Detection of new human papillomavirus sequences in skin lesions of a renal transplant recipient and characterization of one complete genome related to epidermodysplasia verruciformis-associated types

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Human papillomavirus (HPV) DNA, originally isolated from patients suffering from the skin disease epidermodysplasia verruciformis (EV), and a growing number of related sequences have recently been detected in a high percentage of benign and malignant skin lesions of both immunosuppressed and immunocompetent people. HPV L1 DNA fragments (374–389 bp long) from a solar keratosis and a squamous cell carcinoma (SCC) of a renal transplant recipient were amplified, cloned and sequenced. In 54 clones, six different HPV sequences were identified. One of these six corresponded to the known type HPV-8 and two (RTRX3 and RTRX7) have been described previously in cutaneous lesions of immunosuppressed patients. The remaining three sequences were different from all known HPV types: an HPV-9-related sequence (77 ± 4% identity), an RTRX2-related sequence (82.6% identity), and an HPV-22-related sequence (83.7% identity). These three sequences, representing putatively new HPV types, were named RTRX8, RTRX9 and RTRX10, respectively. RTRX7 was found in the majority of clones from both lesions. The complete genome of RTRX7 (7731 bp) was cloned as six overlapping subgenomic fragments, generated by nested PCR with DNA extracts from the SCC. RTRX7 showed a genome organization typical of HPVs associated with EV. The L1 DNA sequence differed by 15% from the corresponding region of its closest known relative, HPV-12; thus, RTRX7 can be regarded as a new HPV type. RTRX7 DNA could not be detected by Southern blot hybridization with the homologous probe, indicating that the DNA concentration was below one copy per 10 cells in the investigated SCC.

Introduction

Human papillomaviruses (HPVs) represent a large group of DNA viruses causing proliferative epithelial lesions at various anatomical sites (Pfister & Fuchs, 1994). Members of HPV group A are primarily associated with benign and malignant lesions in the anogenital tract. A further group was originally isolated from characteristic skin lesions of patients suffering from the rare hereditary disease epidermodysplasia verruciformis (EV) (Jablonska et al., 1972). These viruses are therefore referred to as EV-associated. Chan et al. (1995) classified them as group B1 papillomaviruses.

Prior to the introduction of highly sensitive PCR techniques, these HPV types were only rarely detected in cutaneous specimens from patients without a history of EV (Pfister, 1992). With increasingly sensitive methods, a broad spectrum of HPV types has been found in skin tumours of both immunosuppressed and immunocompetent people (Soler et al., 1993; Stark et al., 1994; Shamanin et al., 1994, 1996; Tieben et al., 1994). In some studies, EV-associated HPV types and related sequences were detected in a high percentage of cutaneous specimens (Berkhout et al., 1995; de Jong-Tieben et al., 1995). More than 30% of the PCR-positive samples were shown to harbour more than one HPV type. Using nested PCR with degenerate primers, Berkhout et al. (1995) revealed six unknown HPV sequences (RTRX1–RTRX6) related to EV

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The GenBank accession numbers of the sequences RTRX7–RTRX10 reported in this paper are U85660–U85663, respectively.
types in skin lesions of renal transplant recipients, and another such sequence, named ICPX1, in tumours of an immunocompetent patient. Complete genomes of these putatively new HPV types from the EV-associated subgroup are not yet available. The discrepancy between the usually negative results of Southern blot hybridizations for EV-associated HPVs, and their high prevalence in investigations by nested PCR, suggest persistence at low concentrations in cutaneous neoplasia of non-EV patients.

Recent reports by the present authors describe an immunosuppressed renal transplant recipient with numerous skin tumours and reveal, in addition to other types, a new HPV DNA sequence which was closely related to the EV-associated HPV-12 (Höpfli et al., 1996, 1997). This sequence was named RTRX7. These findings encouraged close examination of one malignant and one premalignant skin lesion from this patient for the range of HPV types detectable therein, and studies to determine the entire nucleotide sequence of RTRX7 by cloning overlapping subgenomic fragments generated by nested PCR.

Methods

**Biopsy material, DNA preparation and PCR amplification of an L1 gene fragment.** DNA was extracted from frozen material of one solar keratosis from the neck of an immunosuppressed renal transplant recipient and of one incipient squamous cell carcinoma (SCC) from his cheek using the QIAamp Tissue kit (Qiagen) according to the manufacturer’s instructions. Amplification of HPV-specific DNA was carried out using nested PCR and degenerate primer pairs CP65/CP70 and CP66/CP69 (Table 1) designed by Berkhou et al. (1995). For cloning purposes, a BamHI cleavage site was added to the 5’ end of the CP66 oligonucleotide sequence and an EcoRI site was added to the 5’ end of CP69. The reaction mixtures (total volume 50 µl) of both external and internal PCR contained 50 mM KCl, 10 mM Tris–HCl (pH 8.8), 2.5 mM MgCl₂, 0.005 % gelatine, deoxynucleoside triphosphates (0.2 mM each), 0.9 µM forward primer, 0.9 µM backward primer and 2.5 U of the proofreading Expand High Fidelity polymerase (Boehringer). In the external PCR, 10 µl cellular DNA extract (0.5 µg) was used as input. Five cycles each running for 1 min at 95 °C, 1.5 min at 50 °C and 2 min at 72 °C were followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C. For the internal PCR reaction, 3 µl of the first-step PCR product was used as input. A further amplification was performed in 30 cycles (1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C). To avoid contamination of the samples during DNA preparation and PCR processing, the suggestions of Kwok (1990) were strictly followed. With each set of PCR reactions, three aliquots of distilled water were processed in the same way as the template DNA in order to monitor any possible DNA carry-over. In addition, DNA was extracted from several EV-HPV-negative biopsies and subjected to PCR as negative controls of the DNA preparation procedure.

**Cloning and sequencing of the amplimers.** After digestion with BamHI and EcoRI, PCR products were ligated to BamHI/EcoRI-cleaved, dephosphorylated pUC18 vector DNA. These constructs were transformed into competent E. coli DH5α using the CaCl₂ method (Sambrook et al., 1989). Plasmid DNA was isolated from transformants using QIAprep® Miniprep kit (Qiagen). Clones containing inserts of the expected size were identified by double cleavage with BamHI and EcoRI. Both strands of all inserts exhibiting the correct size were sequenced using the T7 Sequencing kit (Pharmacia) and [α-35S]dATP (Amersham). Samples were electrophoresed in 8% polyacrylamide–7 M urea gels, washed with 10% acetic acid, dried and exposed to Hyperfilm–max films (Amersham).

**Sequence and phylogenetic analyses.** Analyses were performed as described previously (Wieland et al., 1997). Briefly, translation of nucleotide sequences and sequence alignments were performed with the sequence analysis software MacVector 6.0 and AssemblyLIGN 1.0 (Oxford Molecular Group). The HPV L1 sequences determined were aligned to 111 published HPV reference sequences (Meyers et al., 1996). The percentage of matching nucleotides of aligned sequences of CP66/CP69 amplimers was calculated. The same was done later with all open reading frames (ORFs) of RTRX7.

Phylogenetic analyses were performed with the Heidelberg Unix Sequence Analysis Resources (HUSAR), German Cancer Research Center, Heidelberg, Germany. Sequences to be analysed were aligned using the HUSAR program Clustal V; the program ClusTree was then used to calculate an unrooted phylogenetic tree according to the neighbour-joining method of Saitou & Nei (1987). The branch lengths represent the percentage divergence along the respective branches. Bootstrapping was employed on all data sets analysed to evaluate confidence levels for the groupings of a tree (1000 bootstrap replicates for each grouping of a tree: Felsenstein, 1985).

**Cloning of the complete RTRX7 genome.** The complete genome of RTRX7 was cloned in six overlapping fragments, named I1–I6, in order of their amplification (Fig. 1). The subgenomic fragments I2–I6 were generated by nested PCR with DNA extracts from the SCC. Cycling conditions were the same as those applied for the CP66/CP69 amplimer (I1). Starting from I1, 17–22 nucleotide (nt) primers for the amplification of subsequent fragments were derived from the sequences of the previously amplified fragments, creating overlaps of 50–120 nt in the RTRX7-specific sequence. Primers for the other end of a new fragment were degenerate, but mainly oriented at the HPV-12 sequence, which is the closest relative of RTRX7 known to date. The primers were chosen to be as far as possible from sites conserved among the EV-associated HPVs. In particular, non-conserved residues at the 3’ end of the primer annealing sites were avoided. Restriction enzyme cleavage sites were attached to the 5’ ends of both internal primers. The forward primer carried an XhoI site and the backward primer a BamHI site. The fragment I6 was amplified with RTRX7-specific primers derived from sequences within I4 and I5, respectively (Fig. 1). Both internal primers carried a SfiI cleavage site in this case. Table 1 shows the exact sequences of the PCR primers used, their annealing sites and the size of the amplified subgenomic fragments. PCR products were analysed in 1% agarose gels stained with ethidium bromide. Bands of the expected size were excised from the gel, and DNA was purified using the QIAquick Gel Extraction kit (Qiagen). The fragments were inserted into pUC18 and transformed into E. coli DH5α. The longer fragments were sequenced using the primer walking strategy which has been described in detail by Delius & Hofmann (1994). All oligonucleotides used for PCR amplification or sequencing were purchased from Eurogentec.

**Southern blot analysis.** Cellular DNA (10 µg) extracted from the RTRX7-positive SCC was digested with HindIII, a non-cutter of the RTRX7 genome. Another 10 µg was digested with XhoI, a single cutter of RTRX7. Purified amplimers (0.15, 1.5 and 15 pg) generated by nested PCR with the I2 primers (Table 1) were each added to 10 µg sonicated salmon sperm (SSS) DNA and used as sensitivity standards corresponding to 0.1, 1 and 10 genome equivalents of RTRX7 per cell. All samples were electrophoresed in 1% agarose, denatured and transferred by vacuum to a nylon membrane (Hybond-N; Amersham) in an alkaline buffer. Thirty ng of the I2 PCR product was labelled with [α-35S]dATP using the Gibco Random Primers DNA Labelling system and this served as a homologous
Table 1. Oligonucleotide primers employed for PCR amplification of subgenomic fragments I1–I6 of RTRX7

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence* (3’ → 5’)</th>
<th>Reference sequence†</th>
<th>Size of amplimer (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1–EXT FW (CP65)</td>
<td>CAR GGT CAY AAY AAT GGY AT</td>
<td>HPV-8 nt 6832–6851 (Berkhout et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>I1–EXT BW (CP70)</td>
<td>AAY TTT CGT CCY ARA GRA WAT TGR TC</td>
<td>HPV-8 nt 7298–7273 (Berkhout et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>I1–INT FW (CP66)</td>
<td>CGC GGATCC AAT CAR MTG TTT RTT ACW GT</td>
<td>HPV-8 nt 6862–6881 (Berkhout et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>I1–INT BW (CP69)</td>
<td>CGC GAATTC GWT ACA TCG AYA TCR AAC</td>
<td>HPV-8 nt 7250–7231 (Berkhout et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>I2–EXT FW</td>
<td>AAM GGT TGG TNT GGG CCT GCA</td>
<td>HPV-12 nt 6133–6153</td>
<td></td>
</tr>
<tr>
<td>I2–EXT BW</td>
<td>CAA TCC TCT AGT AAG GAG GGG</td>
<td>RTRX7 nt 7173–7153</td>
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<tr>
<td>I2–INT FW</td>
<td>CGC TCTAGA AGT GST GTC TTT TTT G</td>
<td>HPV-12 nt 4351–4372</td>
<td></td>
</tr>
<tr>
<td>I2–INT BW</td>
<td>CGC GGATCC TAC GTG CCT TAA ATA TTC C</td>
<td>RTRX7 nt 7069–7051</td>
<td></td>
</tr>
<tr>
<td>I3–EXT FW</td>
<td>CYY CCT GAT GTT CTC AAT AAA G</td>
<td>HPV-12 nt 7250–7231</td>
<td></td>
</tr>
<tr>
<td>I3–EXT BW</td>
<td>TGT GTT CTG CCT ATC ATC TGT A</td>
<td>RTRX7 nt 6306–6340</td>
<td></td>
</tr>
<tr>
<td>I3–INT FW</td>
<td>CGC TCTAGA AGT GST GTC TTT TTT G</td>
<td>HPV-12 nt 4414–4432</td>
<td></td>
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<tr>
<td>I3–INT BW</td>
<td>CGC GGATCC TAC GTG CCT TAA ATA TTC C</td>
<td>RTRX7 nt 6338–6321</td>
<td></td>
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<tr>
<td>I4–EXT FW</td>
<td>ATA AAT GCA ATG AAC CCC TCC T</td>
<td>RTRX7 nt 7139–7160</td>
<td></td>
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<tr>
<td>I4–EXT BW</td>
<td>GTA AYT CCT CTT CAC AAA RCA G</td>
<td>HPV-12 nt 762–741</td>
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<tr>
<td>I4–INT FW</td>
<td>CGC TCTAGA AGA GGA TTG GCA GTT AGG A</td>
<td>RTRX7 nt 7165–7183</td>
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</tr>
<tr>
<td>I4–INT BW</td>
<td>CGC GGATCC AGC CTG TGG TAT AAT TGT</td>
<td>HPV-12 nt 739–722</td>
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<tr>
<td>I5–EXT FW</td>
<td>GTA MAA CTG GAC AAT GGG AAG</td>
<td>HPV-12 nt 3258–3275</td>
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<tr>
<td>I5–EXT BW</td>
<td>ACT TCT AGG AAC AAG TGA GGG</td>
<td>RTRX7 nt 6438–6418</td>
<td></td>
</tr>
<tr>
<td>I5–INT FW</td>
<td>CGC TCTAGA CTC CTG TCA CCA GCT CC</td>
<td>HPV-12 nt 3303–3319</td>
<td></td>
</tr>
<tr>
<td>I5–INT BW</td>
<td>CGC GGATCC GTC AAT ACT GTG GGG GT</td>
<td>RTRX7 nt 4616–4600</td>
<td></td>
</tr>
<tr>
<td>I6–EXT FW</td>
<td>ACG GCT GGA AAG GAG GTC</td>
<td>RTRX7 nt 625–644</td>
<td></td>
</tr>
<tr>
<td>I6–EXT BW</td>
<td>TGT TCT ACT GGA TGC TGG TCG T</td>
<td>RTRX7 nt 3482–3461</td>
<td></td>
</tr>
<tr>
<td>I6–INT FW</td>
<td>CGG GTCGAC GGC ACT GTA AGC ATT TGT AT</td>
<td>RTRX7 nt 645–665</td>
<td></td>
</tr>
<tr>
<td>I6–INT BW</td>
<td>CGG GTCGAC TGC GGC TTG TGG TGA CTG</td>
<td>RTRX7 nt 3440–3423</td>
<td></td>
</tr>
</tbody>
</table>

* Restriction enzyme cleavage sites at the 5′ end of the internal primers are printed in italics. The following IUB codes are used in the oligonucleotide sequences: Y represents C/T; R represents A/G; M represents A/C; W represents A/T; S represents C/G; and N represents A/C/G/T.
† Nucleotide positions from the genomes of HPV-8 and -12 are given according to the numbering by Fuchs et al. (1986) and Delius & Hofmann (1994), respectively.

Results

Spectrum of HPV types in two skin lesions

Nested PCR products obtained with DNA from one solar keratosis and one SCC, which had earlier been shown to contain RTRX7 (Höpfel et al., 1997), were cloned in E. coli. Twenty-eight clones from the keratosis and 26 clones from the SCC contained inserts of the expected size and were sequenced. In the keratosis, five different HPV sequences could be detected, RTRX7 was present in 13 clones, HPV-8 was found...
The complete genome of RTRX7

The complete genome of RTRX7 was 7731 bp long. The position 1 bp was determined by alignment to the HPV-8 sequence (Fuchs et al., 1986). The so-called early region covered 52.6% of the genome and contained the five ORFs E6, E7, E1, E2 and, within E2 in another frame, E4 (Fig. 1b). There was no E5 ORF in RTRX7. The late region represented 40.7% of the total genome length and consisted of the two ORFs L2 and L1, which code for structural proteins in other papillomaviruses. A short non-coding region (NCR) was located between the ORFs L1 and E6. The organization of the ORFs in RTRX7 was collinear to the genomes of known EV-associated HPVs (Fuchs & Pfister, 1997). The exact positions of the genomic regions and their coding capacities are summarized in Table 3. Each ORF of RTRX7 was compared to the respective ORFs of all the other members of the closely related HPV-5 group of viruses (HPV-5, -8, -12, -19, -20, -21, -24, -25, -36 and -47). HPV-12 was always the closest relative in all cases.

Genome organization of RTRX7

The complete genome of RTRX7 was determined by PCR using outward-directed specific primers binding to the known CP66/CP69 fragment (data not shown). The sensitivity of PCR using tissue material is known to be significantly reduced with increasing length of the PCR product (Ohara et al., 1992; Tieben et al., 1993). Therefore, five additional overlapping subgenomic fragments were amplified and subsequently cloned into pUC18 (Fig. 1a). For one end of the desired PCR products, the external and internal primers were derived from the previously determined RTRX7 sequence, beginning with the CP66/CP69 amplimer sequence designated I1 (Fig. 1a). The external and internal primers for the other end were degenerate. XbaI and BamHI were used for the cloning of I2–I5 because they were non-cutters of the closest relative of RTRX7, HPV-12. Assuming that the RTRX7 genome persisted in closed circular form in the investigated SCC, the last and longest fragment, I6, was amplified with RTRX7-specific primers derived from the I4 and I5 sequences, respectively. XbaI and BamHI digestion of the I6 PCR product showed cleavage sites of both enzymes within this fragment. I6 was therefore reamplified with SalI linkers at both internal primers.

Fragments I1–I6 were amplified from the SCC which harboured three HPV L1 sequences (RTRX7, RTRX8 and RTRX10). It was therefore possible that I1–I6 could have originated from different HPV genomes. However, the 100% sequence identities in the 56–120 bp overlaps between adjacent fragments allowed a clear assignment of the six PCR fragments to the genome of one HPV type. Moreover, identical sequences were obtained with each primer set. This indicates that the primers which were absolutely homologous to the previously determined RTRX7 sequences strongly favoured type-specific amplification. Alignments of the sequences of I1–I6 to 74 complete HPV genomes identified HPV-12 as the closest relative in all cases.
New HPV sequences from an immunosuppressed patient

Fig. 2. Phylogenetic analysis of the new HPV L1 sequences RTRX7–RTRX10. The phylogenetic unrooted tree was calculated according to the neighbour-joining method of Saitou & Nei (1987) (see Methods). The four novel HPV L1 sequences RTRX7–RTRX10 (337–349 bp) and 26 HPV L1 group B1 reference sequences (Myers et al., 1996) of the corresponding lengths were used in the analysis. Bootstrap values above 500 are shown. The scale bar indicates percentage divergence along the branches. Of the group B1 reference sequences shown in the tree, HPV-19, -25, -20, -14 and -21 belong to cluster a2; HPV-5, -36, -47, -12, -8 and ICPX1 to cluster a1; HPV-24, RTRX5, X4 and X2 are not classified; HPV-17, -15, RTRX3, HPV-9 and -37 belong to cluster b1; and HPV-22, RTRX1, HPV-23 and -38 to cluster b2. HPV-49 and RTRX6 are not classified (Myers et al., 1996). RTRX7 was assigned to cluster a1, RTRX8 to cluster b1, and RTRX10 to cluster b2.

Table 3. Genomic organization of RTRX7

<table>
<thead>
<tr>
<th>Genomic region</th>
<th>Nucleotide position</th>
<th>Coding capacity in amino acids from first methionine</th>
<th>Sequence identity with HPV-12 (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First nucleotide</td>
<td>First ATG</td>
<td></td>
</tr>
<tr>
<td>E6 ORF</td>
<td>195</td>
<td>204</td>
<td>157</td>
</tr>
<tr>
<td>E7 ORF</td>
<td>622</td>
<td>667</td>
<td>103</td>
</tr>
<tr>
<td>E1 ORF</td>
<td>917</td>
<td>965</td>
<td>606</td>
</tr>
<tr>
<td>E2 ORF</td>
<td>2697</td>
<td>2727</td>
<td>511</td>
</tr>
<tr>
<td>E4 ORF</td>
<td>3289</td>
<td>3326†</td>
<td>229†</td>
</tr>
<tr>
<td>L2 ORF</td>
<td>4312</td>
<td>4345</td>
<td>532</td>
</tr>
<tr>
<td>L1 ORF</td>
<td>5894</td>
<td>5921</td>
<td>516</td>
</tr>
<tr>
<td>NCR</td>
<td>7472</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Identities with the HPV-12 sequence are given as percentage of matched nucleotides or matched amino acids in a pair of aligned sequences. DNA sequence identities are given first followed by amino acid sequence identities in parentheses.
† The E4 ORF does not contain an ATG codon. Therefore, the position of the splice acceptor site for the E1E4 transcript is indicated instead of the first ATG; coding capacity is calculated from this site.

greatest similarity to published EV-associated HPV types. ORF E4 was the most type-specific region in the RTRX7 sequence, and major gaps had to be introduced to achieve its optimal alignment. It had a higher GC content than any other region of the genome (56.9% compared to 42.6% in the complete genome). ORF E4 did not contain an ATG translation initiation codon. The deduced RTRX7 E4 amino acid sequence appears to be proline-rich (22.7%), which probably makes it a hydrophobic protein with a complex structure. Divergence between the E2 ORF of RTRX7 and that of closely related types was mainly restricted to the central segment coinciding with E4 in another reading frame.

RNA processing signals

Several potential splice donor (SD) and splice acceptor (SA) sites occurred in RTRX7 at positions conserved among EV-associated HPVs (Fig. 1c). Their activity in RNA processing has been demonstrated in HPV-5, -8 and -47 (Kiyono et al., 1989; Stubenrauch et al., 1992; Haller et al., 1995). The first SD was detected at position 4 within the NCR of RTRX7. This site
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Fig. 3. Alignment of the NCR sequences of RTRX7 and HPV-8. Conserved residues are marked by an asterisk below the aligned sequences. The nucleotide position number is given for the first and the last nucleotide of each line, respectively. Numbering of the HPV-8 sequence was done according to Fuchs et al. (1986). The potential E2 binding sites P1–P4, the degenerate palindrome P, the AT stretch of the early promoter's TATA box, and the conserved sequence motifs M33 and M29 are boxed; potential binding sites of AP1 and NF1 are indicated in bold; the translation stop codon of L1 and the start codon of E6 are printed in bold and are underlined. The arrowhead suggests the position of the base exchange in M33 which has been shown by Horn et al. (1993) to reduce enhancer activity in HPV-8.

NCR of RTRX7

The NCR of RTRX7 showed an organization of sequence motifs typical of EV-associated HPVs (Fig. 3; Krubke et al., 1987; Ensser & Pfister, 1990). With 463 bp between the translation termination codon of L1 and the start codon of E6, this region was relatively short compared to the NCR of mucosal HPVs. It contained four copies of palindromic E2 binding sites ACCN\_GGT at positions 7477 (P1), 7567 (P2), 7726 (P3) and 22 (P4), respectively. Another copy was located at position 7400 (P0) at the 3' end of the L1 ORF. A more degenerate palindrome, ACCN\_GTT (P\_G), appearing in the NCR sequences of HPV-5, -8 and -36, was found at a homologous position in RTRX7 at nt 7629. A seventh potential E2 binding site, which can also be found in HPV-5 but not in HPV-8 or -12, existed far from the NCR at position 3523 in the E4 ORF. The motifs M29 and M33 (Krubke et al., 1987) and the AP1 binding site were also conserved: compared to HPV-8, there were five nucleotide exchanges in M33, one in M29 and one in the AP1 site (Fig. 3). Furthermore, RTRX7 showed six potential NF1 binding sites spread between P2 and the M29 motif. A 30 bp long AT stretch was located at position -40 relative to the putative E6 protein translation start codon. It consisted of three TAAA blocks framed by short A/T alterations (Fig. 3).

Southern blot analysis

In a reconstruction experiment with HPV-8 plasmids, the Southern blot procedure allowed the detection of 0-1 genome
equivalent of HPV per cell, if 10 µg DNA was used as input (data not shown). In order to estimate the concentration of RTRX7 DNA in the biopsy material, 10 µg cellular DNA was digested with a non-cutter of RTRX7 and 10 µg with a single-cutter of RTRX7. The tumour DNA was then subjected to homologous Southern blot hybridization with purified labelled 12-PCR products (Table 1). Although the sensitivity standard for 0·1 genome equivalent per cell was visible, there was no RTRX7-specific signal, in either the non-cut lane or the single-cut lane (data not shown).

Discussion

The analysis of cloned PCR products revealed five different EV-HPV L1 sequences in a premalignant keratosis and three different EV-HPV DNAs in an SCC from a renal transplant recipient. Previous analyses of these biopsies by direct sequencing of PCR products (Hopf et al., 1997) identified only the RTRX7 sequence, which also prevailed in the majority of clones analysed here. This shows that cloning of PCR products is necessary for a comprehensive evaluation of clinical samples. Of the newly cloned sequences, three (RTRX8–RTRX10) were previously unknown. Alignments with published HPV sequences and phylogenetic analyses allowed their classification as HPV group B1 (EV-HPV) sequences. RTRX7–RTRX10 were distributed into different clusters of group B1 according to Myers et al. (1996) (Fig. 2). These findings underline the great variety of HPV types associated with premalignant and malignant skin lesions of immunosuppressed patients.

The 246 bp at the 3’ end of the determined L1 sequence fragments of RTRX8, RTRX9 and RTRX10 overlap with the 291 bp comprising the MY09–MY11 fragment, which was shown by Chan et al. (1995) to be sufficient for the typing of HPV sequences. RTRX8, RTRX9 and RTRX10 showed only 77·4, 82·6 and 83·7% sequence identity, respectively, with their closest relative (Table 2). These three isolates are therefore likely to represent new papillomavirus types, but before they can be declared new types their complete genomes need to be cloned.

The entire genome of RTRX7 was cloned from the SCC in six overlapping fragments generated by nested PCR. RTRX7 was closely related to HPV-12, but their DNA sequence identity did not exceed 90% in the complete L1 ORF (Table 3), indicating that RTRX7 can be considered a new HPV type (van Ranst et al., 1996). According to its similarity with HPVs from the EV-associated group and its genomic organization, RTRX7 can be designated as an EV type, although it has never been detected in a patient with EV thus far.

The fact that overlapping subgenomic fragments from all regions of the RTRX7 genome could be cloned from one biopsy suggests that the viral DNA persists in an episomal state in this SCC. However, the possibility cannot be excluded that PCR products arose from molecules with different physical states or which were differently integrated into the cellular genome. In malignant skin tumours of EV patients, a mixture of monomeric episomes, oligomeric molecules and genomes with deletions or duplications has sometimes been observed (Ostrow et al., 1987; Yabe et al., 1989; Deau et al., 1991). All RTRX7-positive clones of PCR products from respective genomic sites of the virus were identical, showing that any noteworthy intratype variety, deletions or duplications are very unlikely in the examined SCC. Episomal persistence of DNA is the rule for HPVs from the EV group in both benign and malignant lesions of EV patients (Orth, 1987) and in the few tumours from non-EV patients analysed by Southern blotting (Lutzner et al., 1983; Kawashima et al., 1990; Obalek et al., 1992).

The RTRX7 NCR sequence shared the features of HPV-5, -8 and -36 NCRs according to Enser & Pfister (1990). Its AT stretch was 5 bp shorter than that of HPV-8 and 16 bp shorter than that of HPV-12. The reduced size of this element in RTRX7 represented the main difference from the NCR of HPV-12. Horn et al. (1993) found the activity of the late promoter of HPV-8 to be reduced to 15·4% when a T → G point mutation was introduced into the M33 enhancer motif. A T → C exchange occurred in RTRX7 at exactly the same position (Fig. 3). These results give rise to speculation about a reduced activity of the late promoter in RTRX7.

The oncogenic potential of the sequences RTRX7, RTRX8, RTRX9 and RTRX10 remains to be substantiated. Only small amounts of RTRX7 DNA were present in the SCC examined by Southern blot. Hybridization with a homologous probe was used to demonstrate that the RTRX7 DNA concentration was lower than one copy per 10 cells in this biopsy. Although the tumour to stroma cell ratio in the investigated material is unknown, there was probably less than one viral genome copy per cancer cell. Low-level concentrations of viral DNA in EV-HPV-positive skin tumours from non-EV patients seem to be quite common. For this reason, viral DNA has previously been detected only rarely in such cases when Southern blotting was used. Shamamin et al. (1994) examined a set of skin cancer biopsies from immunosuppressed patients by Southern blot and by sequencing of PCR products, and found that the samples containing EV-associated and related HPV types were mostly negative by Southern blot analysis. Considering that RTRX7 was not detected even in a Southern blot experiment with a homologous probe, it is very likely that the negative results in Southern blots are due to the low concentration of viral DNA, and not to the use of inappropriate probes. Persistence of viral DNA at concentrations below one genome copy per cancer cell suggests that the EV-HPV DNA is not necessary for the maintenance of the malignant state. Nevertheless, there may be a kind of “cross-talk” operating between HPV DNA-containing tumour cells and their HPV-negative neighbour cells. EV-HPV DNA might, for example, induce the production of cytokines or other messenger substances, stimulating the growth of the adjacent cells.

Knowledge of the complete genome sequence of RTRX7
allows a more direct evaluation of its oncogenic potential by detection of transcripts of the viral oncogene E6 in skin cancers, or by analysis of the activity of the E6 promoter. Furthermore, viral oncoproteins could be expressed in keratinocytes to test for immortalization and transformation.

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References


Shamanin, V., zur Hausen, H., Laverne, D., Proby, C., Leigh, I. M.,
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