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Molecular Cloning and Characterization of a Unique Type of Human Papillomavirus from an Immune Deficient Patient

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Several papillomas from a single patient who exhibited an unusual immune deficiency syndrome were analyzed for the presence of specific human papillomavirus (HPV) types. Preliminary analysis indicated that the HPV DNA species present in each of these tissues was quite unlike any of the previously characterized HPV types. In order to more rigorously analyze the HPV from this patient we have isolated the HPV DNA by molecularly cloning it into a bacteriophage lambda vector and have constructed a detailed restriction endonuclease map. Comparative hybridization studies using S1 nuclease analyses showed 6% or less nucleotide sequence homology of this viral DNA with HPV types 1, 2, 3, 4, 5, 6, or an HPV-11, molecularly cloned in this laboratory. Moreover, Southern blot analyses under stringent hybridization conditions revealed little, if any, hybridization to HPV types 1, 2, 4, 5, 7, 8, 10, 11, HPV-EV isolated from a patient with epidermodysplasia verruciformis (EV), or 2 previously described HPVs (HPV-P and HPV-PW) related to HPV-3. There was, however, a very weak sequence homology detected with HPV-6 and an extremely weak homology to HPV-3. No filter hybridization was observed with the recently characterized HPVs 9 or -12 to -24. These data accumulatively indicate that the HPV species from this immunosuppressed patient represents a new, hitherto unidentified HPV type.

While only a handful of apparently benign human papillomaviruses (HPV) are found in the general population, many variants have recently been identified in patients with immune deficiencies. Some of these rare and unusual HPVs seem also to have some oncogenic potential based upon their close association with some malignant tissues. Human papillomavirus type 5 (HPV-5) has been found to be associated with both benign and malignant tumors of patients with epidermodysplasia verruciformis (EV) [1–3], as has HPV-EV in urogenital carcinomas [4], HPV-6 in invasively growing condylomata acuminata [5], and HPV-11 with verrucus carcinoma [6] (also see below). HPV-6 and HPV-11 have both been found in some cervical cancers as well [7]. HPV-5 also has the ability to transform mouse cells in vitro and form tumors in athymic nude mice.* Moreover, a large proportion of cervical and vaginal dysplasia have HPV involvement [8–10]. This evidence points strongly to an etiologic role of some HPVs in human neoplasias. While not all HPV types are associated with malignancy, the incidence of papillomas and associated malignancies is particularly marked in patients who are immunologically compromised as in those having EV [2,11,12] and renal transplant patients [13,14].† We have been involved in an ongoing study in which various tissues from patients with distinct medical histories are examined for the presence of HPV nucleotide sequences. In an effort to begin to understand the biology of these viruses, detected HPV DNAs are then characterized with respect to known HPV types. During the course of these studies, we examined 4 papillomas from a single patient with an unusual immune deficiency, multiple carcinomas, and other viral infections. We report here the molecular cloning and characterization of a novel HPV DNA isolated from this patient.

PATIENT HISTORY

Patient RCM is a 53-year-old heterosexual Caucasian male with a 2-year history of eruptive verruca vulgaris and condylomata. He also has a history of multiple squamous cell carcinomas of the face and forehead first diagnosed in 1972 and treated with x-rays from 1977 to June of 1982. Approximately in 1980 several cutaneous infections appeared. Skin examination revealed multiple erythematous, hyperkeratotic lesions on the face, forehead, and dorsa of the hands (Fig 1*a*). The lesions on the forehead were consistent with actinic keratoses. The lesions on the hands were multiple verrucae. There were also verrucae on the plantar aspect of the right foot. Groin examination revealed extensive maceration on an erythematous base, pustules, scattered erythematous papules, and hyperkeratotic plaques along the medial aspect of the upper thigh and the distal intertriginous groin area (Fig 1*b*, *c*). There was a marked amount of a purulent yellowish-green discharge around the hyperkeratotic plaques. KOH of the groin was negative on admission. There were also marked erythematous, scaly plaques on the abdomen, some with annular configuration. KOH of the annular lesions was positive for hyphae. There was maceration of the toe webs which also showed fungal hyphae on KOH exam. Over the upper back and lower abdomen were many small, umbilicated papules consistent with molluscum contagiosum. The patient does not have Kaposi's sarcoma nor had he received any blood transfusions prior to treatment for the above conditions. Biopsy specimens for molecular studies were verruca vulgaris from the plantar aspect of the right foot and the dorsal aspect of the right fourth finger, and condyloma accuminata of the right and left medial thighs.

Admission CBC revealed a hemoglobin of 9.2 g% (nl, 11.0–15.0), hematocrit of 28% (nl, 35.0–45.0), MCV of 77.9 μm^3 (nl, 75.0–85.0), and MCH of 25.6 pg (nl, 24.0–28.0). Random SMA6 with creatinine was essentially within normal limits and SMA12 revealed a slightly low protein of 5.8 g/dl (nl, 6.0–8.0), albumin of 3.4 g/dl (nl, 3.8–5.0), and a random glucose of 104 mg/dl (nl, 60–100) but was otherwise within normal limits. Reticulocyte count upon admission was 1.1 and serum iron and total iron binding capacity were markedly low, 5 and 151 μg/dl (nl, 35–178 and 254–466), respectively. Rapid plasma reagent (RPR) was not reactive. Urinalysis was within normal limits and blood cultures showed no growth. Virus cultures obtained from the intertriginous lesions of the groin were positive for herpes simplex virus. Screening for hepatitis B was negative. Serum immunoglobulin re-

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Abbreviations:
EV: epidermodysplasia verruciformis
HPV: human papillomavirus
LIF: Leukocyte inhibitory factor
nl: normal limit(s)
SDS: sodium dodecyl sulfate

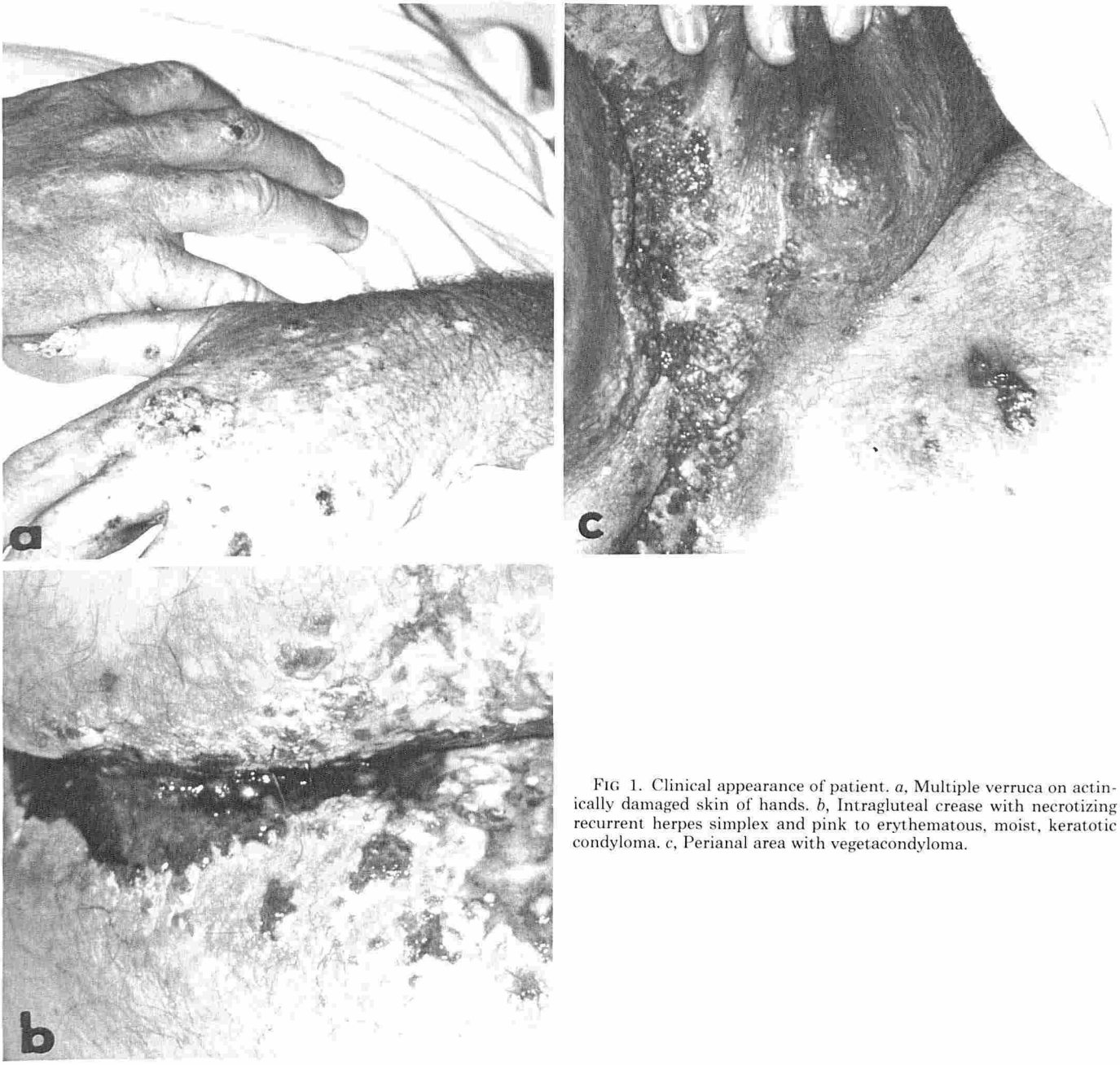

FIG 1. Clinical appearance of patient. *a*, Multiple verruca on actinically damaged skin of hands. *b*, Intragluteal crease with necrotizing recurrent herpes simplex and pink to erythematous, moist, keratotic condyloma. *c*, Perianal area with vegetacondyloma.

vealed a normal IgG and IgA but slightly decreased IgM of 37.8 (nl, 56–352). Direct Coombs was negative. Wedge biopsy from the intertriginous area of the groin showed changes consistent with herpes virus infection. Chest x-ray revealed mild cardiomegaly but no specific adenopathy. CT of the abdomen revealed a slight splenomegaly, absent left kidney (a congenital condition), and 2 bladder diverticuli, but did not reveal any periaortic adenopathy. Direct immunofluorescence examination of a biopsy from the intertriginous area was negative for all immunogobulins and complement. Bone marrow examination revealed granulocytic and megakaryocytic hyperplasia but was otherwise unremarkable. Immunologic parameters included active rosetting of 28% (nl, 36%), total rosettes of 53% (nl, 60%), suppressor rosettes by the Raji cell method of 3% (nl, 35%) indicating low levels of total and interactive T cells. T-cell deliniation by Leu 2 and Leu 3 revealed a low helper to suppressor ratio of 1.35. (After treatments which included baths, Zovirax ointment, and transfer factor, the patient was lost to follow-up for several months at which time he was readmitted for treatment with a Leu 2 to Leu 3 T-cell ratio of 0.49.) DNA synthesis following mitogenic stimulation with both phyto hemagglutinin (PHA) and concanavalin A revealed a reduced response, and leukocyte inhibitory factor (LIF) production (lymphokines) to PHA and PPD and Candida were all negative. Except for the extent of the warts, they appeared clinically normal. However, histology was not performed at the time of biopsy and the patient is not currently available for further study. In any event, histology may have been difficult to interpret due to the several types of simultaneous skin infections present. Clinical photographs of the patient appear in Fig 1. Based upon symptoms, it would appear that the immune deficiency probably began or at least was present by 1980, 2 years prior to our rigorous diagnostic studies.

MATERIALS AND METHODS

DNA Extraction

Total cell DNA was extracted as described previously [1]. Briefly, tissue was treated with sodium dodecyl sulfate (SDS)-pronase at 60°C for 30 min and then fully digested at 37°C followed by the addition of potassium acetate to 1.4 M at 4°C. After clearing, the DNA in the supernatant was concentrated by the addition of ethanol and treated with RNase A followed by SDS-pronase digestion, phenol-$CHCl_3$ (1:1; v/v) and $CHCl_3$ extraction, and finally ethanol precipitation.

Molecular Cloning of Viral DNA

Total cell DNA and lambda phage Charon 27 DNA were each cleaved with Bam HI restriction endonuclease, ligated, packaged in vitro and screened by filter hybridization under conditions of lowered stringency [15] with labeled HPV-6 DNA as described previously [6]. Amplified viral DNA was separated from the vector DNA by cleavage with Bam HI followed by 2 rounds of sucrose gradient (1 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 2 μg/ml ethidium bromide; 10–40% sucrose; SW 27 rotor; 26K rpm; 24 h at 4°C) centrifugation. Following extraction with water-saturated butanol, the purified DNA was concentrated with ethanol [16].

Restriction Endonuclease Analysis

Treatment of DNA with restriction endonucleases, followed by agarose gel electrophoresis and transfer to nitrocellulose filters were performed as described previously [1].

DNA:DNA Hybridization

Filters were hybridized by incubation in solutions containing 50% formamide plus 10% dextran sulfate (high stringency) or 30% formamide plus 10% dextran sulfate (low stringency) as described previously [1].

In order to determine the extent of hybridization, liquid hybridization followed by S1 nuclease analysis were performed as described previously [17]. Molecularly cloned viral DNAs were separated from the cloning vector by treatment with the appropriate restriction endonuclease, denatured by heating at 100°C for 5 min, and incubated in the presence of a heat-denatured, nick-translated DNA [1] in 0.6 M NaCl, 40 mM Tris-HCl pH 7.2, and 2 mM EDTA at 68°C for 2 h to a Cot (DNA conc × time in mol·s/liter) value of 1.0. Samples were then analyzed for degree of renaturation to the labeled probe by treatment with S1 nuclease followed by acid precipitation. DNA probes were 10–15% double-stranded initially and hybridized to a maximum of 63–73% with the positive control DNAs.

RESULTS

Total DNA extracts from 4 papillomas from patient RCM were analyzed for the presence of HPV DNA sequences by nitrocellulose filter hybridization under stringent conditions with several standard HPV DNAs. Little or no hybridization was observed with labeled HPV-1-2, -4, or -5 (data not shown). However, native, unintegrated HPV DNA was detected in each of the tissue samples with labeled HPV-6 and to a lesser degree with labeled HPV-3 (Fig 2). The possibility that both of these HPVs might be present was tested by treating a sample with a combination of the restriction endonucleases Bam HI and Hind III followed by gel electrophoresis and stringent filter hybridization to HPV-6 and HPV-3 (Fig 2). In each case 3 identical viral DNA bands were detected, although hybridization was much less intense with labeled HPV-3, indicating that only a single viral DNA was present.

It was, however, noted that the intensity of the bands detected even with labeled HPV-6 was far less than we would have expected from the amount of viral DNA observed by ethidium bromide staining of these electrophoretic gels. Assuming that the HPV detected in this patient was only distantly related to HPV-6, we undertook the molecular cloning of its DNA so that further characterization could proceed. We achieved this objective by treating total cellular DNA with Bam HI, a restriction endonuclease which cleaved this HPV DNA at a unique site (R. Ostrow, unpublished observation), and then ligating the HPV DNA with Charon 27 lambda phage DNA treated with the same enzyme. Following in vitro phage packaging and screening of plaques by filter hybridization to labeled HPV-6 under less stringent hybridization conditions, molecularly cloned viral DNA, hereafter referred to as HPV-26, was amplified for further studies.

HPV-26 DNA was excised from the vector by treatment with Bam HI and purified by rate zonal centrifugation. The purified HPV-26 was labeled by nick translation and hybridized under stringent hybridization conditions to standard HPV DNAs on nitrocellulose filters (Fig 3). Only a weak hybridization was

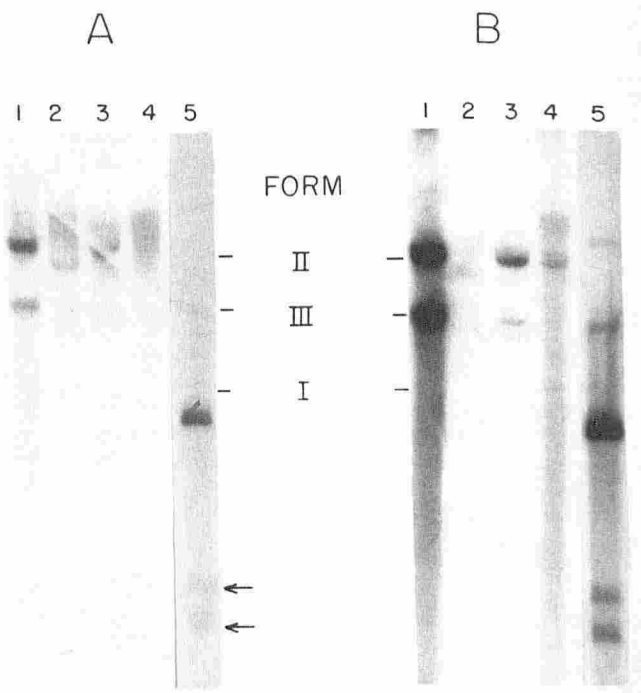

FIG 2. Hybridization of cellular DNA to HPV-3 and HPV-6. Equal amounts of total cellular DNA was electrophoresed, transferred to nitrocellulose filters, and hybridized under stringent conditions to labeled (*A*) HPV-3 or (*B*) HPV-6, each labeled to about the same specific activity, followed by washing and autoradiography. *Lanes 1–4*: DNA extracts from each of the 4 tissue samples studied. *Lane 5*: DNA extract of lane 1 was treated with a combination of Bam HI and Hind III restriction endonucleases. *Form I* DNA is where native superhelical viral DNA migrates, *form II* where nicked, relaxed viral DNA migrates, and *form III* where linear unit length viral DNA migrates. Slight variations in the migration of the native DNA forms can occur depending upon the quality and amount of total DNA extract applied to any one channel. Some faint bands are indicated with *arrows*.

detected with the large, and perhaps the small, Bam HI/Eco RI fragments of HPV-6. This correlates well with the preliminary hybridization data of the cellular DNA extracts. No significant hybridization as observed under these conditions with HPV-1, -2, -3, -4, -5, -7, -8, -10, HPV-P [18], HPV-PW [18], HPV-EV [4], or an HPV-11 molecularly cloned in a similar fashion from a penile verrucous carcinoma [6; R. Ostrow, unpublished data]. Our HPV-11 DNA hybridized 100% by S1 nuclease analysis and had a restriction endonuclease map similar to that of HPV-11 obtained from L. Gissmann [19].

By currently accepted criteria [20], two HPV DNAs are considered to be of different types if they share less than 50% nucleotide sequence homology as measured by cross hybridization of the HPVs and analyzed with S1 nuclease. The relationship of HPV-26 to all of the above HPV DNA standards, with the exception of HPV-7, -8 and -10, was tested by liquid hybridization followed by S1 nuclease analysis. No hybridization above 6% normalized was observed with any of these DNAs (data not shown). HPV-8, -12, and to a lesser degree, HPV-9 share similar homology to HPV-5, and HPV-10 shares significant nucleotide homology with HPV-3 [21–24]. Very recently

we conducted stringent hybridization of HPV-26 with fully or partially molecularly cloned HPV-13 [25], HPV-16 and -18 [26], and HPVs-9, -12, -14, -15, -17, and -19 to -24 isolated from EV patients (G. Orth, personal communication). We observed no significant hybridization (data not shown). However, all of these HPV DNAs were not available for in-depth comparative analysis at this time. HPV-25 isolated from an EV patient is not yet available for comparison, although its restriction endonuclease map differs greatly from that of HPV-26 (G. Orth, personal communication) (Fig 4). Based upon these relationships, HPV-26 is unique and, under the criteria now used for typing HPVs [20], should be referred to as a separate HPV type.

DISCUSSION

Papillomas and associated neoplasias appear more prevalent in patients exhibiting immune deficiency syndromes. Whether HPV is a passenger virus in such cases or plays an etiologic role in the immune deficiency remains to be determined. In this case, we have isolated a previously undescribed HPV DNA genome from a patient suffering from such a malady. While many distinctions remain, the lowered T-cell count and reversed helper/suppressor ratios are quite similar to those observed in patients with acquired immunodeficiency syndrome [27]. Recently, many new HPV-5 related subtypes as well as other distinct subtypes have been identified in EV patients (G. Orth and H. Pfister, personal communications). Our own studies† have shown immune suppressed renal transplant patients to be afflicted with papillomaviruses sharing nucleotide sequence homology with several different HPV DNA standard probes. It may be that in patients having immune difficiencies there is a higher likelihood of mutational or recombinational events which lead to the formation of new HPV types. On the other hand, the immune deficiencies of these patients may permit the infection and replication of human papillomaviruses that are rare and have relatively low infectious potential in the normal population. These rare viruses may also coincidently contain a higher oncogenic potential as witnessed by the increased incidence of malignancies in EV and renal transplant patients. Based upon the correlation for some HPVs with immune deficiencies and associated malignancies [2,11–14,26], it may be appropriate at this time to examine malignancies and other tissues of immune deficient patients for the presence of HPVs like HPV-26. Although there is no record that our patient had papillomas prior to his onset of cutaneous malignancies, we cannot rule out the possibility that a subclinical papilloma infection may have been present. Indeed, in apparently normal skin of patients with EV, we have observed the presence of HPV-5 viral DNA (R. Ostrow, unpublished data). It is also possible that the radiation treatments may have impaired the patient's immune system thus permitting the various viral and microbial infections. The study of warts from patients with various immune deficiency syndromes may lead to the discovery of many more diverse HPV variants.

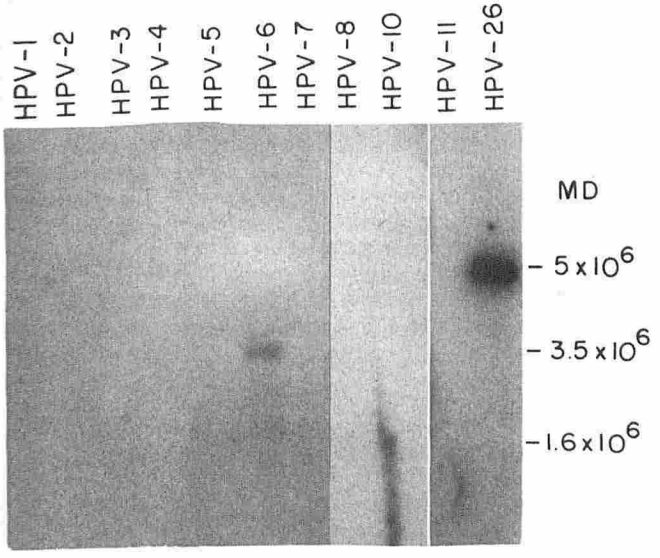

FIG 3. Hybridization of molecularly cloned HPV-26 to previously characterized HPV DNAs. Molecularly cloned HPV-26 DNA was excised from the vector DNA, labeled by nick translation and hybridized under stringent hybridization conditions to approximately 40 pg each of various standard HPV DNAs (which had been excised from their respective vectors) on nitrocellulose filters as indicated. The molecular weight in megadaltons (*MD*) of the unit length HPV genome and that of the 2 Bam HI/Eco RI fragments of HPV-6 are shown on the right. The smaller of the two fragments is just barely visible in this exposure.

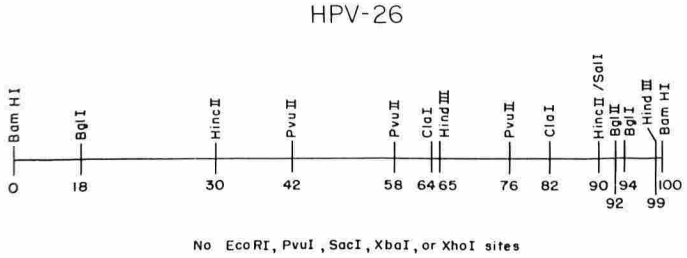

FIG 4. Restriction endonuclease map of HPV-26. Molecularly cloned HPV-26 was treated with restriction endonucleases individually or in combination, electrophoresed on agarose gels, and visualized either with ethidium bromide staining or by transfer to nitrocellulose filters followed by stringent hybridization to labeled HPV-26. Units represent percentage of the genome size with the unique Bam HI site as an arbitrary starting point.

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