*HI cleavage sites. Thus, a related virus, perhaps HPV-3 or a strain of HPV-EV that has a single *Bam*HI cleavage site, appears to be present in these six tumors. Until recently, it was not possible to detect HPV DNA in condylomas. Two recent reports present evidence for the presence in condylomas of HPVs unrelated to HPV-1 and HPV-2; further characterization of these HPVs was not presented (2, 3).

Our findings thus demonstrate the presence of HPV sequences in human urogenital cancers. We emphasize that these findings do not establish a causal relationship between the HPV sequences and the urogenital cancers; for example, HPVs could be passenger viruses. However, there are several reasons to think that a causal relationship may exist. Studies with papillomavirus-induced carcinomas in animals and the occasional conversion of human papillomas to cancer were discussed previously. Most interesting, human urogenital cancer displays the epidemiology of a venereal disease (5). Urogenital condylomas display a similar epidemiology. Our findings of similar HPV-specific sequences in benign condylomas and carcinomas of the vulva and cervix are of additional interest because of reports that vulva condylomas account for up to 5% of carcinomas of the vulva (19) and that cervical squamous cell papillomas occasionally convert to carcinomas (20).

In contrast to other DNA tumor viruses, it appears that papillomavirus genomes exist mainly in an episomal rather than an integrated state in bovine papillomavirus-transformed cells (11, 21–23). Thus our finding that the HPV genomes present in urogenital carcinomas appear to exist mainly as multiple copies of free viral DNA molecules is consistent with the possible involvement of these viruses in the malignant process. We point out, however, that although we can detect as few as 0.2 copies of HPV DNA per cell in reconstruction experiments, it is possible that 1 copy per cell of integrated viral DNA would remain undetected due to the unknown degree of contamination of tumor tissue with normal cells. In addition, we have not as yet ruled out the possibility that HPV DNA is integrated as tandemly repeated copies. Further support for a role of HPVs in cancer is provided in the recent reports that HPV-5 genomes isolated from the benign warts of two patients with EV were present as free viral DNA in squamous cell carcinomas of the same two patients (24). In a similar brief report, HPV-5b-related sequences were found in a squamous cell carcinoma of an EV patient (6).

We thank Drs. W. S. M. Wold, D. P. Grandgenett, and G. Chinnadurai for critical reading of the manuscript and C. E. Mulhall for editorial assistance. This work was supported by National Cancer Institute Grant 1 RO1 CA-28689, Specialized Cancer Center (CORE) Grant 5 P30 25843, and Contract NO1 CP 4-3359. M.G. is the recipient of Research Career Award 5 KO6 AI-04739 from the National Institute of Allergy and Infectious Diseases.

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