The discovery of novel viruses has often been accomplished by using hybridization-based methods that necessitate the availability of a previously characterized virus genome probe or knowledge of the viral nucleotide sequence to construct consensus or degenerate PCR primers. In their natural replication cycle, certain viruses employ a rolling-circle mechanism to propagate their circular genomes, and multiply primed rolling-circle amplification (RCA) with φ29 DNA polymerase has recently been applied in the amplification of circular plasmid vectors used in cloning. We employed an isothermal RCA protocol that uses random hexamer primers to amplify the complete genomes of papillomaviruses without the need for prior knowledge of their DNA sequences. We optimized this RCA technique with extracted human papillomavirus type 16 (HPV-16) DNA from W12 cells, using a real-time quantitative PCR assay to determine amplification efficiency, and obtained a 2.4 × 10^4-fold increase in HPV-16 DNA concentration. We were able to clone the complete HPV-16 genome from this multiply primed RCA product. The optimized protocol was subsequently applied to a bovine fibropapillomatous wart tissue sample. Whereas no papillomavirus DNA could be detected by restriction enzyme digestion of the original sample, multiply primed RCA enabled us to obtain a sufficient amount of papillomavirus DNA for restriction enzyme analysis, cloning, and subsequent sequencing of a novel variant of bovine papillomavirus type 1. The multiply primed RCA method allows the discovery of previously unknown papillomaviruses, and possibly also other circular DNA viruses, without a priori sequence information.

In the past, the discovery of novel viruses could be accomplished only when the virus was abundantly present or when the virus could be propagated in cell culture. This usually involved sucrose or cesium chloride density gradient centrifugation, and subsequent isolation, cloning, and sequencing of the viral genome. Related DNA viruses could then be found by low-stringency Southern blot hybridization. This approach allowed the identification of human papillomavirus type 16 (HPV-16) in a cervical carcinoma by using DNA hybridization with related HPV genotypes from cutaneous and condylomatous warts (14). Once sequence information for a number of related viruses was determined, part of an unknown virus genome, even when present in minute quantities, could be amplified through PCR by using consensus or degenerate primers specific to a conserved part of the genome. For circular DNA viruses, the complete genome could subsequently be characterized by using inverse PCR. Since these strategies were based on hybridization between a primer with a known sequence and a template with an unknown but necessarily related complementary sequence, this strategy allowed the discovery only of (at least moderately) related viruses. This leads to a progressive characterization of different members of a known virus island, but it would not allow detection of new, divergent virus archipelagos.

A technique that does not suffer from this drawback is representational difference analysis, which couples repeated rounds of subtractive hybridization with PCR to exponentially amplify PCR products that correspond to the genetic differences between an otherwise related pair of a noninfected sample and a virus-infected sample. This very laborious technique recently led to the discovery of the causative agent of Kaposi's sarcoma (human herpesvirus 8) (5) and of transfusion-transmitted virus (TTV), a previously unknown circular circovirus (26).

Another method for discovering novel viruses takes advantage of host immunologic recognition of an exogenous virus. Immune serum is used to screen a random-primed cDNA expression genomic library created from an infected specimen. While it is also laborious, this method was used in the discovery of hepatitis C virus (7).

In nature, replication of circular DNA molecules such as plasmids or viral genomes frequently occurs through a rolling-circle mechanism. Rolling-circle amplification (RCA) is a novel technology which mimics this molecular amplification machinery. RCA has been used as a laboratory method for the amplification of small circular DNA templates (<200 nucleotides in size) via prolonged extension of oligonucleotide primers (16, 22). In multiply primed RCA the polymerization process is primed by exonuclease-resistant random hexamers that bind at multiple locations on the circular template DNA, generating multiple replication forks (11). These random hexamer primers eliminate the requirement for custom primers and thus for (even limited) knowledge of the sequence to be am-
plified. The method utilizes bacteriophage φ29 DNA polymerase, a high-fidelity enzyme with a strong strand-displacing capability, high processivity (>70,000 bases per binding event), and proofreading activity (15). Furthermore, this enzyme is very stable, with linear kinetics at 30°C for over 12 h, eliminating the need for thermal cycling. When the polymerase elongates one of the hexamers and reaches the 5' end of a downstream elongated hexamer primer, the strand displacement activity of the φ29 DNA polymerase causes displacement of this downstream extended hexamer. This generates a single-stranded product of complementary concatemers. The random hexamers will bind to the new recognition sites on this single-stranded product, and continued elongation and strand displacement result in branching and eventually in the release of double-stranded DNA concatemers. By using multiply primed RCA, circular DNA templates can be exponentially amplified, up to 10³-fold (25). The reaction products are high-molecular-weight, linear, double-stranded, tandem-repeat copies of the input template that can subsequently be used for DNA sequencing of either strand, restriction endonuclease digestion, and other methods used in cloning, labeling, and detection (11). Kits using this method for preparation of circular DNA templates for direct use in cycle sequencing are commercially available (25). Multiply primed RCA has already been used for the amplification of circular DNA templates, such as phage and plasmid DNAs with variable insert size (25). Other RCA variants have been employed for the genotyping of single nucleotide polymorphisms through ligation of circularizable DNA probes (23, 28) and for whole bacterial or human genome amplification via multiple-displacement amplification (10, 12).

Papillomaviruses (PVs) are a large group of species-specific epitheliotropic viruses that cause epithelial proliferations in a wide variety of animal hosts. More than 100 different HPV types, causing a wide spectrum of pathologies, have been isolated (30). A comparable PV genotype variety has not yet been detected in animal species, although PVs have been found in most vertebrate species investigated. A major impediment to the discovery of new PVs is the absence of a conventional cell culture system for in vitro viral propagation. During the last few years, numerous new HPV types have been detected and partially characterized through degenerate-primer PCR methods. Primers were developed based on alignments of previously characterized HPV types, usually of the L1 gene, which is the most conserved region of the PV genome. Using this technique, it has been shown that healthy human skin harbors multiple and diverse HPV types, suggesting a commensalic nature of these viruses (2). Recently it has been shown that many animal species also harbor multiple PVs on healthy skin, and the PV types that were detected were closely related to their human counterparts (3). Since the degenerate primers used to investigate these animal samples were developed for the detection of human cutaneous PVs, it is possible that PV types that are less similar to the human types were not detected by this method. In general, this technique will always be limited to detecting PVs that show a rather high degree of similarity to previously characterized PV types. The actual diversity of PV types on healthy animal skin might therefore be much broader than what can be detected by degenerate-primer PCR.

In search of a technique that does not suffer from this drawback, we tested whether we could amplify the complete circular double-stranded DNAs of PVs, without any need for prior knowledge of their sequences, by using multiply primed RCA.

MATERIALS AND METHODS

PV samples. As a source of HPV-16 genomic DNA, we used the human cervical keratinocyte cell line W12, which is known to contain approximately 100 copies of the HPV-16 genome, mainly in episomal form (29). A biopsy of a fibropapillomatosus wart from a bovineudder served as a sample that putatively contained a PV of unknown genotype.

DNA extraction. Total DNA was extracted from W12 cells by using the QiAamp DNA blood minikit (Qiagen) according to the manufacturer's blood and body fluid protocol. For extraction of DNA from the bovine tissue, biopsy material was finely minced and the tissue was digested overnight at 50°C in 500 μl of digestion buffer (10 mM Tris, 0.5% sodium dodecyl sulfate [pH 7.4]) with 500 μg of PCR-grade protease K (Roche Diagnostics Belgium). The digest was deproteinized twice by phenol-chloroform-isomyl alcohol extraction, followed by chloroform extraction, and DNA was recovered by ethanol precipitation and then air dried and resuspended in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The concentration of total DNA in both samples was determined by spectrophotometric measurement (BioPhotometer; Eppendorf).

Multiply primed RCA. After a denaturation step in which the circular DNA template is rendered single stranded, exonuclease-protected random hexamer primers anneal to the template DNA at multiple sites and are extended by the φ29 DNA polymerase. When the DNA polymerase reaches a downstream extended primer, strand displacement synthesis occurs. Secondary priming events can subsequently occur on the displaced product strands of the initial RCA step. Iteration of this process results in exponential, isothermal amplification and generates linear, double-stranded, high-molecular-weight, tandem-repeated copies of the template DNA (11, 25) (Fig. 1).

Multiply primed RCA was performed with the TempliPhi 100 amplification kit (Amersham Biosciences) according to the manufacturer's instructions. One microliter of extracted DNA from W12 cells (containing 0.03 μg of total DNA) or from the bovine tissue (3.9 μg of total DNA), or water (negative control), was transferred into a 0.5-ml tube with 5 μl of TempliPhi sample buffer, containing exonuclease-protected random hexamers. The samples were denatured at 95°C for 3 min and afterwards were placed on ice. A premix was prepared on ice by mixing, for each sample, 5 μl of TempliPhi reaction buffer, containing salts and dNTPs, 100 nmol of TempliPhi enzyme mix, containing the φ29 DNA polymerase and exonuclease-protected random hexamers in 50 μl of 50 mM potassium chloride. Alternative protocols were tested, with premixes containing 900, 450, 225, or 112 μM extra dNTPs. After mixing by vortexing, 5 μl of premix was added to the cooled samples. The reaction mixtures were incubated overnight (approximately 16 h) at 30°C. Afterwards, the reaction mixtures were put on ice, subsequently heated to 65°C for 10 min to inactivate the φ29 DNA polymerase, and stored at −20°C until further analysis.

Restriction enzyme analysis. Two microliters of the W12 multiply primed RCA products was digested with 10 U of BamHI, a single cutter of the HPV-16 complete genome. Two microliters of the bovine tissue multiply primed RCA product was digested with 10 U of EcoRI, BamHI, HindIII, Sall, and HincII. The same amount of nonamplified extracted DNA that was used as input material for the multiply primed RCA was subjected to restriction enzyme digestion with the same enzymes as used for restriction enzyme analysis of the corresponding RCA products: 1 μl of W12 extracted DNA was digested with 10 U of BamHI, and 1 μl of bovine tissue extracted DNA was digested with EcoRI. Restriction digests were separated on a 0.8% agarose gel and visualized by ethidium bromide staining.

Quantification of HPV-16 in W12 extracted DNA and multiply primed RCA products. The HPV-16 DNA concentrations in the W12 extracted DNA and in the multiply primed RCA products thereof were determined by using a real-time quantitative PCR assay. A specific HPV-16 E6 forward primer (5'-GCACAGACGTGCAAAACATACTACA-3') and reverse primer (5'-CCGCCAAGACGAACTGCTAGTATACCT-3') and a dually labeled fluorescent probe (FAM-5'TGTACGTGCAAGACAGTATTACTGGACGCT-3'TAMRA) were designed by using Primer Express software (Applied Biosystems). Reaction mixtures contained 0.25 μM forward and reverse primers, 0.1 μM probe, 20 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and water to a final volume of 35 μl. Five microliters of target DNA was added, and reactions were performed in a spectrophotometric thermal cycler (ABI Prism 7700 sequence detection; Applied Biosystems) with an amplification profile of 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Samples that were tested were
dilutions of the W12 extracted DNA and of the multiply primed RCA products. An HPV-16 dilution series was prepared with DNA extracted from the SiHa cervical carcinoma cell line, which contains a single HPV-16 genome integrated into the chromosomal DNA (4). This dilution series was included in each run for generating a standard curve. As a no-template control, we used water which had been subjected to the same multiply primed RCA procedure as the W12 samples. All samples, standards, and the no-template control were tested in triplicate, and the average of the replicate measurements was used for analysis. The data were analyzed with the sequence detector version 1.7 software system (Applied Biosystems), and the cycle values were translated into HPV-16 DNA concentrations by interpolation on the standard curve. For each amplification protocol, the multiply primed RCA was performed five times, and the HPV-16 DNA concentration was determined for each product. The average amplification efficiency for the different RCA protocols was calculated by dividing the average HPV-16 DNA concentration of the RCA products by the HPV-16 DNA concentration of the input W12 extracted DNA, and a 95% confidence interval was determined based on the five repeat measurements.

To measure the degree of enrichment of the HPV-16 viral DNA compared to cellular genomic DNA, the copy numbers of the human β-globin gene in the W12 extracted DNA and in the multiply primed RCA products thereof were determined by real-time quantitative PCR. This was done with specific β-globin forward and reverse primers (5'-ACACAACCTGGTTCACAGC-3' and 5'-TGGTCTCTTAACTCCTGCTG-3', respectively) and a dual-labeled fluorescent probe (VIC-5'-CCACCAACTTCAACAGTTTCA-3'-TAMRA) (Applied Biosystems). Five microliters of target DNA was added to 35 μl of a mix containing 0.3 μM forward and reverse primer, 0.1 μM probe, 20 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and water. Reactions were performed under the same conditions as for the HPV-16 real-time quantitative PCR assay, and the same dilution series of SiHa extracted DNA (two copies of β-globin per cell) was included in each run for generating a standard curve. All samples (dilutions of the W12 extracted DNA and multiply primed RCA products thereof), standards, and the no-template control were tested in triplicate, and the averages of these replicate measurements were used for analysis as described above. The average amplification efficiency of cellular genomic DNA with the different multiply primed RCA protocols was calculated by dividing the average β-globin concentration in the RCA products by the β-globin concentration in the input W12 extracted DNA, based on five repeat measurements for each amplification protocol. To determine the relative degree of enrichment of the HPV-16 viral DNA for each amplification protocol, the average fold amplification of the HPV-16 viral sequences was divided by the average fold amplification of the β-globin sequence.

Isolation and cloning of complete PV genomes. BamHI cuts the HPV-16 genome once, resulting in a single band of approximately 7.8 kb on an agarose gel. Ten microliters of W12 multiply primed RCA product was digested overnight with 100 U of BamHI and run on a 0.8% agarose gel, and the appropriate band was cut out from the gel. The DNA was isolated from the agarose gel slice by using GeneClean II (Bio 101 Systems/Obiogene) and ligated into dephosphorylated BamHI-cut pUC18, using the Rapid DNA ligation kit (Roche Diagnostics Belgium). After transformation of One Shot MAX Efficiency DH5α-T1 Escherichia coli (Invitrogen), the bacteria were incubated for blue-white colony screening on agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Ten white colonies were screened, and two contained an insert of the correct size (approximately 7.9 kb). Both clones were analyzed by sequencing with universal M13 forward and reverse primers. Sequencing was performed on an ABI Prism 3100 genetic analyzer (Perkin-Elmer Applied Biosystems). Chromatogram sequencing files were inspected with Chromas 2.2 (Technelysium), and similarity searches were performed by using the National Center for Biotechnology Information WWW-BLAST (basic local alignment search tool) server on GenBank DNA database release 132.0 (1).

Ten microliters of multiply primed RCA product of the bovine tissue sample was digested with 100 U of EcoRI, resulting in a single band of approximately 8 kb which was cut out from the gel. GeneClean II was again used to isolate the DNA from the gel slice, and the Rapid DNA ligation kit was used to ligate it into dephosphorylated EcoRI-cut pUC18. One Shot MAX Efficiency DH5α-T1 E. coli cells were transformed and incubated on X-Gal-containing agar plates for blue-white colony screening. Ten white colonies were screened, and five contained an insert of the appropriate size. These five clones were sequenced with the universal M13 forward and reverse primer and analyzed as described above.

RESULTS

Optimization of the multiply primed RCA protocol by using HPV-16 from W12 cells. Different protocols for multiply primed RCA were tested on extracted DNA from W12 cells containing HPV-16 genomic DNA in episomal form. The products generated with these different test protocols, as well as the nonamplified input DNA, were digested with BamHI, a known single-cutting restriction enzyme for HPV-16 DNA, and separated on an agarose gel to check for the presence of a band of the HPV-16 complete genome size (approximately 7.9 kb). (Fig. 2A). No PV DNA band could be detected in the nonamplified W12 extracted DNA that served as input material or in the product prepared according to the protocol as described in the TempliPhi 100 amplification kit. The multiply primed RCA products that were prepared according to our modified protocols with added dNTPs showed the clear presence of 7.9-kb bands, indicating highly efficient amplification of the HPV-16 DNA. Addition of extra dNTPs at 450 and 900 μM resulted in bands with the highest density. The 7.9-kb DNA band was isolated and cloned, and two clones with the correct insert size were checked by universal M13 primer sequencing. The insert ends showed 100% identity with HPV-16 isolate 16W12E (GenBank accession number AF125673) (17), demonstrating that the amplified and cloned DNA was indeed full-length HPV-16 DNA.

In order to quantify the amplification efficiencies of the

FIG. 1. Schematic representation of the multiply primed RCA method for amplification of the complete circular double-stranded DNA genomes of PVs (A). In a first denaturation step, the PV episomal DNA is rendered single stranded. Exonuclease-protected random hexamer primers (—) can now anneal to multiple sites on this template DNA, after which the d29 DNA polymerase (●) binds (B) and isothermally extends these primers at the 3' end (→) (C). Strand displacement synthesis occurs when the DNA polymerase reaches a downstream extended primer, and hexamer primers can anneal to the displaced single-stranded product strands and will again be elongated by the d29 DNA polymerase (D). Continuation of this process results in exponential amplification of the template DNA, generating linear double-stranded, high-molecular-weight repeated copies of the complete PV genome (E). Digestion of this multiply primed RCA product with a restriction enzyme which has only a single recognition site in the PV genome will result in multiple double-stranded, linear copies of the PV complete genomic DNA (F), which can be visualized as a single band of ca. 8 kb by agarose gel electrophoresis (G). Lane M, DNA molecular size marker (Fermentas).
different multiply primed RCA protocols, HPV-16 DNA concentrations were determined by real-time quantitative PCR (Fig. 2B; Table 1). The nonamplified W12 extracted DNA contained 6.0 × 10⁴ copies of the HPV-16 genome per μl. Multiply primed RCA according to the manufacturer’s protocol resulted in an increase of the HPV-16 DNA concentration of only 172-fold compared to the nonamplified sample. A large increase of the amplification efficiency was seen when extra dNTPs were added to the amplification reaction mixtures, with a maximum amplification of 2.4 × 10⁴-fold for the protocol in which 450 μM extra dNTPs were added.

In order to quantify the relative degrees of enrichment of HPV-16 viral sequences compared to cellular genomic DNA obtained with the different multiply primed RCA protocols, we used a real-time quantitative PCR assay to determine the β-globin DNA concentrations as a measure for cellular genomic DNA (Table 1). Without amplification, the W12 extracted DNA contained 2.4 × 10⁵ copies of the human β-globin gene per μl. Multiply primed RCA according to the manufacturer’s protocol increased the β-globin DNA concentration only 2.4-fold, and although increasing the dNTP concentration in the amplification reaction mixtures did increase this amplification efficiency, the maximum amplification was only 30-fold, with the use of 900 μM of extra dNTPs. The relative enrichment of HPV-16 viral sequences compared to cellular genomic DNA (β-globin) was maximally 2.8 × 10³-fold, using the protocol in which 450 μM extra dNTPs were added.

Detection, isolation, and typing of PV from bovine tissue by using multiply primed RCA. Extracted DNA from a bovine tissue sample was subjected to amplification with the optimized multiply primed RCA protocol, using 450 μM extra dNTPs. Whereas no DNA was visible on agarose gel electrophoresis after EcoRI digestion of the nonamplified bovine tissue DNA sample, restriction digestion of the RCA product with EcoRI, BamHI, and HindIII revealed a single fragment of approximately 8 kb on agarose gel electrophoresis, consistent with a full-length PV genome. Digestion with HincII resulted in three distinct fragments, with fragment sizes that added up to 8 kb.

### Table 1. Amplification efficiency of multiply primed RCA performed on W12 extracted DNA with different amplification protocols

<table>
<thead>
<tr>
<th>Amplification protocol (μM added dNTPs)</th>
<th>Avg HPV-16 amplification</th>
<th>Avg β-globin amplification</th>
<th>Relative enrichment of HPV-16 viral DNA</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>1.7 × 10⁻²</td>
<td>2.4</td>
<td>71</td>
</tr>
<tr>
<td>112</td>
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<td>4.5</td>
<td>4.9 × 10²</td>
</tr>
<tr>
<td>225</td>
<td>1.5 × 10⁻⁴</td>
<td>8.5</td>
<td>1.5 × 10³</td>
</tr>
<tr>
<td>450</td>
<td>2.4 × 10⁻⁴</td>
<td>8.5</td>
<td>2.8 × 10³</td>
</tr>
<tr>
<td>900</td>
<td>1.7 × 10⁻⁴</td>
<td>30</td>
<td>5.7 × 10³</td>
</tr>
</tbody>
</table>

FIG. 2. Amplification efficiency of multiply primed RCA performed on W12 extracted DNA by using different amplification protocols. (A) Restriction enzyme analysis of W12 extracted DNA before and after multiply primed RCA. One microliter of nonamplified sample, containing 0.03 μg of total DNA, was digested with BamHI (lane 1). Multiply primed RCA was performed with 1 μl of input W12 extracted DNA (0.03 μg of total DNA), either according to the TemplPhi 100 amplification kit instructions (lane 2), or by alternative protocols with addition of extra dNTPs at a concentration of 112 μM (lane 3), 225 μM (lane 4), 450 μM (lane 5), or 900 μM (lane 6). As a negative control, 1 μl of water was used as input for the multiply primed RCA, using the amplification protocol with 450 μM extra dNTPs (lane 7). For all RCA products, 2 μl of product was digested with BamHI. Lane M, DNA molecular size marker (Fermentas). (B) The HPV-16 concentrations in the nonamplified sample and the different RCA products were determined by real-time quantitative PCR, and the amplification compared to the nonamplified sample was calculated. The data represent averages from five experiments, and 95% confidence intervals are indicated.

FIG. 3. Restriction enzyme analysis of the bovine tissue DNA before and after multiply primed RCA. One microliter of the nonamplified DNA, containing 3.9 μg of total DNA, was digested with EcoRI (lane 1). Multiply primed RCA with 450 μM extra dNTPs was performed with 1 μl (3.9 μg) of input bovine tissue DNA, and 2 μl of the RCA product was digested with EcoRI (lane 2), BamHI (lane 3), HindIII (lane 4), SalI (lane 5), and HincII (lane 6). As a negative control, 2 μl of multiply primed RCA product of water was digested with EcoRI (lane 7). Lane M, DNA molecular size marker (Fermentas).
SallI did not cut the product (Fig. 3). This matches the restriction pattern of bovine PV type 1 (BPV-1) but not that of any of the other bovine PV types whose complete genome has been determined. To further characterize the amplified DNA, a larger volume (10 μl) of multiply primed RCA product was digested with EcoRI. Subsequently, the 8-kb DNA fragment was isolated and cloned, and clones with the correct insert size were analyzed by M13 primer sequencing. After removal of the plasmid sequence, a 1,394-bp contig of the forward and reverse sequence was constructed, which showed 99% identity with BPV-1 isolate 307 (GenBank accession number J02044) (6) and was colinear with the region between bp 1393 and 2786 of this sequence (with the EcoRI restriction site at positions 2113 to 2117), demonstrating that the complete genome of a BPV-1 variant was successfully amplified and cloned from the tissue sample.

**DISCUSSION**

The present study shows that multiply primed RCA is a promising technique for amplification and isolation of novel human and animal PVs without the need for prior knowledge of their sequences. In our experiments, multiply primed RCA with the unmodified TempliPhi kit according to the manufacturer’s protocol resulted in only a very low amplification efficiency (172-fold). Although up to 10^7-fold amplification can be accomplished when this protocol is used with 1 ng of purified pUC18 DNA (25), apparently the amplification efficiency is significantly lower when more diluted and larger circular DNA molecules are used as input material. We were able to achieve an improved amplification efficiency of the input DNA, up to 2.4 × 10^3-fold, by using a modified protocol with added dNTPs. For all protocols tested, the amplification efficiency was much higher for the circular HPV-16 DNA than for the β-globin gene, demonstrating the preferential amplification of circular DNA templates. With the optimized amplification protocol using 450 μM extra dNTPs, cellular genomic DNA (β-globin) was amplified only 8.5-fold, resulting in a relative enrichment of the viral sequences of 2.8 × 10^3-fold. Information about the exact concentration of dNTPs in the TempliPhi amplification kit was not released to us by the manufacturers, but since this kit is designed to allow direct sequencing of amplified products, dNTPs would have to be present in low concentrations so that they are depleted during the amplification. The addition of extra dNTPs might make the amplification products generated with our protocol unsuited for direct sequencing without previous purification steps, since an excess of dNTPs could interfere with the sequencing reaction. Since we designed this protocol for the detection of viral DNA genomes with unknown sequences, sequencing of the RCA-amplified products will require a cloning step in any case. From our optimization experiment we conclude that 450 μM extra dNTPs is the optimal dNTP concentration to be added, since this resulted in the highest amplification efficiency for the PV sequences and gave only limited nonspecific amplification of cellular genomic DNA, thereby resulting in the highest degree of relative enrichment of HPV-16 sequences.

The amplification products were successfully used for restriction enzyme analysis and cloning, thereby allowing characterization of the virus. The sequence data obtained from the cloned RCA product are sufficiently reliable, since the δ29 DNA polymerase has an associated 3'-5' exonuclease proof-reading activity resulting in an error rate of 3 × 10^-6 to 5 × 10^-6 (25, 15), which is about 100-fold lower than that of the Tag DNA polymerase used in most PCRs (13) and is comparable to that of DNA polymerase mixtures which are commonly used in long PCRs for high-fidelity amplification of long stretches of DNA (8).

Since no prior knowledge of the virus sequence is required for the use of this technique, it would allow the discovery of new PVs which are very divergent from previously characterized genotypes. Because many viruses have a circular DNA genome or a genome that is circularized in some stage of their life cycle, this technique could prove to be more generally useful in the isolation of novel viruses. Viruses with circular double-stranded DNA include not only PVs (7.2 to 8.6 kb) but also polyomaviruses (4.7 to 5.3 kb), plant reverse-transcribing pararetroviruses such as caulimoviruses (6.8 to 8.2 kb) and badnaviruses (7.5 to 8 kb), polydnaviruses (2 to 28 kb), baculoviruses (80 to 230 kb), and ascoviruses (140 to 180 kb). Single-stranded DNA viruses with a circular genome include the circovirus TTV (3.8 kb), geminiviruses (2.5 to 3 kb), nana- viruses (1.3 kb), and bacteriophages such as M13 (6.4 kb) and δX174 (5.4 kb). All of these virus families could therefore possibly be genome amplified by using multiply primed RCA. The hepadnaviruses, such as hepatitis B virus, have a partially double-stranded genome (3.2 kb) with an incomplete plus strand, leaving 15 to 60% of the molecule single stranded, and a minus strand that, while being complete, contains a nick at a unique site approximately 225 bp from the 5’ end of the positive strand in mammalian hepadnaviruses. Upon entry into a liver cell, the viral core particle is translocated into the nucleus, and the viral DNA is repaired by a virion DNA polymerase, giving rise to a covalently closed circular DNA (18), at which stage this DNA could also be prone to amplification by multiply primed RCA. The linear genomes of many DNA viruses are known to become circularized at certain stages of infection. For instance bacteriophage lambda has a linear double-stranded DNA genome (48 kb) which circularizes after infection. Some viruses with linear DNA genomes that contain inverted terminal repeats are joined head to tail around the inverted terminal repeats after infection to form circular episomes from which replicative (circularly permuted) concatamers can be produced. Single-stranded DNA viruses such as the adeno-associated viruses use this strategy (24). Also, herpesviruses such as Epstein-Barr virus, varicella-zoster virus, human herpesvirus 8, and the simian herpesvirus saimiri, which have large linear double-stranded DNA genomes, are known to exist as multicopy circular episomes in latently infected cells (9, 19–21). Even retroviruses, with a linear RNA genome, might have circular DNA replicative intermediates or by-products when the ends of the linear cDNA become joined, either through homologous recombination between the two long terminal repeats (LTRs), forming one-LTR circles, or through intramolecular (end-to-end) ligation of the linear human immunodeficiency virus type 1 cDNA, forming two-LTR circles. The presence of episomal circular DNA forms of human immunodeficiency virus type 1 is currently being investigated as a surrogate diagnostic marker capable of detecting residual ongoing human immunodeficiency virus replication in patients.
undergoing antiretroviral therapy (27), and multiply primed RCA could prove to be a valuable technique for preferential amplification of this circular viral DNA.

In summary, we optimized a multiply primed RCA protocol by using HPV-16 DNA from W12 cells, obtaining an amplification efficiency of 2.4 × 10^4-fold. We demonstrated its usefulness in the detection and isolation of PVs without any need for prior information about their nucleotide sequences. To our knowledge, we are the first to report the use of multiply primed RCA for the detection and isolation of a complete viral genome. Given the widespread existence of circular viral DNA forms, a much broader use of this novel technique, in the isolation of many different viral genomes or even to increase the sensitivity of existing PCRs, could be envisioned.

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