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(54) Title:
PURIFIED HUMAN PAPILLOMAVIRUS

(57) Abstract
A live, infectious stock of a new human papillomavirus, HPV 83, is provided. HPV 83 is related to but distinct from HPV MM7 and HPV LVX82. Methods of using HPV 83 to develop and test HPV vaccines and HPV antiviral agents are also provided.
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PURIFIED HUMAN PAPILLOMAVIRUS

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Institute of Allergy and Infectious Disease, Grant No. AI31494.

This application claims priority to U.S. Application No. 09/135,241, filed August 17, 1998, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the field of virology and control of viral disease. In particular, the invention provides a purified, infective stock of a human papillomavirus, useful for testing and developing vaccines and other therapeutic agents.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application in parentheses in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications is incorporated by reference herein.

Genital tract infections with human papillomaviruses (HPVs) are manifested in many ways, from an asymptomatic carrier state, to the commonly recognized disease condylomata acuminata (genital warts), and to overt malignancy. Infections with certain genital HPVs, such as HPV types 6 and 11 cause external genital warts, a condition characterized by marked epithelial
proliferation and a low risk of dysplastic changes. In contrast, other genital HPVs including types 16, 18, and 31 are highly associated with dysplastic cervical lesions. These dysplasia-associated, or "high risk" HPV types can be detected in external genital warts removed from immunosuppressed patients. About half of the lesions from immunosuppressed patients contain a high risk HPV type, often in a mixed infection with a "low risk" type such as HPV type 6 or 11.

For an HPV to be assigned a type number, the entire genome must be cloned and three gene regions (E6, E7, and L1) must be sequenced and compared to known types. With the advent of consensus primer PCR amplification for detection of HPV DNA in clinical specimens, it has become clear that there are a significant number of HPVs in addition to the numbered types. The most commonly used consensus PCR system uses degenerate primers located in a conserved region of the L1 (major capsid) gene to amplify a segment of about 450 base pairs. A number of HPV sequences have been found which do not match well with any of the known (numbered) HPV types. These are usually designated by an arbitrary name assigned by the group that first recognized it. In some cases, the same or closely related sequences are independently reported by different groups and given different names. While these almost certainly represent novel HPV types, they are designated by these "unconventional" names until the full genome is cloned and characterized. Several of the uncharacterized HPVs have been identified in specimens from immunocompromised patients.

One such partially characterized HPV is HPV MM7 (also called Pap 291) (Manos, M. M., Waldman, J., Zhang,
T. Y., Greer, C. E., Eichinger, G., Schiffman, M. H., and Wheeler, C. M. (1994) *J. Infectious Diseases* 170(5), 1096-1099). This virus type is closely related to HPV LVX82 (Ong, C. K., Bernard, H. U., and Villa, L. L. (1994) *J. Infectious Diseases* 170(5), 1086-1088), and the two are sometimes referred to as HPV MM7/LVX82. Infection with this HPV type appears to be relatively common compared to other genital HPVs. All published studies that have identified HPV MM7/LVX82 have been performed on cervical lavage or cervical biopsy samples rather than in DNA extracted from genital warts. In one study, HPV MM7/LVX82 was identified in 20 of 338, or 5.9% of cervical samples from patients with low grade dysplasia, and 1 of 43, or 2.3% of high grade dysplasia samples. In a study of cervical HPV acquisition by college women, incident infection with HPV MM7/LVX82 (called Pap 291 in this study) was identified in 15 of 529, or 2.8% of women followed over a period of two years, making it one of the more common types acquired (Ho, G. Y. F., Bierman, R., Beardsley, L., Chang, C. J., and Burk, R. D. (1998) *New England J. Med.* 338(7), 423-428).

In another study, 22 HPV types were identified in cervical lavage samples of human immunodeficiency virus (HIV)-seropositive women, and seven types in lavage samples from HIV-seronegative women (Shah K. V., Solomon, L., Daniel, R., Cohn, S., and Vlahov, D. (1997) *J. Clin. Microbiol.* 35(2), 517-519). Overall, 104 of 150, or 69.3% of HIV-seropositive women had detectable HPV compared to 19 of 72, or 26.4% of HIV-seronegative women. The most prevalent HPV type detected in both groups of women was HPV MM7/LVX82, accounting for 20 of 150, or 13.3% of HIV-seropositive women and five of 72,
or 6.9% of HIV-seronegative women. Of the 123 women with detectable HPV of any type, HPV MM7/LVX82 was the most common type detected, present in 20% of samples.

The development of vaccines against human papillomaviruses (HPV) has been hampered by the lack of a conventional culture system because HPV completes its life cycle only in fully differentiated human tissue. To overcome this obstacle, the athymic mouse xenograft system has been used to study the pathogenesis of HPV 11, and to attempt to develop neutralizing assays for vaccine development (Kreider, J. W., M.K. Howlett, A.E. Leure-Dupree, R.J. Zaino and J.A. Weber. (1987) J. Virol. 61, 590-593; Kreider, J. W., Howlett, M. K., Lill, N. L., Bartlett, G. L., Zaino, R. J., Sedlacek, T. V., and Mortel, R. (1986) J. Virol. 59, 369-376). In this method, an extract from HPV-infected tissue is used to infect human foreskin fragments, which are then implanted under the renal capsules of athymic mice. After ascertaining that an implant is HPV infected, the virus contained in the implant is propagated by additional rounds of xenograft implantation into athymic mice. Recently, HPV 40 has been produced in this system (Christensen N. D., Koltun, W. A., Cladel, N. M., Budgeon, L. R., Reed, C. A., Kreider, J. W., Welsh, P. A., Patrick, S. D., and Yang, H. (1997) J. Virol. 71(10), 7337-7344). Production of an isolate consistent with HPV MM7/LVX82 was also reported. However, an infectious stock of HPV MM7/LVX82 was not obtained. HPV 16 has been produced using a similar system, implanting into mice with severe combined immune deficiency (SCID).

From the foregoing discussion, it is clear that live, infectious stocks of a variety of types of human papillomavirus are needed in order to produce and test
broad spectrum vaccines against these viruses. It is particularly important that such a vaccine is targeted to commonly found HPV types, such as the MM7/LVX82. At present, only a small number of HPV types, HPV 11, HPV 40 and HPV 16, are available as infectious stocks. Accordingly, it is an object of the invention to provide a live, infectious stock of HPV MM7/LVX82. It is a further object of the present invention to provide vaccines directed against HPV MM7, HPV LVX82 and similar types, as well as methods for testing the ability of those vaccines to elicit an appropriate immune response against the selected HPV types.

SUMMARY OF THE INVENTION

According to one aspect of the present invention, a live, infective stock of a human papillomavirus (HPV) of type HPV MM7/LVX82 is provided. This virus stock is substantially free of other HPV types. The live, infective HPV stock comprises a novel human papillomavirus, referred to herein as HPV 83. HPV 83 is related to, but distinct from HPV MM7 and HPV LVX82.

According to another aspect of the invention, an isolated nucleic acid comprising the genome of an HPV MM7/LVX82 type is provided. In a preferred embodiment, the genome is an HPV 83 genome. In a particularly preferred embodiment, the genome comprises the sequence of SEQ ID NO:1 or a sequence that specifically hybridizes with SEQ ID NO:1.

According to another aspect of the invention, a recombinant expression vector is provided, in which is inserted a nucleic acid sequence comprising part or all of the aforementioned HPV MM7/LVX82 genome. Preferably,
the vector comprises portions of the genome that encode
HPV proteins, including E1, E2, E4, E5, E6, E7, L1 or L2. An antigenic epitope, or a virus-like particle, produced
by expression of part or all of this genome is also
provided.

According to another aspect of the invention, a vaccine for immunization against human papillomavirus is
provided, comprising the antigenic epitope or virus-like
particle produced by expression of the HPV MM7/LVX82
genome or part thereof.

According to another aspect of the invention, a method of testing a candidate HPV vaccine for its ability
to elicit a HPV-neutralizing immune response in a mammal
(human or animal) is provided. The method comprises: (a)
providing serum from a mammal immunized with the
candidate vaccine; (b) providing a live, infectious stock
of HPV of type MM7/LVX82 (preferably HPV 83); (c)
contacting an implantable tissue with the infectious HPV
83, in the presence or absence of the serum; (d)
implanting the tissue into an animal host in which the
implantable tissue has been determined to grow, and HPV
infection of the tissue to occur over a pre-determined
time period; and (e) comparing HPV infection in the
implanted tissue contacted with the infectious HPV in the
presence of the serum with HPV infection in the implanted
tissue contacted with the infectious HPV in the absence
of the serum. A reduction in HPV infection in the
presence of the serum is indicative of the ability of the
candidate vaccine to elicit a HPV-neutralizing immune
response in the mammal.

According to another aspect of the invention, a method is provided for testing antiviral agents directed
against HPV, particularly HPV of the MM7/LVX82/83 type.
The method comprises (a) providing a live, infectious stock of HPV of the MM7/LVX82 type; (b) contacting an implantable tissue with the infectious HPV, in the presence or absence of the candidate antiviral agent; (c) implanting the tissue into an animal host in which the implantable tissue has been determined to grow, and HPV infection of, and replication in, the tissue to occur over a pre-determined time period; and (d) comparing HPV infection or replication in the implanted tissue contacted with the infectious HPV in the presence of the candidate antiviral agent with HPV infection or replication in the implanted tissue contacted with the infectious HPV in the absence of the candidate antiviral agent, a reduction in HPV infection in the presence of the agent being indicative of the ability of the candidate antiviral agent to reduce or prevent HPV infection or replication in the mammal.

These and other features and advantages of the present invention will be described in greater detail in the description and examples set forth below.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Fig. 1.** Sequence alignment of HPV 83 consensus primer region (bases 6695-7146 of SEQ ID NO:1) with two previously described HPVs designated MM7 (SEQ ID NO:2) and LVX 82 (SEQ ID NO:3). Positions in which HPV 83 differs from one or both sequences are shown in bold. Regions corresponding to the degenerate primers are indicated by a line above the sequence.

**Fig. 2.** Predicted genetic map of the HPV 83 genome (SEQ ID NO:1), showing the major putative early and late open reading frames, early and late polyadenylation signals, and the location of the
consensus primer 452 base pair region within the L1 open reading frame.

Fig. 3A. Possible E5 ORFs contained in the region between the end of E2 and the beginning of L2 in HPV 83. One potential ORF that extends beyond the early polyadenylation signal is not shown. The putative protein labeled E5a contains no methionine residues and E5b has its only methionine near the carboxy terminus. The numbers to the left of the ORF arrows indicate the three reading frames.

Fig. 3B. The sequence (SEQ ID NO:11) of the putative 47 amino acid E5 protein made from the E5c ORF above is shown. Shown below is the BPV 1 E5 protein (SEQ ID NO:12). No alignment is performed because the two proteins have little true sequence homology, but they are of similar size and both contain a long hydrophobic stretch (boxed).

Fig. 4. Sequence of a portion of the E1/E4, E5 amplifier (SEQ ID NO:13) showing the splice site at the predicted location. The E1 start codon is underlined. The AG splice donor is at nucleotide 733 and the CA splice acceptor is at nucleotide 3226, as indicated. The genomic sequences surrounding the splice site (SEQ ID NO:14 and SEQ ID NO:15) are shown above the amplifier sequence.

Fig. 5A. Predicted origin of HPV 83 DNA replication (SEQ ID NO:16) shown aligned with several other papillomavirus ori sequences (HPV 11 ori is SEQ ID NO:17; HPV 16 ori is SEQ ID NO:18; HPV 18 ori is SEQ ID NO:19). The sequences on the ends are E2 binding sites (ACCN6GGT) and the middle sequence is the E1 binding site. Each dot represents one nucleotide.

Fig. 5B. Position of several predicted cis
elements in the HPV 83 LCR. The sites shown are those with the greatest degree of homology to consensus sequences for those sites as calculated by the MATInspector program (Quandt et al., 1995).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

With reference to nucleic acids, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived; or, if the nucleic acid comprises an entire genome (such as a viral genome), it has been isolated from a virus particle in which it naturally occurs, and inserted into a heterologous DNA molecule. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term
"substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules. As used herein, the term "antigenic epitope" refers to a sequence of a protein that is recognized as antigenic by cells of the immune system and against which is then directed an immune response, such as an antibody response, for example.

As used herein, the term "open reading frame" refers to a length of nucleic acid sequence, between a translation start codon and any one or more of the known termination codons, which can be translated potentially
into a polypeptide sequence. An open reading frame is sometimes referred to herein as a "coding region", since it has the potential to encode a polypeptide.

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. Alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the present invention utilizes the BLAST programs used to query nucleotide sequence similarity in GenBank and other public databases may be used (Altschul et al., Nucl. Acids Res. 25: 3389-3402, 1997). The same software was used to determine protein sequence homologies.

Alternatively, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids.

Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein.
Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to hybridization between single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989, supra):

\[ T_m = 81.5°C + 16.6\log [\text{Na}^+] + 0.41(\% \text{G+C}) - 0.63 (\% \text{formamide}) - 600/\#\text{bp in duplex} \]

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the \( T_m \) is 57°C. The \( T_m \) of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. In a preferred
embodiment, the hybridization is at 37°C and the final wash is at 42°C, in a more preferred embodiment the hybridization is at 42°C and the final wash is at 50°C, and in a most preferred embodiment the hybridization is at 42°C and final wash is at 65°C, with the above hybridization and wash solutions. Conditions of high stringency include hybridization at 42°C in the above hybridization solution and a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in a DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression cassette.

As used herein, the term "expression vector" refers to a plasmid, viral vector, or other vector comprising a sequence desired to be expressed, or an insertion site for such sequence, operably associated with any and all 5' and 3' regulatory sequences necessary for expressing the desired sequence. The terms "expression" or "expressing" refer to the production of an encoded product of the sequence desired to be expressed, which is usually a protein or peptide, but which could be an RNA molecule.

As used herein, the term "capsid coding region" refers to that region of a viral genome that contains the DNA or RNA code for protein subunits that are packaged into the protein coat of the virus particle.

The term "virion" refers to a virus particle
that contains both the capsid protein and the viral genome. Accordingly, a virion is an infectious particle. The term "virus-like particle" refers to a particle that comprises only the viral capsid, and therefore is not infectious.

II. Description

An infectious stock of HPV 83, a genital human papillomavirus related to HPV MM7/LVX82, is provided in accordance with the present invention. The preparation of this infectious stock is summarized below and described in detail in Examples 1 and 2. The successful propagation of a substantially pure stock of HPV 83 using the mouse xenograft system is surprising and unexpected, in view of the failure of others (e.g., Christensen et al., 1997, supra) to propagate an HPV MM7/LVX82 type using the same system. Without intending to be limited by any explanation, it is believed that the inventor's success in propagating a substantially pure HPV MM7/LVX82 type resulted from repeated screenings of samples from immunosuppressed patients (accompanied by numerous failures in identifying a propagatable HPV MM7/LVX82 type), until a particular sample was collected comprising a sufficiently high titer of the virus to enable its successful propagation in the athymic mouse xenograft system.

To identify and isolate additional genital HPV types for use in development of vaccines and elucidation of HPV biology, condylomata acuminata lesions containing a high copy number of HPV and detectable L1 major capsid protein were used to prepare infectious virus stocks. Human foreskin fragments were infected with the virus
preparations and implanted under the renal capsules of athymic mice. After five months of growth, implant tissue was removed and processed for studies to detect HPV infection. Evidence of HPV infection was noted in some of the implants, but in contrast to HPV 11-infected epithelium, the implants derived from the new virus preparations contained a lesser degree of acanthosis; less developed koilocytosis; and a reduced number of preserved nuclei in the hyperkeratotic material within the cyst lining.

The L1 consensus region was amplified by polymerase chain reaction (PCR) from implant DNA and sequenced. Alignment of the amplified sequences with those in the HPV sequence database showed that the 452 bp amplifier was closely related, but not identical to HPV LVX82 and HPV MM7 (also called Pap 291). Within the 452 bp region, the HPV 83 sequence differed at four positions from the HPV LVX82 sequence and at six positions from the HPV MM7 sequence (Fig. 1). However, translation of all three sequences revealed an almost identical 150 amino acid sequence, indicating that HPV 83, HPV MM7 and HPV LVX82 are sequence variants of a single HPV type, classified as HPV 83 as described below.

The presence of virions of the new isolate, HPV 83, in the implants was verified by immunohistochemical detection of L1 major capsid protein and by demonstration of virion particles by electron microscopy. A second extract was made from one of the new implants and used to successfully propagate HPV 83.

Several lines of evidence indicate that the HPV 83-infected implants were substantially, if not completely, free of other HPV types. First, a total of eight implants were examined by PCR and subsequently
sequenced; all amplimers contained the identical HPV 83 sequence. Second, the low stringency Southern blot using probes of HPV types 6, 11, 16, and 18 yielded bands adding up to the approximate size of an HPV genome. These bands did not match the PstI pattern of any common genital HPV type. Third, the high stringency Southern blot using the HPV 83 whole genomic probe yielded a PstI restriction pattern identical to that of the low stringency blot. Fourth, the DNA in situ assay was negative for HPV types 6, 11, 16, 18, 31, 33, and 35. Finally, the hybrid capture assay was negative for the 14 types commonly found in genital tract infection.

The entire genome of HPV 83 was amplified by PCR and cloned. The sequence of the HPV 83 genome is set forth herein as SEQ ID NO:1, and the genome depicted schematically in Fig. 2. Viral proteins and open reading frames of HPV 83 are set forth herein as follows:

E6: SEQ ID NO:4
E7: SEQ ID NO:5
E1: SEQ ID NO:6
E2: SEQ ID NO:7
E4: SEQ ID NO:8
L2: SEQ ID NO:9
L1: SEQ ID NO:10
E5c: SEQ ID NO:11

The DNA sequence of HPV 83, while typical of papillomaviruses in general, has several unique features. First, HPV 83 has the largest genome of all sequenced HPVs and it is larger than all but a few animal papillomaviruses (e.g., deer papillomavirus, 8,374 base pairs, and canine oral papillomavirus, 8,607 base pairs). The size difference is primarily due to a relatively long region between the end of the E2 ORF and the beginning of
the L2 ORF (the "E5" region) and a comparatively long LCR. The long E5 region of HPV 83 does not contain an easily identified E5 ORF. The most likely candidate for an authentic E5 coding sequence occupies only about one quarter of the E5 region. However, because of the sequence surrounding the potential initiation site and the general similarity of this candidate HPV 83 E5 protein to the BPV 1 E5 protein, it is likely to be the authentic product. The putative HPV 83 E5 protein lacks two features of the BPV 1 E5 protein. First, the HPV 83 protein contains no cysteine residues and so cannot exist as a disulfide-linked dimer as is thought to be important for the BPV protein. Second, the HPV 83 protein does not contain a glutamine residue adjacent to the hydrophobic domain, a feature critical to the function of the BPV protein.

Neither of the two most closely related HPV types (HPV 61 and 72) has an apparent E5 ORF, including a BPV-like E5. It is unlikely that any of the other potential ORFs in the E5 region of HPV 83 encode any proteins unless there is an unrecognized splice site that joins one of these sequences with an upstream ORF. However, amplification and cloning of the E1^E4, E5 cDNA did not reveal any unexpected splice sites.

The HPV 83 E4 ORF also varies from other HPVs in the way that the E1^E4 spliced product (possibly the only authentic gene product of the E4 ORF) is constructed. For example, in HPV 11, the E1^E4 splice occurs in the fifth codon downstream from the E4 initiation codon. In contrast, the HPV 83 splice acceptor is at nucleotide 3,226, in the 15th codon downstream from the most proximal in-frame methionine codon at nucleotide 3,191. This difference raises the
possibility that an E4 gene product that is significantly
different from the E1^E4 gene product could be produced.

Despite its increased size, the HPV 83 LCR does
not contain any recognizable novel cis elements. In
fact, the HPV 83 LCR has fewer of some elements (e.g., E2
binding sites) than most HPVs and lacks other features
(e.g., YY-1 sites and glucocorticoid responsive elements)
that are common in many HPVs. HPV 83 does appear to have
all of the essential elements such as the probable origin
of DNA replication and standard promoter elements.

HPV 83 is a relatively prevalent genital tract
papillomavirus in a variety of patient populations,
including patients with HIV infection. Based on the
known epidemiology of HPV 83 and the structure of the
putative E7 protein, HPV 83 should be considered to be at
least an intermediate risk HPV type.

Because HPV 83 (and HPV MM7/LVX82, as
previously reported) appears to be a relatively common
genital type, especially in immunocompromized women, the
number of cases of cervical abnormalities caused by this
type is likely to be relatively large. A preferred
method of reducing the incidence of genital tract HPV
infection is development of effective vaccines. It is
likely that type-specific vaccines will be required to
protect against the many HPVs known to exist. To prepare
effective type-specific vaccines, it is necessary to
identify, sequence, and establish neutralization assays
for prevalent genital HPV types. Recently identified and
uncharacterized HPVs such as HPV MM7/LVX82 and HPV 83 are
clearly of medical importance, given their prevalent
distribution, especially in immunocompromised women. In
addition to the well characterized genital HPVs such as
HPV types 6 and 16, many of these newly identified types
should also be included in effective vaccines.

Live, infective stocks of HPV 83 are prepared by grinding the infected mouse tumor tissue in an appropriate buffer, e.g., phosphate-buffered saline, then freezing the tissue suspension. The live, infective HPV 83 stock provided in accordance with the present invention is used to produce and/or test a vaccine, or a component of a multi-target vaccine, to elicit neutralizing antibodies against different HPV types.

Such vaccines are expected to elicit an immune response against HPV 83 and variants thereof, such as HPV MM7 and HPV LVX82, among other variants which have not yet been identified.

Inf ective stocks of HPV 83 can be used in any standard methodology to test candidate vaccines directed to HPV. In a preferred embodiment, the neutralization assay in athymic mouse xenografts described by Bryan et al. (J. Med. Virol. 53: 185-188, 1997) is used. The steps are as follows. First, HPV 83 virions are purified by cesium gradient centrifugation from the HPV-infected foreskin tissue described above. The protein content of the HPV 83 preparation is determined by measuring the total protein content of the preparation, then estimating the percentage of the major capsid (L1) protein in the preparation using SDS polyacrylamide gel electrophoresis and immunoblotting with anti L1-serum.

To prepare polyclonal antisera against HPV 83, rabbits are immunized with an appropriate amount, e.g., 10 ng, of non-denatured virions emulsified in an adjuvant, such as Freund's adjuvant, followed by four booster immunizations of an equivalent amount of antigen in incomplete adjuvant, given at two-week intervals after the first immunization. Sera from the rabbits are
collected about nine weeks after immunization. The amount of anti-HPV 83 IgG in the rabbit serum is quantified by capture ELISA (enzyme-linked immunosorbent assay), using HPV 83 virus-like particles (VLPs), prepared as described below.

To perform the neutralization assay, foreskin tissue is obtained from routine circumcision, the dermis is removed, and the remaining epidermal tissue is cut into small fragments. Tissue fragments are added to a tube containing medium in which is dispersed a known quantity of HPV 83 virions, along with various dilutions of the antiserum (including a control in which no antiserum is added). Following a short incubation at 37°C, the fragments are implanted under the renal capsules of athymic mice. To ensure HPV infection adequate for detection, mice are not killed until 10 weeks after implantation. Implants are then removed from the mice, measured and placed in formalin solution for preparation of paraffin-embedded sections.

For the DNA in situ assay to detect the presence or absence of HPV 83 infection, the sections of foreskin implants are deparaffinized and processed with the Digene Tissue Hybridization Kit (Digene Diagnostics, Beltsville MD) according to manufacturer’s instructions. Following deparaffinization, sections are hybridized with a biotinylated HPV 83 DNA probe. Detection of the hybridized probe is performed by incubation of slides with a streptavidin-alkaline phosphatase conjugate, followed by reaction with a colored product-forming substrate. Cells positive for HPV DNA are identified as purple nuclear-staining of differentiated epithelial cells in the foreskin implants by light microscopy. Neutralization of HPV 83 is demonstrated by an absence of
HPV DNA in the DNA in situ hybridization assay.

HPV 83 virions are also used to screen for antiviral agents (see, e.g., Kreider et al., Antiviral Res. 14: 51-58, 1990). This is accomplished using the mouse xenograft system in a manner similar to that described above for identifying neutralizing antibodies. Infection and implantation is carried out in the presence or absence of a candidate antiviral agent, and the effect of the agent on subsequent infectivity and replication of the virus in the graft tissue is observed.

The genome of HPV 83 has been cloned and sequenced in accordance with the present invention. This material is used to produce an HPV 83 vaccine, for use alone, or as part of a multi-component HPV vaccine. A preferred method for developing such a vaccine is to subclone a segment of the genome encoding a particularly antigenic component of HPV, such as the major (L1) capsid protein, and thereafter expressing capsids or virus-like particles in a suitable eucaryotic expression system, such as vaccinia virus or baculovirus, among others. Expressed capsids or VLPs are purified and used as vaccines to elicit a neutralizing immune response in persons at risk of HPV infection (for a review of HPV VLPs and their uses, see Hagensee & Galloway, Papillomavirus Report 4: 121-124, 1993).

The following specific examples are provided to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way.

EXAMPLE 1
Identification and Characterization of HPV 83 and Propagation in Athymic Mouse Xenograft System
To identify and characterize additional genital HPV types for elucidation of HPV pathogenesis and development of vaccines, an extract was prepared from genital lesions removed from immunosuppressed patients, pregnant women, and from patients with large lesions containing L1 protein. This example describes the identification of a new HPV isolate (originally referred to as HPV IU, later referred to as HPV 83) from this source, and its successful propagation in the athymic mouse xenograft system.

**MATERIALS AND METHODS**

**Preparation of virus extract from tissue.**

Thirty-three excision biopsies of exophytic condylomata acuminata lesions were performed as previously described (Brown et al., J. Infectious Diseases 170: 759-765, 1994). All of the biopsy samples were analyzed by Southern blot analysis or hybrid capture for detection of HPV DNA (Brown et al., J. Clin. Microbiol. 31: 2667-2673, 1993). Twenty-seven of the lesions were removed from patients with conditions known to be associated with defects in cellular immunity. Nine of the lesions were removed from patients who were iatrogenically immunosuppressed following transplantation of kidney or liver; seven were removed from patients infected with the human immunodeficiency virus (HIV); and eleven were from pregnant women. The remaining six patients were not immunosuppressed or pregnant, but had unusually large genital lesions, thus providing abundant infected tissue.

Lesions from these 33 patients were combined in a large polypropylene tube with 100 ml of Solution 1, (50 mM NaPO₄ (di/mono) pH 8.0, 200 mM NaCl, 10 mM EDTA) prechilled to 4°C. Using a Biosonik IV tissue homogenizer
(Bronwill, Rochester, NY) on the lowest setting, the
tumor tissue was homogenized to a thick suspension and
the extract chilled on ice. The tissue extract was
centrifuged for five minutes at 5000 X g at 4°C. The
supernatant was collected and stored on ice. An
additional 50 ml of Solution 1 was added to the pellet,
and ultracentrifugation was repeated as above. The two
low speed supernatants were combined and diluted with an
additional 50 ml of Solution 1, and ultracentrifuged at
20,000 X g at 4°C for two hours to pellet HPV. The
supernatant was discarded, and the resulting high speed
pellet was suspended in 5 ml of Solution 2 (50 mM NaPO₄
(di/mono) pH 7.4, 10 mM NaCl, 10 mM EDTA).

Half of this suspension derived from the high
speed pellet was reserved ("high speed pellet"), and to
the other half, CsCl was added to a density of 1.34 g/ml.
Centrifugation was performed at 50,000 X g at 20°C for 18
hours. A faint band was visualized by shining a small
light up through the bottom of the tube. This band was
removed using an 18 gauge needle. Additional Solution 2
plus CsCl was added to a density of 1.34 g/ml, and
centrifugation was repeated. A visible band was removed
and dialysis was performed against three changes of
phosphate buffered saline, pH 7.4, and the resulting
banded virus preparation was stored at -70°C.

**Infection and implantation of tissue into
athymic mice.** Infection of human foreskin fragments and
growth in athymic mice was performed as described
originally by Kreider et al. (J. Virol. 59: 369-376,
1986, 1987) with slight modifications as previously
described (Brown et al., Virology 214:, 259-263, 1995).
Experiments were performed using the two preparations
described above (high speed pellet suspension and banded
-24-

virus), using eight athymic mice for each virus preparation. Additional experiments were performed using extracts from tumors resulting from the first experiments to demonstrate propagation of virus in the athymic mouse xenograft system. Mice were sacrificed 150 days after implantation of foreskin fragments.

**Light microscopy, immunohistochemistry, and electron microscopy.** A portion of each implant was placed in zinc formalin, and paraffin-embedded sections were prepared. One section was stained with hematoxylin and eosin. Histologic evaluation of sections was performed, using uninfected implant tissue and HPV 11-infected human foreskin implants grown in athymic mice for 150 days for comparison (Brown et al., Virology 201: 46-54, 1994). Additional sections were deparaffinized and used to detect L1 protein with a rabbit polyclonal antiserum raised against a bacterially expressed HPV 11 trpE/L1 fusion protein, as previously described (Brown et al., Virology 1994, supra). This antiserum has been shown in our laboratory to react with L1 protein from several HPV types. Preimmune rabbit serum was used as control serum. Antibody binding was detected using the Vectastain ABC Kit (Novocastra, Newcastle upon Tyne, UK). Positive cells for L1 protein were identified by brown staining of nuclei in differentiated keratinocytes. For electron microscopy, a portion of an implant was fixed in 3% glutaraldehyde, and the tissue was embedded and sectioned. Sections were stained with uranyl acetate.

**Hybrid capture assay and Southern blots.** DNA was extracted from implants as previously described (Brown et al., J. Clin. Microbiol. 1993, supra). The hybrid capture assay, marketed as ViraType Plus® by Digene Diagnostics, Beltsville, MD was used to detect HPV
sequences in the implants (Id.). HPV probes were used in two pools whose composition was based on the association of each type with genital tract malignancy. Probe group A contained the "low risk" HPV types 6, 11, 42, 43, and 44 while probe group B contained the "high risk" HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56.

For Southern blots, genomic DNA from an implant that contained histologic features of HPV infection was digested with one of several restriction endonucleases: PstI, BamHI, HincII, EcoRI, or no added restriction endonuclease. The probes used in the first Southern blot (low stringency) were a mixture of HPV types 6b, 11, 16, and 18, all cut from pUC 19 and gel purified. Southern blots were performed as previously described (Brown et al., J. Clin. Microbiol. 1993 supra). For the low stringency blot, prehybridization, hybridization, and washes were performed at 55°C. A second Southern blot was performed at high stringency conditions (65°C hybridization and wash) using the PCR-amplified whole genome of the new isolate, HPV 83, as a probe (see below).

Consensus primer PCR, cloning and sequencing.

PCR was performed using the primer pair My09/My11 to amplify HPV sequences in genomic DNA extracted from a total of eight athymic mouse implants (two from the initial experiments and six from the propagation experiments) (Manos et al., Cancer Cells 7: 209-214, 1989). A total of 250 ng of DNA from each implant was used as template for each of eight individual PCRs. The resulting 452 base pair (bp) amplimers were cloned into pCR3.1 (Invitrogen, Carlsbad, CA) and DNA sequencing was performed.

Amplification and cloning of the entire HPV 83
genome and DNA in situ assay. To amplify and clone the genome of HPV 83, total DNA was isolated from an implant containing histologic features suggesting HPV infection. PCR was performed using the Extend Long PCR Kit as instructed by the manufacturer (Boehringer Mannheim, Indianapolis, IN). This PCR system uses two polymerases, one without proofreading (Taq) and one with proofreading (Pwo). The primer pair was designed using the 452 bp sequence amplified with L1 consensus primers. The primers were 5' CAG TAG GAT CCT TAT GAT GGC TTG TAT TTT GGG AGG TTG AT (SEQ ID NO:20) and 3' GCT ATG GAT CCT TTT TAG GGG CAG GGG CGG AAG GA (SEQ ID NO:21). Conditions for PCR were 94°C for 30 seconds, 66°C for 30 seconds, and 68°C for twelve minutes, for a total of 38 cycles.

Agarose electrophoresis revealed a band of approximately 7.9 kilobase pairs (kb) which was excised from the gel and purified with Gene Clean as instructed by the manufacturer (Bio 101, Vista, CA).

The 7.9 kb amplified DNA fragment was cloned into the Expand Vector 1 (Boehringer Mannheim) as directed by the manufacturer. This cloning method facilitates cloning of large PCR products by size selection of PCR fragments provided by the lambda packaging system, eliminating cloning of small DNA fragments. To prepare a probe for DNA in situ hybridization, the fragment was removed from the vector by BamHI endonuclease digestion and gel purification. Nick translation of the fragment was performed with the BioNick kit (Life Technologies, Gaithersburg, MD) to biotinylate the probe. DNA in situ hybridization was performed using this biotinylated probe with the PathoGene Kit as directed by the manufacturer (Enzo Diagnostics, Farmingdale, NY). Positive cells were
identified by blue staining of nuclei in differentiated keratinocytes.

RESULTS

**Gross and microscopic characterization of HPV-infected implants.** In an effort to propagate additional HPV types, an extract was made from 33 lesions known to contain large quantities of HPV DNA and HPV L1 capsid protein. Hybrid capture analysis of the high speed pellet and the banded virus preparation showed that abundant viral DNA was present for the A and B probe groups. Probe group A contained the "low risk" HPV types 6, 11, 42, 43, and 44 while probe group B contained the "high risk" HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56. Each of these preparations were used to infect human foreskin fragments which were then implanted into athymic mice. Mice were sacrificed 150 days after implantation of foreskin tissue, and implants were removed from the mice. Four implants from the first experiment, two using the high speed pellet and two using banded virus, appeared much larger with a rougher texture than the other twelve implants. The larger tumors appeared to adhere to the kidney surface and extend outward, clearly demarcated from the mouse kidney tissue. When cut transversely, the implants were thin walled and contained solid, dry cellular debris.

Uninfected and HPV 11-infected implants grown in athymic mice were used as controls for comparison of histology. In the uninfected control implants grown for 150 days, the epithelial tissue consisted of a single basal layer with four to six additional layers of flattened intermediate squamous cells and a thin granular layer. Cellular debris and keratinized material was
present in the center of the implant. No nuclei were present in the material in the center of the implant. In contrast, the HPV 11-infected implant demonstrated marked epithelial thickening (acanthosis). The parabasal layer was expanded and involved approximately one-third of the epithelium. Papillomatosis (papillary projections of the epidermis forming a microscopically undulating surface) was present, with invagination from the underlying stroma by fibrovascular supports. The suprabasal layer of the HPV 11-infected implant contained well developed koilocytes containing nuclei that were enlarged two to three times compared with nuclei of suprabasal cells in uninfected implants. Marked parakeratosis (retention of nuclei in cells of the stratum corneum) was observed as well as accumulation of cellular debris and hyperkeratotic material within the implant. In addition, nuclei were present in abundance in the shed material in the center of the implant.

In implants infected with HPV 83, there were areas of mild parabasal hyperplasia with increased mitotic activity. While epithelial thickening was noted compared to the uninfected implant, HPV 83 implants were significantly less acanthotic than the HPV 11 implant after the same 150 days of growth. In addition, hypergranulosis (an abundance of granular cytoplasmic structures) and mild parakeratosis were present. Similar to the HPV 11-infected implant, the epithelium was abnormal throughout, with marked nuclear enlargement in the suprabasal and subcorneal zones. Nuclei were at least three times enlarged compared to normal suprabasal cell nuclei. Koilocytic vacuole development was present although not nearly as flagrant as was seen in the HPV 11-infected implant. For subcorneal cells, a
higher nuclear to cytoplasmic ratio was observed in the HPV 83-infected implant compared to the HPV 11-infected implant. Basal cell crowding was seen, occasionally very focal in nature. In addition, parakeratosis was observed as well as accumulation of cellular debris and hyperkeratotic material within the implant. In contrast to the HPV 11-infected implant, very few nuclei were present in the center of the HPV 83-infected implants.

In summary, the main histologic differences between the implants infected with HPV 83 and the HPV 11-infected implant were 1) a lesser degree of acanthosis in the implants infected with the new virus preparation; 2) less developed koilocytosis in the implants infected with the new virus; and; 3) a markedly reduced number of preserved nuclei present in the hyperkeratotic material within the implants infected with HPV 83 compared to HPV 11-infected implants.

Expression of HPV capsid antigen and whole virus. Immunohistochemical assays were performed using a polyclonal antiserum against a bacterially-expressed HPV 11 trpE-L1 fusion. Dark brown staining of nuclei of differentiated keratinocytes was seen in implants containing the histologic abnormalities described above. By analogy with other papillomavirus systems, this observation suggests that the most differentiated cells were making capsid protein and were likely to be producing virus particles.

Electron microscopy was performed to verify the production of virions. Electron micrographs revealed abundant particles of approximately 50 nm in the nuclei of numerous differentiated cells. These particles resembled typical icosahedral papillomavirus. Both apparently full (appearing dense and dark) and empty
(appearing less dense) capsids were observed.

Detection and characterization of HPV 83 DNA.

Hybrid capture analysis was performed as a screen for
common genital HPV types on DNA purified from HPV 83-
infected implants with abnormal histology. These studies
were negative with both the A and B probe groups,
indicating that a common genital HPV type was not present
in the new implants.

Southern blot analysis was performed at low
stringency using a mixture of HPV types 6b, 11, 16, and
18 genomic DNAs as probes. The low stringency Southern
blot analysis of PstI-digested DNA from implants infected
with HPV 83 showed faint bands of approximately 2900,
1600, 1200, 900, 800 bp after exposure of the
autoradiogram for seven days. As controls, a high copy
number sample containing HPV 6c and a low copy number
condylomata acuminata lesion containing HPV 16 were
included in the blot. The faint bands seen in the seven
day exposure did not correlate with any PstI restriction
patterns for HPV types commonly associated with genital
warts.

L1 consensus primers were used to amplify HPV
sequences from the new implants and the amplimers were
cloned and sequenced. Alignment of the amplified
sequences with those in the HPV sequence database showed
that the 452 base pair PCR product was nearly identical
to HPV LVX82 (Ong et al., J. Infect. Dis. 170: 1086-1088,
1994) and HPV MM7 (also called Pap 291) (Manos et al.,
1994, supra), two uncharacterized (and unnumbered) HPVs
(Fig. 1). Eight independent clones from both initial and
passaged implants were sequenced and were identical to
each other. All eight differed at four positions
(outside of the degenerate primer region) from the HPV
LTX82 sequence and at six positions from the HPV MM7 sequence. However, translation of all three sequences yielded an identical 150 amino acid sequence (with the exception of one change encoded in the primer region).

It is therefore likely that HPV LTX82, HPV MM7, and the new isolate, HPV 83, represent sequence variants of a single yet unnumbered HPV type.

Using PCR primers extending outward from the L1 consensus primer region, the entire genome of HPV 83 was amplified, cloned and sequenced (Fig. 1; SEQ ID NO:1). The full length DNA was then used as a probe for high stringency Southern blotting. A similar PstI pattern was visualized in the high stringency blot as was seen on the low stringency blot, with an obvious doublet present of approximately 800 and 900 bp. The sum of PstI restriction fragments was very close to 7900 bp, with bands corresponding to approximately 2900, 1600, 1200, 900, 800 and 500 bp. Digestion with BamHI resulted in a single band at 7.9 kb, though sequencing revealed that the HPV 83 genome is actually about 8100 base pairs, one of the largest HPV genomes. Digestion with HincII resulted in a dark, wide band at approximately 4 kb, suggesting that two bands of nearly equal size were generated from episomal HPV DNA. This indicated the presence of two HincII sites in the HPV 83 genome. EcoRI digestion resulted in a high molecular weight band similar to the band present when no endonuclease was added, suggesting that no EcoRI site was present.

To demonstrate the presence of HPV 83 DNA and to exclude the presence of other common genital HPV types, implants were analyzed by DNA in situ hybridization using the whole genomic HPV 83 probe, or one of three probe mixes consisting of HPV types 6 and
11, HPV types 16 and 18, and HPV types 31, 33, and 51. Only hybridization with the HPV 83 probe resulted in positive signals. No positive signals were present in sections hybridized with the other HPV probe groups, suggesting an absence of these other HPV types in the new implants.

**Propagation of HPV 83.** To verify that HPV 83 could be propagated in the athymic mouse xenograft system, an extract was made from an implant from the initial experiment and used to infect a second human foreskin. Fragments of this foreskin were implanted into 16 athymic mice. Mice were sacrificed after 150 days of growth and implants were removed. Of the 32 passaged implants, 20 were significantly larger than the remaining implants and had the same outward extension and demarcation from kidney tissue as appeared in the HPV 83-infected implants from the initial experiment. The remaining 12 implants were spherical and smooth, with the typical appearance of uninfected foreskin implants.

Histological analysis of these larger passaged implants was similar to that of the original group of HPV 83 implants. Histologic analysis of the smaller implants resembled that of uninfected implants grown in athymic mice. As described above, the DNA sequences of the 452 bp amplimers from passaged, HPV 83-infected implants were identical to those from the original implants.

**EXAMPLE 2**

**Further Characterization of HPV IU and its Classification as Human Papillomavirus Type 83**

Example 1 describes the propagation of a novel genital HPV type using the athymic mouse xenograft system and cloned the genome of this HPV. In this example, we
describe the nucleotide sequence and unique properties of the 8,104 base pair genome of this virus, now classified as HPV 83.

5 MATERIALS AND METHODS

Cloning and sequencing of HPV 83 DNA. The full-length HPV 83 genome was amplified from a human foreskin implant recovered from an athymic mouse as described in Example 1. The sequence of the HPV 83 insert was determined by starting in the vector sequence at both ends and "walking" through the insert using primers designed from the most recent sequence in that direction. Sequencing was done on an ABI Prism Model 377 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). A total of 32 sequencing primers were used for the primary sequence determination. The overlapping sequence fragments were assembled manually and confirmed from the complementary strand. Several additional primers were used to clarify sequence ambiguities. In addition, segments in the E5 region and LCR were amplified and subcloned from infected tissue and sequenced to verify the sequence obtained from the original clone.

Once assembled, the sequence was analyzed for homology to other HPVs using the basic local alignment sequence tool (BLAST) software (Altschul et al., Nucl. Acids Res. 25: 3389-3402, 1997). The same software was used to determine protein sequence homologies. The long control region was searched for cis elements using the MATInspector software (Quandt et al., Nucl. Acids Res. 23: 4878-4884, 1995).

Southern Blots. DNA was extracted from foreskin implants and Southern blots performed as
previously described in Example 1, except that a 0.6% agarose gel was used. A mixture of cloned, linearized HPV 11 and HPV 83 DNA was labeled with $\alpha^{32}$P dATP by nick translation and used as a probe for the blot.

**Amplification and cloning of the E1^E4, E5 spliced transcripts.** Total RNA was isolated as previously described from the viable capsule tissue on an athymic mouse human foreskin xenograft infected with the HPV 83 extract and grown for four months (Brown et al., Virology 222: 43-50, 1996). Reverse transcription was performed followed by a polymerase chain reaction using primers designed to amplify a predicted E1^E4 spliced transcript (5' primer GTGTGAAGACGCAGACCTAA, position 635-654, SEQ ID NO:22; 3' primer GAATGGCTGTTGTTGTGGTT, position 4044-4063, SEQ ID NO:23).

**RESULTS**

**Sequence.** Sequencing of the cloned viral DNA revealed a genome of 8,104 base pairs, making it the largest among characterized genital HPVs. The sequence is set forth herein as SEQ ID NO:1 and is also available in Genbank under accession number AF151983. The sequence and clone were submitted to the Referenzzentrum für humanpathogene Papillomviren (Heidelberg, Germany) and the virus was assigned the number HPV 83. To confirm the larger size of the HPV 83 genome, DNA was purified from HPV 11- or HPV 83-infected human foreskin implants, digested with Bam HI (a single cut enzyme for both DNAs) and run on an agarose gel. A Southern blot of that gel demonstrated that the HPV 83 genome is slightly larger than that of HPV 11. A BLAST homology search showed that HPV 83 is most closely related to HPV 61 (71.2% homology) and HPV 72 (71.4% homology). HPV 61 and HPV 72 were also
identified in mucosal lesions, but have not been found commonly in surveys of normal or abnormal genital specimens. This relationship places HPV 83 in the papillomavirus homology group A3; the sequence fragment of HPV 83 called MM7 had been tentatively placed in group A3 and this sequence confirms that assignment (see the Los Alamos papillomavirus sequence compendium at http://cds.lanl.gov/HTMLFILES/HPVcompintro04.html for current homology information and for summaries of papillomavirus sequences and homologies).

Examination of the sequence for potential genes showed the typical complement of papillomavirus open reading frames, with the exception of E5 (see below). The putative open reading frames are summarized in Table 1.

**Table 1: Location of Putative HPV 83 ORFs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start</th>
<th>First ATG</th>
<th>Stop</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>8071</td>
<td>1</td>
<td>444</td>
</tr>
<tr>
<td>E7</td>
<td>414</td>
<td>420</td>
<td>713</td>
</tr>
<tr>
<td>E1</td>
<td>694</td>
<td>718</td>
<td>2673</td>
</tr>
<tr>
<td>E2</td>
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<tr>
<td>E5*</td>
<td>4046</td>
<td>4112</td>
<td>4255</td>
</tr>
<tr>
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* Tentative designation, see text

Table 2 shows the relatedness of putative HPV 83 proteins to the analogous proteins of several other papillomavirus types.

**Table 2: Homology of putative HPV 83 ORFs with those of other HPVs**
-36-

<table>
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<th>E6</th>
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* The putative HPV 83 E5 protein has no sequence homology to any other HPV E5 protein.

As expected, HPV 83 proteins are predicted to be most closely related to HPV 61 or 72 proteins. Most HPV 83 proteins also were closely related to homology group A4 HPV types 2A, 27 and 57 proteins. The relationship with other common HPV types such HPV 11 and HPV 16 was substantially less.

**E7 protein homology.** Based on available epidemiologic evidence, it is not certain whether HPV 83 should be classified as a high cancer risk HPV type or as a low risk type. HPV 83 DNA (identified in those studies as either MM7, LVX82, or PAP291) has been found in some dysplastic cervical lesions and at least one cervical cancer as well a number of specimens from patients with normal cytology. Examination of the sequence of the putative E7 protein may provide some insight into the predicted biological behavior. Two regions of the E7 protein have been shown to be critical for transformation and these regions are homologous to the adenovirus E1A protein conserved regions (CR) 1 and 2. In mutational analyses of the E7 amino terminal region homologous to
CR1, 4 individual residues have been defined as critical for immortalization or transactivation. Eight of the first 17 amino acids of HPV 83 and HPV 16 E7 proteins, including the critical residues 2, 10, 13, and 17, are identical and two others are conservative changes. Both HPV 11 and HPV 18 E7 proteins share similar numbers of amino acids with the HPV 16 protein, but neither is a perfect match at all 4 critical positions. An alignment of the critical pRB binding region (analogous to CR2) of HPV 83 E7 with those of other HPV types shows the presence of an aspartic acid residue just before the characteristic L-X-C-X-E motif. The presence of this an aspartic acid residue in that position is more common in high risk HPV types than in low risk types (e.g., HPV 6/11, which contains a glycine at that position) and has been associated with increased affinity for pRB binding and increased transforming activity.

**E5 ORF region.** One of the regions of HPV 83 DNA that accounts for the larger size compared to some other HPVs is the region between the end of the E2 ORF and the beginning of the L2 ORF that usually contains the E5 ORF plus some cis elements, such as the early polyadenylation signal. In HPV 83 DNA this distance is 592 base pairs compared to 384 base pairs in HPV 16, 330 base pairs in HPV 18, and 330 base pairs in HPV 31.

However, the comparable distance is 582 base pairs in HPV 11 and 549 base pairs in HPV 6, so some types do contain "E5" regions of similar length. Despite this elongated region, HPV 83 DNA contains no ORF in this area that encodes a potential E5 protein with any homology to a known papillomavirus E5 protein. A search of this region for potential ORFs reveals four stretches of sequence that could encode proteins of less than 100 amino acids.
each. One of these potential ORFs extends beyond the early polyadenylation signal and so is unlikely to be authentic. The three remaining ORFs are shown in Fig. 3A. Note that E5b and E5c are in the same reading frame and are separated by a single stop codon. E5a contains no methionine residues while E5b has a single methionine residue 22 amino acids from the carboxy terminus. E5c contains a methionine residue 23 amino acids from the amino terminus, potentially encoding a 47 amino acid protein. In addition, the three nucleotides immediately upstream from the methionine codon are ACT, similar to the ACC sequence that is preferred for ribosome internal initiation. While this potential protein has no homology to any known E5 protein, it does contain a large hydrophobic domain (16 consecutive residues of leucine, isoleucine, or valine) similar to the 44-amino acid bovine papillomavirus type 1 E5 protein (13 consecutive residues of leucine, phenylalanine, or valine (Fig. 3B). We propose that the region labeled E5c is likely to encode the authentic HPV 83 E5 protein.

**Characterization of the E1^E4, E5 transcript.**

To further characterize the predictions based on the sequence, we identified a potential splice donor at nucleotide 733 and splice acceptor at nucleotide 3226 that would be utilized to produce an E1^E4,E5 transcript. The E1^E4,E5 transcript is produced in abundance in tissue infected with HPV 11 and several other HPV types. This assumption predicts that PCR primers located at nucleotide 635 and 4063 should produce a cDNA amplifier of 935 base pairs from the spliced transcript. If there were other splicing events involving some of the other potential ORFs in the E5 region, additional products might be produced. PCR primers located at 635 and 4063
were used to copy and amplify RNA purified from human foreskin implants infected with HPV 83 stock virus. An amplimer of the predicted size (935 base pairs) was visualized by ethidium bromide staining of an agarose gel. The amplimer was excised from the gel, cloned into pCR3.1 (Invitrogen Corp., Carlsbad, CA), and sequenced to confirm the location of the splice site. The sequence of the cloned cDNA around the splice site as well as the genomic sequences surrounding the splice donor and acceptor sites are shown in Fig. 4.

**The long control region (LCR).** The sequence between the end of the L1 ORF and the beginning of the E6 ORF is called the LCR because it contains many of the cis-acting regulatory sequences that control transcription and replication. The LCRs of HPV types 11, 16, 18, and 31 are 673, 833, 826, and 954 base pairs long, respectively. The LCR of HPV 83 is 851 base pairs in length, making it one of the longest among HPVs. The combination of a long E5 region and a long LCR accounts for nearly all of the additional ~200 base pairs of DNA in HPV 83 compared to other HPVs.

One of the elements present in papillomavirus LCRs is the origin of DNA replication. The origin sequence typically contains two E2 binding sites (ACCN6GGT) with an E1 binding site between (Lu et al., 1993; Mendoza et al., 1995; Sedman and Stenlund, 1995; Spalholz et al., 1993; Sun et al., 1996). The HPV 83 LCR contains three E2 binding sites including sites at 7,958 and 8,037 that contain an E1 binding site between them. This site probably represents the HPV 83 origin of DNA replication. It is shown aligned with other origin sequences in Fig. 5A.

Papillomavirus LCRs also contain multiple
binding sites for transcriptional regulatory factors such as Oct-1, AP-1, and Sp-1 among others. The HPV 83 LCR has many of these putative binding sites that are consistent with those found in other papillomaviruses. These sites include TATA boxes at positions 8061 and 8067, just upstream from the beginning of the E6 ORF at position 8071 and 43 and 37 base pairs upstream from the E6 initiation codon at position 1. The predicted locations of these sites are shown in Fig. 5B. The HPV 83 LCR differs somewhat from other HPV LCRs by having only one E2 binding site near the probable E6 start site, but it is similar to other HPVs because the promoter-proximal E2 site partially overlaps two Sp-1 sites (Rapp et al., 1997). Unlike many HPV LCRs, HPV 83 contains no YY-1 binding sites and no glucocorticoid responsive elements.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.
We claim:

1. A live, infective stock of a human papillomavirus (HPV) of type HPV MM7/LVX82, said stock being substantially free of other HPV types.

2. The live, infective HPV stock of claim 1, which is HPV 83.

3. An isolated nucleic acid molecule comprising a genome of the HPV of claim 1.

4. The nucleic acid molecule of claim 3, wherein the genome specifically hybridizes with SEQ ID NO:1.

5. The nucleic acid molecule of claim 4, wherein the genome comprises SEQ ID NO:1.

6. A recombinant expression vector in which is inserted part or all of the nucleic acid molecule of claim 3.

7. The recombinant expression vector of claim 6, in which is inserted a part of the nucleic acid molecule of claim 3 encoding an HPV polypeptide selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2.


9. The antigenic epitope of claim 8, comprising part or all of a capsid protein.
10. The antigenic epitope of claim 8, comprising part or all of a polypeptide encoded by an E5 open reading frame.

11. A vaccine for immunization against human papillomavirus, comprising the antigenic epitope of claim 8.


13. A vaccine for immunization against human papillomavirus, comprising the virus-like particle of claim 12.

14. An isolated human papillomavirus, which is HPV 83.

15. An isolated nucleic acid molecule comprising a genome of the HPV 83 of claim 14.

16. The nucleic acid molecule of claim 15, wherein the genome comprises a sequence that specifically hybridizes with SEQ ID NO:1.

17. The nucleic acid molecule of claim 16, wherein the genome comprises SEQ ID NO:1.

18. A recombinant expression vector in which is inserted part or all of the nucleic acid molecule of claim 15.

19. The recombinant expression vector of claim 18, in which is inserted a part of the nucleic acid molecule of claim 15 encoding an HPV polypeptide selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2.

21. The antigenic epitope of claim 20, comprising part or all of a capsid protein.

22. The antigenic epitope of claim 20, comprising part or all of a polypeptide encoded by an E5 open reading frame.

23. A vaccine for immunization against human papillomavirus, comprising the antigenic epitope of claim 20.


26. A method of testing a candidate HPV vaccine for its ability to elicit a HPV-neutralizing immune response in a mammal, which comprises:

a) providing serum from a mammal immunized with the candidate vaccine;

b) providing a live, infective stock of a HPV of type MM7/LVX82;

c) contacting an implantable tissue with the infective HPV, in the presence or absence of the serum;

d) implanting the tissue into an animal host in which the implantable tissue has been determined to grow, and HPV infection of the tissue to occur over a pre-determined time period; and
e) comparing HPV infection in the implanted tissue contacted with the infectious HPV in the presence of the serum with HPV infection in the implanted tissue contacted with the infectious HPV in the absence of the serum, a reduction in HPV infection in the presence of the serum being indicative of the ability of the candidate vaccine to elicit a HPV-neutralizing immune response in the mammal.

27. The method of claim 26, wherein the HPV is HPV 83.

28. A method of testing a candidate HPV antiviral agent for its ability to reduce or prevent infectivity or replication of HPV in a mammal, which comprises:

a) providing a live, infectious stock of a HPV of type MM7/LVX82;

b) contacting an implantable tissue with the infective HPV, in the presence or absence of the candidate antiviral agent;

c) implanting the tissue into an animal host in which the implantable tissue has been determined to grow, and HPV infection of, and replication in, the tissue to occur over a pre-determined time period; and

d) comparing HPV infection or replication in the implanted tissue contacted with the infective HPV in the presence of the candidate antiviral agent with HPV infection or replication in the implanted tissue contacted with the infective HPV in the absence of the candidate antiviral agent, a reduction in HPV infection in the presence of the agent being indicative of the ability of the candidate antiviral agent to reduce or prevent HPV infection or replication in the mammal.

29. The method of claim 28, wherein the HPV is HPV 83.
HPV IU (6695) GCCCCCCAGGACATAATAATGGCATTGGATTGGTTATATGAGTATTTGTTTAC
HPV MM7 (1) GCCCAAGGGACATAATATGGCATTGGATTGGTTATATGAGTATTTGTTTAC
LVX 82 (1) GCCCAAGGGTCAATAATAATGGCATTGGATTGGTTATATGAGTATTTGTTTAC
HPV IU (6745) AGTTGTTGAGATACTACCCCGAGTACATACATATTCATCTATCCGTCGCTA
HPV MM7 (51) AGTTGTTGAGATACTACCCCGAGTACATACATATTCATCTATCCGTCGCTA
LVX 82 (51) AGTTGTTGAGATACTACCCCGAGTACATACATATTCATCTATCCGTCGCTA
HPV IU (6795) CACAGGCTAATGATACACATACGGCCTAATTTAAGAGAATACCTCCGCCAC
HPV MM7 (101) CACAGGCTAATGATACACATACGGCCTAATTTAAGAGAATACCTCCGCCAC
LVX 82 (101) CACAGGCTAATGATACACATACGGCCTAATTTAAGAGAATACCTCCGCCAC
HPV IU (6845) ACAGAGGAATATAGCTATTACAGGTATATTTGCAACCTTGGAAATAATACCT
HPV MM7 (151) ACAGAGGAATATAGCTATTACAGGTATATTTGCAACCTTGGAAATAATACCT
LVX 82 (151) ACAGAGGAATATAGCTATTACAGGTATATTTGCAACCTTGGAAATAATACCT
HPV IU (6895) GACCCCTGAAAATTGAGCATACACTCATAGATGAATGAAACATTTATAG
HPV MM7 (201) lACCCCTGAAAATTGAGCATACACTCATAGATGAATGAAACATTTATG
LVX 82 (201) lACCCCTGAAAATTGAGCATACACTCATAGATGAATGAAACATTTATG
HPV IU (6945) ATGAGTGGAAATTTTGGCGGTATACCCCGTTTCCCTCCACACCTGTTGATGAT
HPV MM7 (251) ATGAGTGGAAATTTTGGCGGTATACCCCGTTTCCCTCCACACCTGTTGATGAT
LVX 82 (251) ATGAGTGGAAATTTTGGCGGTATACCCCGTTTCCCTCCACACCTGTTGATGAT
HPV IU (6995) ACCTATCGCTATCCTGAGTCCCGTCTTACCATCCCAAGGAGGTCCTTC
HPV MM7 (301) ACCTATCGCTATCCTGAGTCCCGTCTTACCATCCCAAGGAGGTCCTTC
LVX 82 (301) ACCTATCGCTATCCTGAGTCCCGTCTTACCATCCCAAGGAGGTCCTTC
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HPV MM7 (351) CGCCCGTGGGACTAAAGAGATCTATGGAGCCCTTGGTATTTGTGGAGG
LVX 82 (351) CGCCCGTGGGACTAAAGAGATCTATGGAGCCCTTGGTATTTGTGGAGG
HPV IU (7095) TTGATTTAAGGACAACTATCCACAGATTAGATCGATTTTCTTTGGGC
HPV MM7 (401) TTGATTTAAGGACAACTATCCACAGATTAGATCGATTTTCTTTGGGa
LVX 82 (401) TTGATTTAAGGACAACTATCCACAGATTAGATCGATTTTCTTTGGGa
HPV IU (7145) CG
HPV MM7 (451) CG
LVX 82 (451) CG

Fig. 1
Fig. 3A

HPV 83 MYPLEARGQQAYDLIVIGRDEVQLLLLILILIVIITILYMRLLLHM

BPV 1 MPNLWFLLFLGLVAAMQLLLLLFLLLFFLVLVWDHFECSCGTGLF

Fig. 3B

SUBSTITUTE SHEET (RULE 26)
Fig. 4
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC(6)**: C12N 7/00, 15/37, 15/63; A61K 39/12, 49/00

**US CL**: 435/235.1; 320.1; 536/23.72; 424/204.1. 9.2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1; 320.1; 536/23.72; 424/204.1. 9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please see Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>Y</td>
<td>MANOS et al. Epidemiology and Partial Nucleotide Sequence of Four Novel Genital Human Papillomaviruses. Journal of Infectious Diseases. 1994, Vol. 170, pages 1096-1099, see Figure 1.</td>
<td>3-13, 15-27</td>
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| X         | further documents are listed in the continuation of box C. | See patent family annex. |

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Document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Document member of the same patent family

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Date of the actual completion of the international search: 15 NOVEMBER 1999

Date of mailing of the international search report: 09 DEC 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks

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Form PCT/ISA/210 (second sheet)(July 1992)
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<td>Y</td>
<td>ONG et al. Identification of Genomic Sequences of Three Novel Human Papillomavirus Sequences in Cervical Smears of Amazonian Indians. Journal of Infectious Diseases. 1994, Vol. 170, pages 1086-1088, see Figure 1.</td>
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<td>US 4,814,268 A (KREIDER et al) 21 March 1989, see col. 9, lines 9-29.</td>
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<td>FORSLUND et Al. Human Papillomavirus Type 70 Genome Cloned from Overlapping PCR Products: Complete Nucleotide Sequence and Genomic Organization. Journal of Clinical Microbiology. April 1996, Vol. 34, No. 4, pages 802-809, see entire document.</td>
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<td>WO 91/18294 A (MEDSCAND AB) 28 November 1991, see entire document.</td>
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MEDLINE, DERWENT BIOTECHNOLOGY ABSTRACTS, EAST USPAT, HPV?, PAPILLOMA?, MM7, MM(W)7, LVX82, LVX(W)82, 83, HPV83, HPV(W)83, PAP291, PAP(W)291, CLON7, PCR