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Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis

(Southern blot hybridization/metastasis/warts)

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ABSTRACT **DNA extracted from squamous cell carcinomas from patients with the chronic wart disease syndrome, epidermodysplasia verruciformis, was analyzed for the presence of human papillomavirus (HPV)-specific DNA sequences by Southern blot hybridization analysis. Employing an HPV probe obtained by molecular cloning of viral DNA purified from benign warts from these patients, we have unequivocally identified HPV-specific nucleotide sequences in squamous cell carcinomas from these patients. Restriction endonuclease mapping indicated that the DNA present in the carcinomas was of the same type (type 5) as that found in the benign tumors from these patients and was present as unintegrated, free viral DNA. Moreover, we have demonstrated the presence of HPV-5 DNA in a subcutaneous metastatic tumor from one of these patients. This latter observation essentially eliminates the possibility that the HPV-5 DNA present in the malignant tumors in these patients resulted from cross-contamination from an adjacent benign warty lesion. In addition to wild-type HPV-5 DNA, both the primary and metastatic carcinomas analyzed also contained an HPV-5 DNA species lacking approximately 20% of the HPV-5 DNA genome. These subgenomic forms of HPV-5 DNA could not be detected in benign papillomas from these patients.**

It has been known for some time that papillomaviruses cause benign warty tumors in a number of animal species, including humans. Moreover, in several animal systems papillomaviruses, or components thereof, have been identified associated with malignant tumors occupying similar anatomical sites and suspected of arising from papillomas (1–6). Although papillomaviruses clearly cause benign wart disease in man (2, 7), their involvement or even association with human malignant disease is presently unknown. Certain chronic wart disease syndromes have been known to exhibit a propensity towards malignancy (2, 7, 8) and there has been considerable interest in determining whether human papillomaviruses are, at the very least, associated with, and quite possibly involved in the development of, malignant tumors in these patients. In an effort to resolve this issue we have begun studies on a group of patients exhibiting the wart disease syndrome, epidermodysplasia verruciformis (EV), which is a rare, familial disease characterized by a life-long progression of cutaneous, wart-like lesions to the malignant phenotype (5, 7). This specific wart disease syndrome is of particular interest because of the high incidence (ca. 25%) of patients displaying transition from benign papillomas to frank, multiple cutaneous carcinomas (2, 7, 9).

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Although human papillomavirus (HPV) has been demonstrated to be associated with benign papillomas from patients with EV, no virus has been detected in carcinomas suspected to have arisen from wart tissue in these patients (9-12). Recent studies have in fact demonstrated that benign lesions from EV patients contain two types of HPV, type 3 and type 5 (13). Whereas HPV 3 appears to be present exclusively in those EV patients that do not exhibit malignant conversion, HPV-5 is associated with warts from EV patients that invariably develop carcinomas (13). In addition to a specific type of HPV, other factors appear involved in the development of carcinomas in these patients. For instance, because carcinomas develop on sun-exposed areas of the skin, it has been postulated that ultraviolet light is in part responsible for the transition from papillomas to carcinomas (14). Moreover, many patients exhibit defective cell-mediated immunity (13, 15, 16), which may play a role in their disposition to viral infection and susceptibility to carcinoma.

Because of the lack of apparent virus in carcinomas from patients with EV and our previous success in identifying HPV DNA in papillomas in which no intact virus could be detected (17, 18), we have initiated studies to determine whether HPV-specific DNA can be demonstrated in carcinoma tissue from these patients exhibiting this particular wart disease syndrome. In this communication we demonstrate the presence of HPV-5-specific DNA in cutaneous squamous cell carcinomas in several patients with EV. Moreover, we have demonstrated the presence of HPV-5 DNA in a metastatic lesion, thereby eliminating the possibility that superficial cutaneous carcinoma tissues may be contaminated by virus from adjacent benign papillomas.

MATERIALS AND METHODS

DNA Preparation. Tissue was minced and suspended in 0.2 M Tris·HCl, pH 8.5/0.1 M EDTA/1% $NaDodSO_4$/containing Pronase at 500 μg/ml and incubated at 60°C for 2–4 hr. Diethyl oxidiformate (Eastman) was added to 0.4% and incubation was continued at 60°C for 30 min. The solution was cooled and potassium acetate was added to 1.43 M. After 30 min on ice, the solution was centrifuged for 20 min at 20,000 × *g* at 4°C. The supernatant was concentrated by addition of 2 vol of ethanol. The precipitate was resuspended in 20 mM Tris·HCl, pH 7.0/10 mM EDTA and treated with boiled RNase A at 100 μg/ml followed by incubation with $NaDodSO_4$/Pronase, extraction

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

with phenol/chloroform, 1:1 (vol/vol), and precipitation with ethanol.

Virus Purification. Papilloma tissue was frozen at −70°C, crushed, and ground in a mortar and pestle in 5–10 vol of 50 mM potassium phosphate, pH 6.8/50 mM NaCl containing silicon carbide powder. The 12,000 × *g* supernatant was adjusted to 10 mM $MgCl_2$ and centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 90 min at 4°C to pellet virus. Virus was then banded by CsCl equilibrium centrifugation. DNA was extracted from purified virus by phenol/chloroform after $NaDodSO_4$/Pronase treatment.

Agarose Gel Electrophoresis. HPV DNA samples, or restriction endonuclease fragments thereof (18), in buffer containing 5% (vol/vol) glycerol, 89 mM Tris borate (pH 8.3), 2.5 mM EDTA, and 1% $NaDodSO_4$ were heated at 68°C for 10 min and electrophoresed in 0.9% agarose (Seakem Laboratories, Rockland, ME) at 60 V for 20 hr. Gels were stained with ethidium bromide at 1 μg/ml and destained in water.

DNA fragments were removed from the gel by placing agarose gel slices in acetylated dialysis tubing filled with electrophoresis buffer and electrophoresing the sample for 2 hr at 100 mA. The electric field was reversed for 30 seconds and the solution was loaded onto a DE-52 (Whatman) DEAE-cellulose column in 250 mM NaCl/50 mM sodium acetate, pH 5.0, and washed with the same buffer. DNA was eluted with 1 M NaCl/0.2 M KCl/50 mM sodium acetate, pH 5.0, and concentrated with ethanol.

Molecular Cloning of HPV-5. HPV-5 viral DNA (800 ng) was cleaved at its unique *Bam*HI site and inserted into the *Bam*HI site of pBR322. This was accomplished by treating the cleaved vector DNA with bacterial alkaline phosphatase for 30 min at 65°C, followed by extraction with phenol and ether. Cleaved HPV-5 DNA and plasmid DNA were then mixed and treated with 0.25 unit of phage T4 ligase (Bethesda Research Laboratories) in 66 mM Tris·HCl, pH 7.6/7.5 mM $MgCl_2$/0.4 mM ATP/10 mM dithiothreitol at 4°C for 16 hr. Ligated DNA was used to transform *Escherichia coli* K-12 LE392 (19). Clones containing HPV-5 inserts were then detected by a modification of a previously described filter hybridization technique that allowed greater sensitivity and gave a lower background (20). Colonies on tetracyline-containing agar plates were replicated onto a piece of sterile Whatman no. 1 paper on an agar plate. After overnight growth, the paper was treated successively for 7 min each with 0.5 M NaOH; 1 M Tris·HCl (pH 7.4); 1 M Tris·HCl (pH 7.4); 0.5 M Tris·HCl (pH 7.4); 0.3 M NaCl/0.03 M sodium citrate; and finally ethanol. The papers were then baked *in vacuo* 2 hr at 80°C and hybridized in 50% (wt/vol) formamide/0.75 M NaCl/0.075 M sodium citrate/yeast tRNA at 25 μg per ml/0.2% $NaDodSO_4$ and 2×10^5 cpm of HPV-5 DNA ^{32}P-labeled by nick-translation at 42°C for 18 hr. The papers were washed two times in the reaction buffer lacking labeled DNA at 42°C and two times in 0.3 M NaCl/0.03 M sodium citrate at room temperature. After drying, the papers were autoradiographed at −70°C with Cronex x-ray film, using an intensifying screen. Cloned DNA was amplified by treatment of cultures with chloramphenicol (180 μg/ml) or spectinomycin (300 μg/ml) and lysed in 25% sucrose/50 mM Tris·HCl, pH 8.1, containing lysozyme (6 mg/ml) for 15 minutes at 37°C followed by the addition of EDTA (87 mM, 10 min at 0°C) and 1% $NaDodSO_4$/1 M NaCl to precipitate high molecular weight DNA at 4°C (21). Finally, supercoiled double-stranded DNA was isolated by CsCl/propidium iodide equilib-

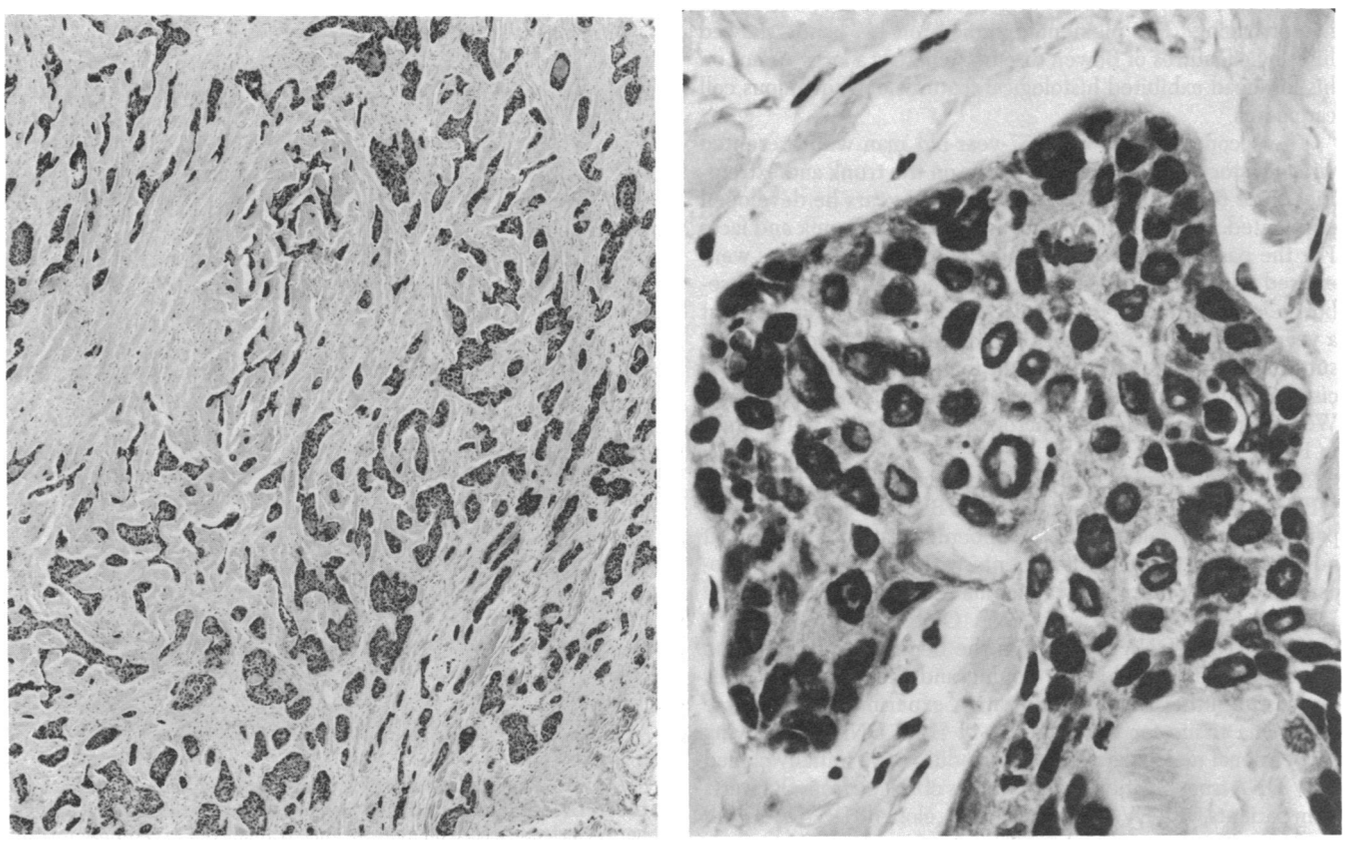

FIG. 1. Histopathology of subcutaneous femoral nodule, patient no. 2. (*Left*) Photomicrograph of subcutaneous metastatic squamous cell carcinoma. Anaplastic keratinocytes within fibrous stroma. (×40). (*Right*) Higher power (×650). Note pleomorphic nuclei, mitotic figures, dense chromatin, increased nuclear-to-cytoplasmic ratio.

rium centrifugation. Dialyzed DNA was then transferred into *E. coli* HB101 by transformation.

Filter Hybridization. DNA fragments were transferred from agarose gels to nitrocellulose filters similar to those previously described (22). After the filters had been heated at 80°C *in vacuo* for 1–2 hr, the sheets were incubated with a solution containing 0.9 M NaCl, 0.09 M sodium citrate, 10 mM EDTA, and 0.05% each of bovine serum albumin, Ficoll 400 (Pharmacia), and polyvinylpyrrolidone at 68°C for 2 hr. The filters were then incubated at 68°C for 3 hr in a solution containing 0.3 M NaCl, 0.03 M sodium citrate, 10 mM EDTA, yeast tRNA at 5 μg/ml, depurinated calf thymus DNA at 10 μg/ml, and 0.02% each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolidone. The filters were then dried under a heat lamp and incubated in a sealable plastic bag at 68°C for 24 hr in the latter solution containing, at 5 ng/ml, heat-denatured nick-translated HPV-5 [^{32}P]DNA (23) exhibiting a specific activity of 1.1×10^9 cpm/μg. Filters were washed at 68°C three times for 15 min each in a solution containing 0.3 M NaCl, 0.03 M sodium citrate, 25 mM sodium phosphate at pH 7.3, 0.1% sodium pyrophosphate, and 0.1% $NaDodSO_4$; once with a 1:2 dilution of the wash buffer for 10 min; and once with a 1:5 dilution of the wash buffer for 1 min. After drying, the filters were autoradiographed as described above.

RESULTS

Patient Case Histories. Two patients with EV that developed squamous cell carcinomas were analyzed for the presence of HPV-specific nucleotide sequences. Patient no. 1 was a 36-year-old man who began to develop wart-like lesions at the age of 9. Within the next several years his wart disease spread, encompassing his trunk, extremities, face, neck, and head. At age 30 two black tumors appeared, gradually enlarged, and began hemorrhaging. The pigmented tumors on his hands showed histologic features of Bowen disease, whereas a large tumor on his forehead exhibited histological features of a squamous cell carcinoma.

The second patient was a 57-year-old man who presented with a history of flat wart-like lesions on the trunk and extremities since 6 years of age. At the age of 18 years he developed pigmented verrucous cutaneous lesions on the trunk and face. For the past several years more than 20 of these lesions were excised and their histopathology showed features in both seborrheic keratoses and Bowen disease (carcinoma *in situ*). About a year ago the patient noted the presence of a hard enlarging subcutaneous nodule on the right femur. The nodule was excised and diagnosed as a metastatic squamous cell carcinoma. We have presented a photomicrograph of this metastatic tumor in Fig. 1.

Analysis of Benign and Primary Carcinoma Tissues for HPV-Related Nucleotide Sequences. Because complete HPV particles could be demonstrated in benign flat-wart tumors but not carcinoma tissue from patients with EV (9–12), we proceeded to analyze primary carcinoma tissue for the presence of HPV-related nucleotide sequences. Our strategy was to first obtain an HPV DNA probe from the benign papillomas from these patients by molecular cloning and then to employ this probe in Southern blot hybridization experiments to analyze carcinoma tissue for the presence of HPV DNA. Employing conventional molecular cloning procedures, we initially cloned HPV DNA from purified virus particles obtained from flat warts from patients with EV. Treatment of the cloned HPV DNA with a variety of restriction endonucleases either singly or in combination confirmed the presence of HPV type 5 in these benign lesions (24). Moreover, only HPV-5 DNA and not HPV-1, -2, -3, or -4 DNAs hybridized to the molecularly cloned HPV DNA under stringent conditions, thereby substantiating its identity as a type 5 HPV (8, 25).

Total DNA was extracted from the primary carcinoma tissues from one of these patients (patient no. 1) and tested for the presence of HPV-5-related DNA sequences (Fig. 2). Primary carcinoma tissue from this patient exhibited bands comigrating with forms I, II, and III of free viral HPV-5 DNA. In contrast to the benign warts from this patient, the primary carcinoma yielded additional bands exhibiting sequence homology to HPV-5 DNA. On the basis of the restriction endonuclease analysis of the HPV DNA from this carcinoma tissue it appears that these additional bands are subgenomic forms of HPV-5 DNA (Fig. 2, see below).

Analysis of Metastatic Squamous Cell Carcinomas for HPV-5-Related Nucleotide Sequences. One of the difficulties with analyzing cutaneous squamous cell carcinomas for HPV-related DNA sequences is the potential contamination of the carcinomas with virus or viral DNA from adjacent wart tissue. Although analysis of the carcinomas by electron microscopy may be insufficient to detect low levels of HPV contamination, procedures such as Southern blot hybridization may be sufficiently sensitive to detect potential HPV contamination and therefore contribute to difficulties concerning the interpretation of positive hybridization data with these cancers. In an effort to obviate this difficulty and unequivocally establish the association of HPV-5 DNA with carcinomas from patients with EV, we have analyzed metastatic squamous cell carcinomas for HPV-5 nucleotide sequences. The data presented in Fig. 3 demonstrate

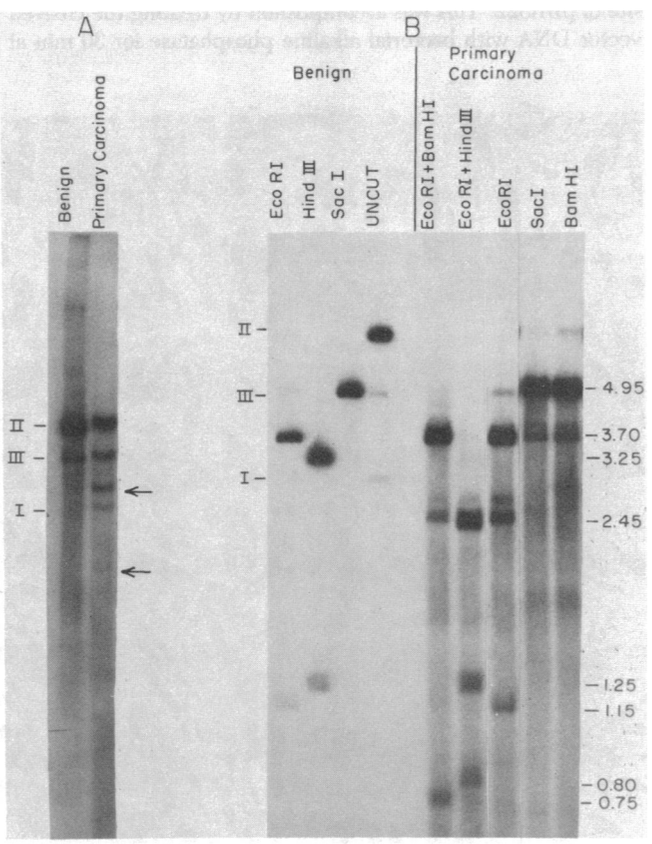

FIG. 2. Papillomavirus DNA in benign and primary carcinoma tissue. DNA was extracted from primary carcinoma tissue from patient no. 1, electrophoresed on agarose gels, transferred to cellulose nitrate paper, hybridized to labeled HPV-5 DNA, and autoradiographed. (*A*) Total cell DNA. (*B*) Restriction endonuclease-treated cell DNA. HPV DNA forms are indicated by roman numerals. The molecular masses of DNA fragments are presented in megadaltons.

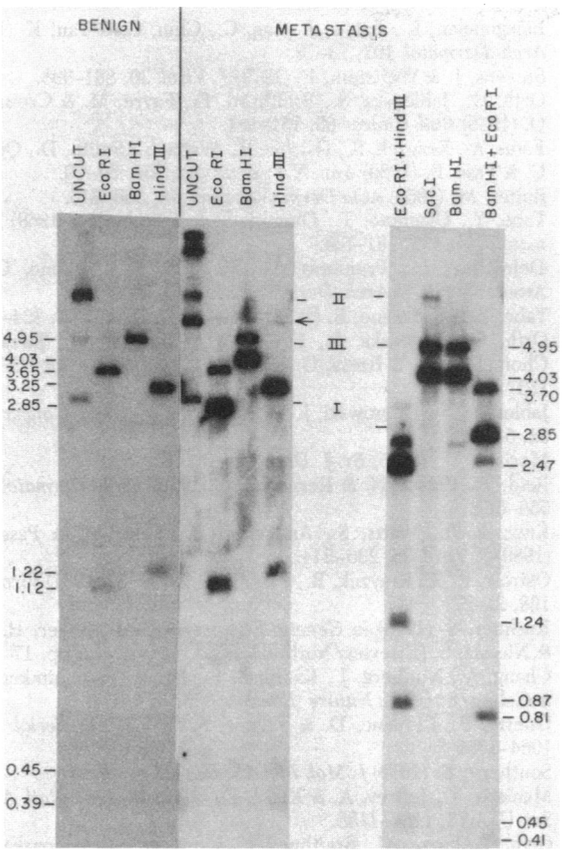

FIG. 3. Papillomavirus DNA in the metastatic carcinoma tissue. Total cell DNA from either a benign wart or a metastatic lesion from patient no. 2 was treated with restriction endonucleases, electrophoresed, transferred to cellulose nitrate paper, hybridized to a ^{32}P-labeled HPV-5 DNA probe, and autoradiographed. The smallest *Hin*dIII fragment (0.39 megadalton) of the benign tumor is just barely visible in the original print of this exposure. Arrow indicates position in uncut metastatic DNA we believe to be form II of this patient's subgenomic HPV-5 DNA. Note slower migrating bands, which may represent either integrated or concatemeric forms of HPV-5 DNA. We have occasionally observed similar bands in benign tissues but not in nearly the abundance seen in this case.

that a metastatic carcinoma from patient no. 2 contains HPV-5 DNA forms present in wart lesions from these patients as well as the subgenomic DNA forms similar to those identified in the primary cutaneous carcinomas. Moreover, restriction endonuclease analysis of the HPV-5 DNA present in the metastatic tumors indicates that it is identical to the HPV-5 DNA obtained from benign warts from these patients (Fig. 3).

Restriction Endonuclease Analysis of HPV-5 DNA from Carcinomas. The HPV DNA forms from these patients have been analyzed by restriction endonuclease treatment to determine their structure with respect to HPV-5 DNA obtained from purified virus. Both *Bam*HI and *Sac* I cleave at a single unique site in HPV DNA, resulting in a single linear form III molecule. However, treatment of the DNA forms from the metastatic carcinoma with these two restriction enzymes yields two linear forms exhibiting masses of 4.95 and 4.05 megadaltons. Further restriction endonuclease analysis indicated that the subgenomic HPV DNA exhibited a deletion of about 0.9 megadaltons (Fig. 4). Restriction endonuclease analysis of the HPV DNA from one of the primary carcinomas exhibited a similar pattern. Both *Sac* I and *Bam*HI treatment of this DNA generates two bands corresponding to the linear DNA of the viral genome and a deletion of 1.3 megadaltons (Fig. 2). *Eco*RI, which cleaves HPV-5 DNA twice, yields the normal HPV-5 DNA restriction pattern as well as an additional band. The band at 1.15 megadaltons is a doublet, whereas the extra band of 2.48 megadaltons appears to result from a deletion of 1.3 megadaltons in the HPV-5 genome (Fig. 2). A mixture of *Eco*RI and *Hin*dIII generated a pattern indistinguishable from DNA obtained from HPV virions. These data collectively indicate that essentially all of the *Hin*dIII B fragment was absent from the subgenomic species of HPV DNA present in the primary and metastatic carcinomas analyzed and that the subgenomic HPV-5 DNAs had undergone a deletion of similar but not identical size corresponding to the same region of the viral genome.

DISCUSSION

We have observed the presence of HPV-5 viral DNA in both primary cutaneous and metastatic carcinomas from two patients with EV. HPV-5 DNA was identified in these malignant tumors by hybridization under stringent conditions to an HPV-5 DNA probe obtained from benign warts and by its characteristic restriction endonuclease cleavage pattern. The studies of the metastatic carcinomas served to eliminate possible contamination of the malignant tumors with HPV-5 present in the benign tumors, a possible criticism of the observations obtained with the cutaneous carcinomas. The results of these hybridization studies establish an association of HPV-5 with carcinomas in patients exhibiting chronic wart disease that can progress to the malignant phenotype. However, many more patients will have to be analyzed in order to firmly establish this association as a rule rather than an exception.

The HPV-5 DNA present in these malignant tumors exhibited several interesting features. First, the bulk of the HPV-5 DNA appeared to be unintegrated in these cells, although integration of a single copy of the HPV-5 genome could not be conclusively ruled out by these studies. Second, a subgenomic species of HPV-5 DNA could be identified in the carcinoma tissue. Although the subgenomic species present in carcinomas from two different patients varied slightly in the size of their deletions, they represented similar nucleotide sequences, in-

FIG. 4. Restriction endonuclease map of HPV-5 DNA and its related subgenomic DNA forms. This map was obtained by treatment of both wild-type viral DNA and molecularly cloned HPV-5 DNA with one or more restriction endonucleases followed by gel electrophoresis and either ethididium bromide staining or filter hybridization. Numbers represent percent of the genome length (4.95 megadaltons) from an arbitrary origin, the unique *Sac* I site. The open box represents the approximate extent of the deletion in the HPV DNA from the cutaneous primary carcinoma of patient no. 1 and the hatched box represents the extent of the deletion of HPV-5 DNA from the subcutaneous metastatic tumor from patient no. 2.

dicating that the same region of the HPV-5 genome was affected in both isolates. It is important to note, however, that subgenomic papillomavirus DNA has not been observed to date in carcinomas in rabbits induced by Shope papillomavirus (unpublished observations). Thus the exact significance of these subgenomic forms and their involvement in transformation is presently unclear. It is entirely possible that the subgenomic HPV DNA represents aberrant forms that do not play any role in oncogenesis. Molecularly cloning the subgenomic form may allow us to obtain further information concerning its structure and biological function.

We must be cautious in our interpretation of these findings of HPV DNA associated with malignant human tumors. Even if further studies on additional patients indicate that this association is a general phenomenon, the issue remains as to the exact involvement of HPV-5 in the development of carcinomas in these patients. Extreme care must be taken to develop the appropriate experiments to conclusively establish the involvement, if any, of HPV-5 in the progression of papillomas to carcinomas in these patients. The development of an appropriate vaccine that reduces wart disease in these patients and correspondingly reduces the incidence of malignant conversion would provide support for the involvement of HPV-5 in the transformation process in these patients.

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