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Coinfection of Human Foreskin Fragments with Multiple Human Papillomavirus Types (HPV-11, -40, and -LVX82/MM7) Produces Regionally Separate HPV Infections within the Same Athymic Mouse Xenograft

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The athymic mouse xenograft system was used to prepare infectious stocks of two additional anogenital tissue-targeting human papillomaviruses (HPVs) in a manner similar to that for the development of infectious stocks of HPV-11. An anal condyloma from a transplant patient was used as material for extraction of infectious virus, and human foreskin fragments were incubated with the virus suspension and transplanted subrenally into athymic mice. Partial viral sequencing indicated that two rare HPV types (HPV-40 and HPVLVX82/MM7) were concurrently present in both the patient condyloma and the foreskin xenografts, and passage of both types was achieved as a mixed infection with HPV-40 predominating. Xenografts that developed from simultaneous infection of human foreskin fragments with HPV-11, -40, and -LVX82/MM7 virions produced regionally separate areas of HPV-11 and -40 infection as determined by in situ hybridization. In addition, in situ hybridization with HPV-40 and HPVLVX82/MM7 DNA probes demonstrated that both of these HPV types were present as adjacent but separate infections within the same anal condyloma of the transplant patient. These studies indicate that multiple HPV types can simultaneously infect genital tissue and that each HPV type predominantly maintains regional separation within the same papilloma.

Production of quantities of infectious human papillomaviruses (HPVs) has proven to be difficult for a variety of technical reasons. The assembly of papillomavirus (PV) virions is intimately tied to the differentiation cycle of epithelial cells, making in vitro production of infectious HPV possible only by using organotypic culture systems (32). The high levels of tissue and species specificity of HPVs also prevent their growth in laboratory animal tissues. One further difficulty with most HPV types, especially those that are strongly associated with epithelial malignancies, is that patient lesions are often virus poor, with as many as 50% of the lesions showing undetectable levels of HPV capsid proteins as determined by immunohistological staining with anticapsid antibody (16, 19, 22, 27). Infectivity models using animal PVs have further demonstrated that there is a substantial inefficiency of infectivity by PVs such that large numbers of virus particles are required to initiate an infection (7, 21, 23, 36).

Refinements to animal models and cell culture systems have provided several methods to produce fully assembled infectious HPVs including HPV-1 (26), HPV-11 (14, 25), HPV-16 (5, 39), HPV-31 (32), and several epidermodysplasia verruciformis-associated HPVs (30). Cell culture techniques using recombinant vaccinia and Semliki Forest viruses have also succeeded in producing infectious bovine PV type 1 (BPV-1) (36, 40). Despite these advancements, however, to date only one system has achieved the successful production and amplification of the large quantities of infectious HPV that are required for reinfection and passage studies (24). This method for production of high-titered stocks of an anogenital tissue-targeting HPV (25) utilized HPV-11-infected genital skin fragments that were transplanted subrenally into athymic (25) and severe combined immunodeficiency (4) mice. The athymic mouse xenograft system has also been used to produce large stocks of several animal PVs including cottontail rabbit PV (10), BPV-1 (10, 18) and BPV-4 (17), and rabbit oral PV (8).

In this study, we report the propagation of two additional anogenital tissue-targeting HPV types that were inadvertently propagated together as a mixed infection in human foreskin xenografts grown in athymic mice. One HPV type was demonstrated to be a newly described anogenital HPV currently known as HPVLVX82/MM7 (3, 20, 34). The second type, HPV-40, is also rare and is associated mostly with benign anogenital lesions (12, 13). Athymic mouse xenografts containing the mixed infections produced predominantly HPV-40 virions, with HPVLVX82/MM7 present at low levels. The production of infectious stocks of additional anogenital tissue-targeting HPV types has enabled us to study the outcome of simultaneous infection of human foreskin fragments with three HPV types (HPV-11, -40, and -LVX82/MM7). We found that simultaneous infection with multiple HPV types led to regionally separate HPV infections within a single xenograft. These studies have important implications with regard to the initiation, detection, and maintenance of multiple HPV infections in individual patients and within individual lesions.

MATERIALS AND METHODS

Isolation of HPV from anal condylomas. Anal condylomas from several patients were excised, and fragments were either fixed in neutral buffered formalin for histological analysis or frozen for later virus extraction. Individual condylomas were screened for the presence of viral antigens with a polyclonal antibody recognizing the PV group-specific antigen (GSA) (Biømeda, Foster City, Calif.). An anal condyloma from a 29-year-old female transplant patient that showed

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strong GSA staining was processed for virus extraction as previously described (25). Virus was isolated from the patient as a crude 10% extract in phosphate-buffered saline to be used in infectivity studies. Virions from a portion of this extract were purified by CsCl density centrifugation, followed by viral DNA extraction and cloning of the HPV viral genome into pUC19 at the *Ban*2 site.

Determination of HPV types and cloning. The viral type present in patient condylomas was determined initially by PCR with the redundant primers MY09 and MY11 (1, 3, 31). Amplified viral DNA was subjected to a set of restriction enzyme digestions, and banding patterns were compared to the published results obtained for 44 different genital HPV MY09/MY11 fragments (3). The MY09/MY11 fragment amplified from viral DNA (extracted from CsCl-banded virus) from the anal condyloma of the transplant patient described above was cloned into pUC19 and sequenced by standard procedures. An exact match with the published sequence fragment of MM7 was obtained. Several full-length viral genomes were cloned into pUC19 at the *Ban*2 and *Kpn*I sites, and the genome was partially sequenced within the L1 region. The sequence of this region (420 bp of L1) was shown to exactly match the published sequence of HPV-40. In addition, the sizes of digests with several restriction enzymes matched predicted fragment sizes calculated from the published sequence (data not shown). No viral clones containing the HPVLVX82/MM7 sequence were obtained in pUC19 after several cloning attempts. As an alternative initial strategy for obtaining the full-length genome of HPVLVX82/MM7, primers were designed from the sequenced L1 fragment around the *Bam*HI site for full-length amplification of the entire viral genome by long-PCR techniques (Boehringer Mannheim Corp., Indianapolis, Ind.). An 8-kb fragment was obtained after long PCR, and subfragments of this PCR product were cloned into pUC19 following restriction digestion with *Pst*I. A plasmid containing a 2.7-kb fragment was used as a probe for detecting HPVLVX82/MM7 DNA by in situ hybridization. The HPVLVX82/MM7 fragment obtained with the MY09 and MY11 PCR primers was used as a probe to confirm that the sequence of the complete viral genome obtained from long PCR prior to cloning was the HPVLVX82/MM7 sequence.

In situ hybridization. In situ hybridizations of anal condylomas and xenografts were conducted under stringent conditions as previously described (8). Biotinylated probes used included HPV-11, HPV-40, and the 2.7-kb *Pst*I fragment of HPVLVX82/MM7 cloned from the long-PCR product as described above.

HPV infection of human foreskin xenografts. (i) Production of stocks of HPV-40 and HPVLVX82/MM7. Two hundred microliters of crude virus stock (subsequently shown to contain both HPV-40 and HPVLVX82/MM7) was mixed with human foreskin fragments, incubated for 1 h at 37°C, and then transplanted subrenally into athymic mice as previously described (25). In an initial experiment, a total of eight mice received two infected foreskin grafts each. Mice were sacrificed at various times between 85 and 200 days postinfection, and the xenografts were measured (length, width, and height) in millimeters. Sections of the xenografts were prepared for histological analysis including hematoxylin and eosin and GSA antibody staining and in situ hybridization with biotinylated HPV-40 and HPVLVX82/MM7 probes as previously described (8, 25). A repeat experiment using 28 athymic mice was set up to produce stocks of infectious HPV and for Southern and PCR analysis for the detection of HPV-40 and HPVLVX82/MM7 DNA.

A second set of experiments was conducted to determine the viral titer of the extract prepared from the patient, as well as to test whether xenograft-derived extract could produce infectious HPV-40 and/or HPVLVX82/MM7. Serial 10-fold dilutions of virus extract were prepared from six small 120-day-old xenografts from the first experiment, as well as from the original patient material. The virus dilutions were incubated separately for 1 h at 37°C with human foreskin chips and were transplanted subrenally as described above. Each dilution point was tested in groups of four mice, with two grafts/mouse for a total of eight grafts per virus dilution. After 120 days of growth, xenografts were removed and examined by hematoxylin and eosin staining and in situ hybridization for the presence of morphological changes and HPV-40 and HPVLVX82/MM7 DNA as described above. Genomic DNA from separate xenografts was also prepared and examined by Southern blotting for the presence of both HPV types.

(ii) Simultaneous infections with HPV-11, HPV-40, and HPVLVX82/MM7. Simultaneous infections of human foreskin fragments with HPV-11 and HPV-40 (with residual HPVLVX82/MM7) were conducted to determine the outcome of infection with multiple HPV types. HPV-11 was included in these mixed-infection experiments because high-titer stocks of this type are available and because HPVLVX82/MM7 virions were present only at very low levels such that they were usually undetectable by in situ hybridization. Human foreskin fragments (2 by 2 by 0.5 mm) were incubated for 1 h at 37°C with a mixed suspension of HPV-11 (1 μl of undiluted stock extract) and HPV-40 (100 μl of undiluted patient material) with residual HPVLVX82/MM7. A total of 14 grafts (seven mice) were attempted; 10 grafts were available for analysis when the mice were sacrificed and the xenografts were retrieved 120 days later. Serial 5-μm-thick sections from individual xenografts were examined by in situ hybridization with HPV-11 and HPV-40 DNA probes (HPVLVX82/MM7 was not examined by in situ hybridization because of the low concentration of infectious virions in the original infection mixture).

Southern blot analysis of viral DNA in the anal condyloma and xenografts. DNA was purified from several xenografts and the anal condyloma for the detection of HPV-40, HPV-11, and HPVLVX82/MM7 DNA. These experiments were designed to determine the relative proportions of the viral types in the xenografts and original patient condyloma. Xenografts were homogenized with a polytron, and the cell debris that was collected by low-speed centrifugation (1,000 × *g*) was used for DNA extraction. The cellular material was digested overnight at 37°C with proteinase K (20 μg/ml) in extraction buffer (0.01 M Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% sodium dodecyl sulfate) and DNA purified by standard protocols. Ten micrograms of cellular DNA was digested with *Pst*I enzyme, then electrophoresed on a 1% agarose gel, and transferred onto Hybond N membranes (Amersham Corp., Arlington Heights, Ill.). Three replicate membranes were prepared and were probed with ^{32}P-labeled HPV-11 full-length probe, HPV-40 full-length probe, and the 2.7-kb *Pst*I fragment of HPVLVX82/MM7. The hybridized membranes were exposed to X-Omat (Eastman Kodak Co., Rochester, N.Y.) films for 10 min (HPV-11 and HPV-40) or for 5 h (HPVLVX82/MM7) by using cassettes with intensifying screens.

RESULTS

Isolation, characterization, and propagation of infectious HPV-40 and HPVLVX82/MM7 virions. An anal condyloma from a transplant patient was used as the source of tissue for the extraction of infectious HPV, as previously described for the extraction of HPV-11 (25). This patient has had three occurrences of condylomas located on the labia and vulva, on the perianal region, and on the cervix. These lesions have been excised surgically in successive years over a 3-year period. Fresh material was obtained from the anal condylomas, and formalin-fixed paraffin-embedded tissue was available from all three treatment periods.

Viral typing was initially achieved by PCR with the redundant primers MY09 and MY11 as described in Materials and Methods. Only one 450-bp HPV product was amplified from the viral DNA extracted from CsCl-banded virus, and restriction enzyme digestion indicated a pattern match for HPVLVX82/MM7. Cloning and sequencing of the PCR product demonstrated an exact match for the MM7 sequence (data not shown). Several viral genomes were cloned from this preparation of DNA, and all clones were unexpectedly found to be HPV-40. Further experiments demonstrated that HPV-40 predominated in the anal condyloma (see below) and therefore that the PCR conditions used with the MY09 and MY11 primers preferentially amplified MM7 despite the predominance of HPV-40 DNA. (Note that PCR with the MY09 and MY11 primers and cloned HPV-40 DNA successfully amplified a 450-bp HPV-40 DNA fragment.) In addition, PCR with DNA

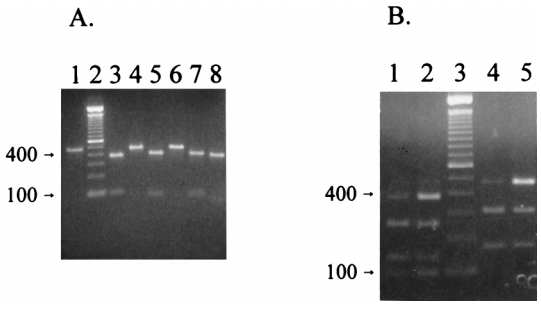

FIG. 1. Restriction digests of PCR products amplified by L1 consensus primers MY09 and MY11 from genomic DNA extracted from two fragments of the anal condyloma and one xenograft. (A) Genomic DNA template from the first fragment of the anal condyloma. Lane 1, uncut PCR product of 452 bp; lane 2, 100-bp markers; lanes 3 to 8, PCR products digested with *Bam*HI (lane 3; 369- and 83-bp fragments), *Dde*I (lane 4; 452-bp fragment), *Hae*III (lane 5; 383- and 69-bp fragments), *Hin*fI (lane 6; 452-bp fragment), *Rsa*I (lane 7; 380- and 72-bp fragments), and *Sau*3A (lane 8; 369-, 63-, and 20-bp fragments). (B) Genomic DNA template from a second fragment of the anal condyloma (lanes 2 and 5), DNA from a xenograft (lanes 1 and 4), and 100-bp markers (lane 3). Restriction digestion of the 452-bp products was with *Bam*HI (lanes 1 and 2) and *Dde*I (lanes 4 and 5). For the expected bands for HPVLVX82/MM7, see above. For HPV-40 *Bam*HI digestion, the expected bands were of 240, 132 and 83 bp; for HPV-40 *Dde*I digestion, the expected bands were of 297 and 158 bp.

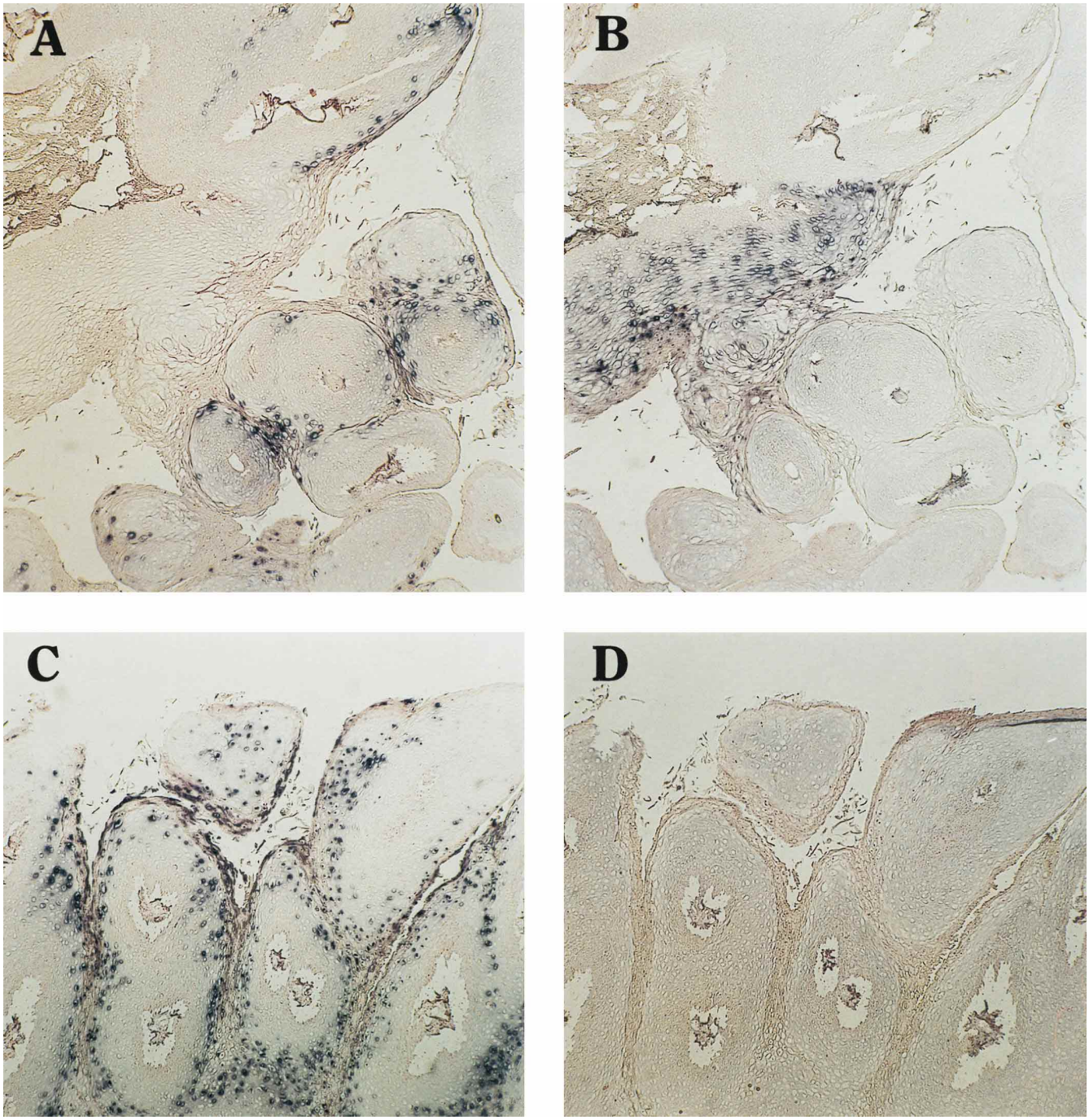

FIG. 2. In situ hybridization of serial 5-μm-thick sections from the anal condyloma with HPV-40 (A and C) and HPVLVX82/MM7 (B and D) biotinylated DNA probes. Two regions (A and B; C and D) are shown. Biotinylated HPV probes were hybridized under stringent conditions and detected with alkaline phosphatase-labeled streptavidin and substrate. Sections were lightly counterstained with nuclear fast red. Magnification, ×70.

extracted from xenografts and from a second preparation of DNA from the patient lesion showed concurrent amplification of both HPV-40 and HPVLVX82/MM7 450-bp fragments (Fig. 1), suggesting that HPV-40 can be amplified provided the level of HPVLVX82/MM7 DNA is below a certain critical level when both types are present concurrently.

Fragments of anal condyloma were placed into phosphate buffered saline and homogenized with a polytron as previously described (9). The crude suspension was used as a source of infectious HPV for infectivity studies using human foreskin chips implanted into athymic mice (25). An aliquot of the crude viral suspension was subjected to CsCl banding for purification and cloning of the HPV as described in Materials and Methods. In the first infectivity experiment, mice were sacrificed at various times between 85 and 200 days postinfection. A second infectivity experiment with the patient extract (28 mice) was conducted to produce stocks of infectious HPV-40 and HPVLVX82/MM7. Xenografts reached a maximum size of 10

TABLE 1. Titration of infectious HPV-40 and HPVLVX82/MM7 virus from patient and xenograft extracts

Dilution of viral extract	Patient-derived[a] extract			Xenograft-derived[b] extract		
	In situ HPV-40[c]	In situ HPVLVX82/MM7[c]	Cyst size[d]	In situ HPV-40[c]	In situ HPVLVX82/MM7[c]	Cyst size[d]
Undiluted	4/4/7	1/4/7[e]	2.9 (0.5)	2/2/2	0/2/2	1.8 (0.3)
10^{-1}	2/3/6	0/2/6	2.5 (0.6)	3/8/8	0/8/8	2.0 (0.2)
10^{-2}	2/5/7	0/5/7	2.3 (0.6)	0/4/8	0/4/8	2.0 (0.3)
10^{-3}	0/4/7	0/4/7	2.4 (0.2)	0/4/8	0/4/8	2.4 (0.3)
10^{-4}	NT[f]	NT	NT	0/7/8	0/7/8	2.2 (0.3)

[a] HPV derived from patient anal condyloma.
[b] HPV derived from first-passage xenografts.
[c] Compound fractions represent number of grafts in situ positive/number of viable grafts/number of grafts attempted (24).
[d] Cyst sizes are determined as the means (standard errors) of geometric mean diameters of xenografts at time of harvest (24).
[e] One graft represents a foreskin orthograft; the remainder are subrenal xenografts. Positive in situ signals for both HPVLVX82/MM7 and HPV-40 DNA were observed only in the orthograft.
[f] NT, not tested.

by 10 by 10 mm (mean geometric mean diameter = 5.5 ± 2.3 mm at 250 days).

Immunohistological and in situ analysis of transplant patient condylomas and xenografts. Histological features of the anal condyloma included areas of koilocytosis typical of low-risk HPV infection (data not shown). In situ hybridization of the patient anal condylomas with HPV-40 and HPVLVX82/MM7 DNA probes revealed predominantly HPV-40-positive nuclei with nonoverlapping and less frequent areas of HPV LVX82/MM7-positive nuclei (Fig. 2). The earlier lesions of the vulva and labia showed a similar distribution of HPV-40 and HPVLVX82/MM7 infection, whereas the most recent condylomas of the cervix revealed only HPV-40 DNA-positive nuclei by in situ hybridization. These data demonstrated that HPV-40 and HPVLVX82/MM7 did not cross-hybridize under the conditions used for the in situ hybridizations. Strong GSA-positive signals were obtained for all three condylomas from this patient (data not shown).

Propagation and passage of HPV-40 and HPVLVX82/MM7 in athymic mice. An extract was prepared from six 120-day xenografts from the first experiment to determine whether HPV-40 and HPVLVX82/MM7 could be propagated and passaged. A titration of the xenograft and patient-derived extracts was included in this study. After 120 days, xenografts were harvested and examined by in situ hybridization with HPV-40 and HPVLVX82/MM7 DNA probes as previously described. The results (Table 1) showed that the patient extract was infectious at dilutions of up to 1:100, whereas the xenograft-derived extract was infectious at a dilution of up to 1:10. Additional mice were used to prepare a larger stock of infectious HPV-40 (and HPVLVX82/MM7). In situ positive signals for HPVLVX82/MM7 DNA were observed only in a foreskin orthograft established on an athymic mouse after infection with the patient extract (Table 1). Xenografts examined showed strong in situ signals to the HPV-40 DNA probe that were comparable to the signals obtained from the patient condyloma (see below).

In situ hybridization analysis of xenografts concurrently infected with HPV-11, HPV-40, and HPVLVX82/MM7. Experiments were designed to determine the outcome of simultaneous infection of human foreskin tissue with HPV-11, HPV 40, and HPVLVX82/MM7. Xenografts were examined at 120 days by in situ hybridization with HPV-11 and HPV-40 DNA probes. Several xenografts showed nonoverlapping areas of infection for HPV-11 and -40 (Fig. 3C and D), whereas other xenografts showed a preponderance of either HPV-40 (Fig. 3A and B) or HPV-11 (data not shown). In a total of eight xenografts examined by in situ hybridization, one had HPV-40 DNA only (Fig. 3A and B), two had only HPV-11 DNA, and five showed nonoverlapping areas of both HPV-11 and -40 DNA (e.g., Fig. 3C and D). All eight xenografts examined showed strong in situ signals. These data demonstrated that there was no detectable cross-hybridization between HPV-11 and HPV-40 under the in situ hybridization conditions described above. In an earlier mixed-infection experiment using higher concentrations of HPV-11, the xenografts showed predominantly HPV-11 DNA as a result of in situ hybridization, with very little HPV-40 DNA (data not shown).

Examination of patient- and xenograft-derived DNA by Southern blotting. Strong in situ signals to the HPV-40 DNA probe in the xenografts (Fig. 3) and passage in xenografts (Table 1) clearly demonstrated successful propagation of HPV-40 virions. In contrast, HPVLVX82/MM7 was observed in only a single orthotopic xenograft by in situ hybridization (Table 1). PCR analysis with redundant primers MY09 and MY11, however, indicated that HPVLVX82/MM7 was detectable in the xenografts (Fig. 1). Southern analysis of DNA from the anal condyloma and selected xenografts was therefore conducted to determine relative viral loads. Of three xenografts initially infected with HPV-40 and HPVLVX82/MM7 virus, all three showed strong hybridization signals to HPV-40 DNA, whereas one showed strong hybridization signals to HPV LVX82/MM7 DNA (Fig. 4). Several xenografts (Fig. 4) showed a higher level of HPVLVX82/MM7 DNA than that obtained from the anal condyloma, whereas others contained no detectable HPVLVX82/MM7 DNA. Densitometric scanning of the Southern blot (Fig. 4C) indicated that the relative concentration of HPVLVX82/MM7 in the anal condyloma was 2 pg/15 μg of DNA (lane I); corresponding concentrations in the xenografts were 72 (lane C) and 29 (lane E) pg/15 μg of DNA. These data indicated that expansion of HPVLVX82/MM7 virus had occurred by passage in the xenograft system but that the level of virion propagation for this type is variable between xenografts.

Of five xenografts established from multiple infections with HPV-11, -40, and -LVX82/MM7, abundant HPV-11 and HPV-40 DNA was detected in three xenografts. HPVLVX82/MM7 DNA was also detected in one of these xenografts, indicating that this xenograft contained all three viral types (Fig. 4). The radiolabeled HPV-11 DNA probe did not cross-hybridize with HPV-40 or HPVLVX82/MM7 DNA, and the HPV LVX82/MM7 DNA probe did not cross-hybridize with HPV-11 or HPV-40 DNA (Fig. 2 and 4 and data not shown). *Pst*I digests of HPV-11 and -40 gave the expected fragments as calculated from published sequences (Fig. 4).

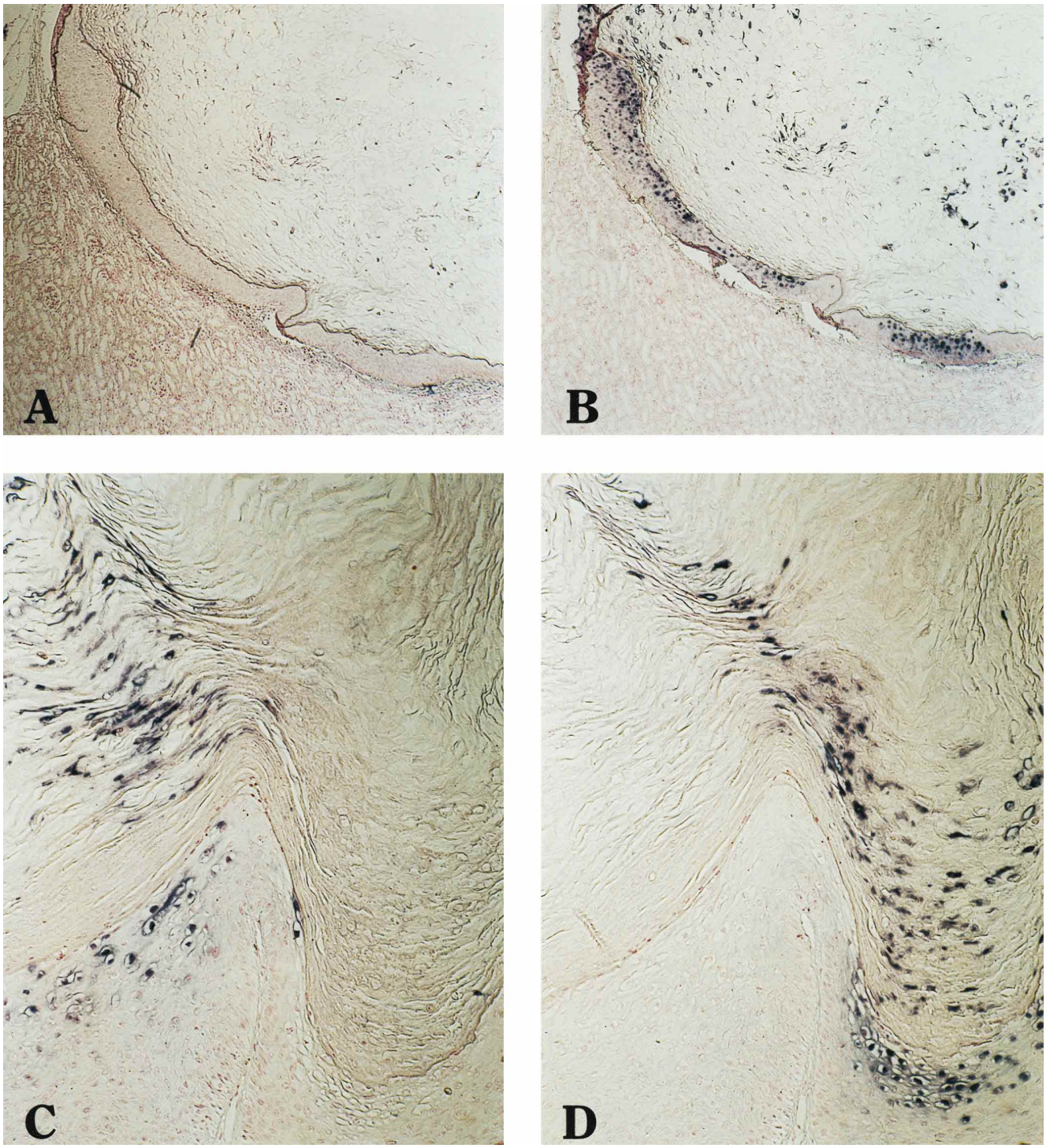

FIG. 3. In situ hybridization of serial 5-μm-thick sections from two human foreskin xenografts with HPV-11 (A and C) and HPV-40 (B and D) biotinylated DNA probes. The first xenograft (A and B) shows strong in situ positive staining with HPV-40 (B) but not with HPV-11 (A) DNA probes. Photomicrographs of the second xenograft (C and D) show sequential sections of an area of the xenograft where both HPV-11 (C) and HPV-40 (D) in situ positive nuclei were observed. Magnification, ×70 (A and B) and ×176 (C and D).

DISCUSSION

Lack of effective methods for producing infectious stocks of HPVs has proven to be a significant obstacle for studying the HPV life cycle. However, recent improvements in animal and cell culture systems have produced infectious HPV virions for a limited number of types (4, 14, 25, 26, 30, 32, 39). The athymic mouse xenograft system remains the most successful method for producing large stocks of infectious virions (4, 24, 25). One limitation of the xenograft system has been that only one of the genital tissue-targeting HPV types has been propagated successfully (25). Recent reports, however, have demonstrated that several additional HPV types can now be ex-

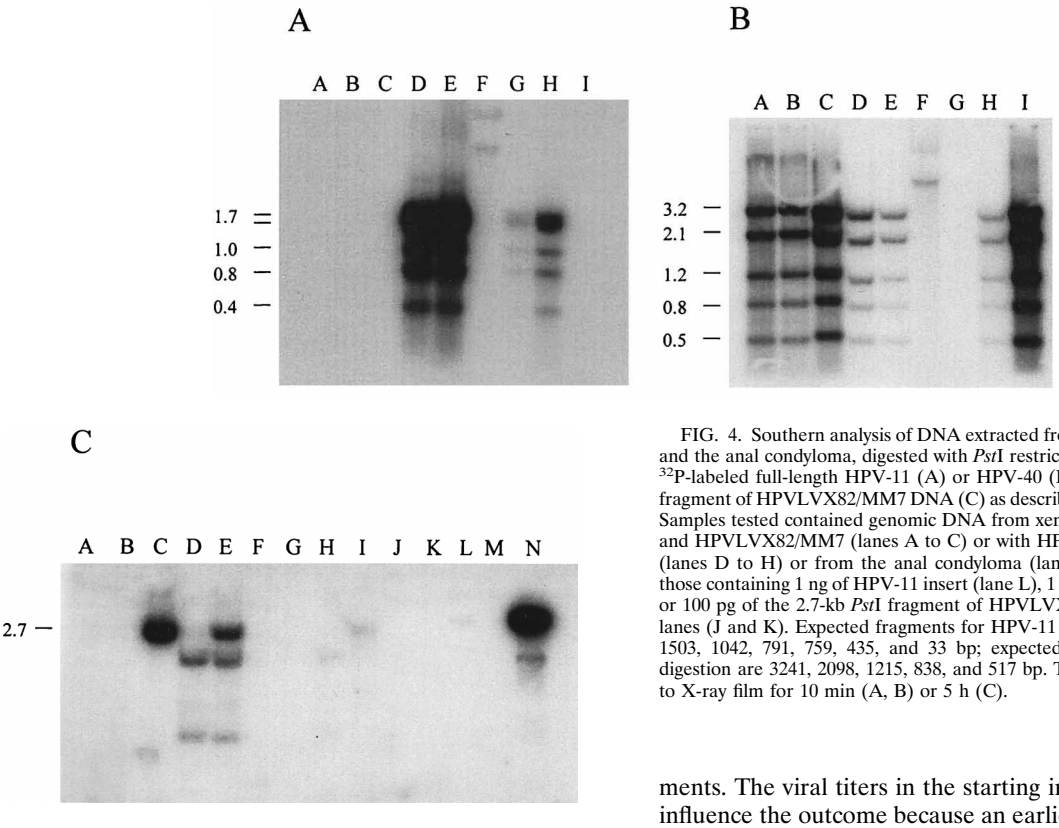

FIG. 4. Southern analysis of DNA extracted from human foreskin xenografts and the anal condyloma, digested with *Pst*I restriction enzyme, and probed with ^{32}P-labeled full-length HPV-11 (A) or HPV-40 (B) DNA or with a 2.7-kb *Pst*I fragment of HPVLVX82/MM7 DNA (C) as described in Materials and Methods. Samples tested contained genomic DNA from xenografts infected with HPV-40 and HPVLVX82/MM7 (lanes A to C) or with HPV-11, -40, and -LVX82/MM7 (lanes D to H) or from the anal condyloma (lanes 1). Control lanes included those containing 1 ng of HPV-11 insert (lane L), 1 ng of HPV-40 insert (lane M), or 100 pg of the 2.7-kb *Pst*I fragment of HPVLVX82/MM7 (lane N) and blank lanes (J and K). Expected fragments for HPV-11 *Pst*I digestion are 1774, 1594, 1503, 1042, 791, 759, 435, and 33 bp; expected fragments for HPV-40 *Pst*I digestion are 3241, 2098, 1215, 838, and 517 bp. The membranes were exposed to X-ray film for 10 min (A, B) or 5 h (C).

panded effectively in the athymic mouse xenograft system (reference 39 and this study).

In this study, we have established infectious stocks of two additional anogenital tissue-targeting HPV types that were identified as HPV-40 and HPVLVX82/MM7. HPV-40 has been detected infrequently in benign anogenital lesions and appears to be a low-risk type (13, 29). HPVLVX82/MM7 has been detected in approximately 4% of HPV-positive cervical samples (31) and has been characterized as a possible "intermediate-risk" HPV type (3, 31, 34). These two HPV types, together with HPV-11, provided us with an opportunity to study the effects of simultaneous multiple HPV infections of human genital tissues by using the athymic mouse xenograft system. These experiments have provided preliminary data that have important implications regarding (i) potential interactions between different HPV types within a single lesion and (ii) potential expansion and/or repression of one HPV type within a lesion containing a dominant or more abundant type.

Simultaneous infection of human foreskin fragments with multiple HPV types could result in several possible outcomes including exclusion or mixed infections that are either overlapping or regionally separate. In situ hybridization results clearly demonstrated that HPV-11 and HPV-40 developed nonoverlapping areas of infection (Fig. 3). These areas may represent the progeny of a single (or small number) basal epithelial cell that was infected during the initial incubation period of virus with foreskin. Since individual basal epithelial cells of the foreskin fragment were exposed simultaneously to infectious virions of all three HPV types (at least theoretically), the above results suggest some level of early exclusion between the viral types. The mechanism of this exclusion could involve genetic differences between the foreskin fragments because foreskin fragments from several patients were used in these experiments. The viral titers in the starting inoculum also appear to influence the outcome because an earlier dual-infection experiment with an excess of HPV-11 led to a predominantly HPV-11 infection (data not shown). Other factors may be involved, however, and it is difficult to explain the large variations in final viral content between individual xenografts (Fig. 3 and 4), considering that each foreskin fragment was exposed to an equal concentration of virions in the virus mixture. In addition, recent experiments suggest that all PVs have a common receptor (15, 33, 37), and it was clear that all foreskin fragments were susceptible to HPV infection.

Southern analyses from the mixed infections demonstrated the expected fragments after *Pst*I digestion for both HPV-11 and HPV-40. These data also confirm that coincidental infections were regionally separate because no recombination (in the form of shared or new bands) between these two types was observed.

We were unable to detect HPVLVX82/MM7 DNA by in situ hybridization with the exception of one small region in a foreskin orthograft. Southern analysis clearly demonstrated that HPVLVX82/MM7 infections were present in the xenografts, and the levels were increased in some xenografts compared to the levels present in the anal condyloma (Fig. 4). These results demonstrate that infection with a less-abundant HPV type can still occur in the presence of an excess of virions from another type. We also detected several smaller bands with the 2.7-kb *Pst*I HPVLVX82/MM7 DNA probe in three xenografts that were also infected with HPV-11 (Fig. 4). These additional bands were not observed in the anal condyloma or in the xenografts infected with HPV-40 and HPVLVX82/MM7, and they were present only in those xenografts that were infected with all three virus types (Fig. 4). However, the smaller bands were not the same size as the predominant HPV-11 *Pst*I fragments of 1.7 and 1.5 kb. In addition, no cross-hybridization of the HPVLVX82/MM7 DNA probe with 1 ng of HPV-11 plasmid DNA was observed (Fig. 4C).

The clear bias in the results of the PCR amplification with the consensus MY09 and MY11 L1 primers was of some interest. These primers preferentially amplified HPVLVX82/MM7 DNA (Fig. 1) despite the predominance of HPV-40 DNA in the anal condyloma (Fig. 2). If HPV-40 had been the less frequent HPV type, it is highly unlikely that we would have detected it by the combined strategies of PCR, in situ hybridization, and viral cloning that were used in this study. We were able to amplify both HPV-40 and HPVLVX82/MM7 concurrently in a second preparation of DNA from the anal condyloma (Fig. 1), but Southern analysis (Fig. 4) showed a much higher level of HPV-40 infection. Thus, under certain conditions, PCR amplification with consensus primers can amplify two HPV types concurrently in a single lesion or sample, a result described by others (35). These observations have implications for HPV detection with consensus L1 primers in lesions with multiple HPV types, especially if one or more of these types is a rare or previously uncharacterized type. If there is a bias in the amplification of an HPV type by PCR, then any downstream analysis of the PCR product, no matter how extensive, will provide insufficient data as to the presence of additional viral types present in the sample. It is possible therefore, that certain HPV types may be underrepresented in epidemiological studies that are based on consensus PCR amplification alone. Previous studies have demonstrated that up to 20% of samples may contain more than one HPV type (2, 6, 11, 28). The incidence of multiple HPV types within a single lesion appears to be increased in papillomas obtained from immunosuppressed patients (6, 38), and these results are compatible with our findings that two HPV types were found in an anal condyloma of the transplant patient described in this study.

In summary, two rare anogenital tissue-targeting HPV types (HPV-40 and HPVLVX82/MM7) have been grown and passaged in the athymic mouse xenograft system. Both of these types were concurrently present in an anal condyloma taken from a transplant patient. Coinfection of human foreskin fragments with these two types plus HPV-11 produced 120-day-old xenografts containing mixed-HPV infections that were regionally type specific as determined by in situ hybridization. PCR amplification with consensus L1 primers preferentially detected the less frequent HPVLVX82/MM7 in the anal condyloma, despite a preponderance of HPV-40. These findings indicate that under certain conditions, some HPV types may not be detected effectively by consensus primer PCR amplification alone. We conclude that HPV coinfection studies of the athymic mouse xenograft system will provide an important model to examine (i) mechanisms for the maintenance of evolutionary stability of HPV types and the generation of potentially new types and/or variants and (ii) potential mechanisms for the exclusion, reactivation, or expansion of one HPV type by a second dominant or more frequent HPV type within the same lesion.

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