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Detection and characterization of human papillomavirus type 45 DNA in the cervical carcinoma cell line MS751

Jochen Geisbill, Ute Osmers and Matthias Dürst

Angewandte Tumorvirologie, Abteilung 0615, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

The cervical carcinoma-derived cell line MS751 was examined for human papillomavirus (HPV) DNA and RNA. A genomic fragment containing both viral and cellular sequences was cloned. Sequence analysis showed that MS751 cells contain a partially deleted HPV-45 genome integrated at a single chromosomal site. HPV sequences from the E6–E7 region are expressed as poly(A) RNA.

Human papillomavirus (HPV) types are known to contribute to the development of cervical cancer. This is documented by numerous epidemiological and experimental studies (Schiffmann, 1994; zur Hausen, 1994). Cancer-associated genital HPVs are represented by types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 68 (de Villiers, 1989, 1994; Jacobs *et al.*, 1995). HPV-16 and HPV-18 are detected more frequently in cervical carcinomas than any of the other HPV types (Bosch *et al.*, 1995; Lörincz *et al.*, 1992). Cell lines derived from cervical cancer are often used as model systems to analyse virus–host cell interactions which may contribute to carcinogenesis. On the basis of DNA and RNA filter hybridization data, cells of the cervical carcinoma-derived cell line MS751 were assumed to contain HPV-18 DNA (Pater & Pater, 1985; Yee *et al.*, 1985).

In this study, we have analysed the HPV sequences in MS751 cells by molecular cloning, DNA sequencing and Northern blot hybridization. The cell line was obtained from the ATCC (HTB 34). First, MS751 genomic DNA was examined for the presence of HPV-18 sequences by Southern blot analysis. DNA was isolated from MS751 cells and C-4 I cells by proteinase K treatment and phenol extraction. C-4 I cells were used for control purposes since these cells are known to contain a single copy of HPV-18 DNA per cell (Schwarz *et al.*, 1985). After restriction endonuclease digestion and electrophoretic separation in a 0·7% agarose gel, DNA was transferred to a GeneScreen nylon membrane (Dupont NEN). Cloned HPV-18 DNA was radiolabelled by the random priming method (Feinberg & Vogelstein, 1983). Hybridization was first performed under stringent conditions [50% formamide, 5 × SSC, 50 mM sodium phosphate pH 6·5, 5 × Denhardt's solution, 0·1 mg/ml tRNA, 1% SDS and 3×10^6 c.p.m./ml denatured gel-purified ^{32}P-labelled HPV-18 DNA ($> 5 \times 10^8$ c.p.m./μg) at 42 °C]. The filter was washed in 2 × SSC, 0·1% SDS at 68 °C and exposed for several days using intensifying screens. Under these conditions, only very faint signals were observed for MS751 DNA (data not shown). This filter was then stripped and reprobed with HPV-18 DNA under conditions of reduced stringency (30% instead of 50% formamide which corresponds to T_m − 36 °C instead of T_m − 18 °C, respectively). The filter was washed in 2 × SSC, 0·1% SDS at 50 °C (T_m −36 °C). For each restriction digest one or several prominent bands and numerous faint bands were evident (Fig. 1*a*). After exposure the filter was washed under stringent conditions (T_m −18 °C) and re-exposed for the same length of time. As expected the signal intensity of the major bands for C-4 I were slightly reduced. In contrast, the signal intensities of the bands for MS751 DNA decreased considerably (Fig. 1*b*). These results indicate that MS751 cells do not contain HPV-18 DNA but rather the DNA of a papillomavirus related to HPV-18.

For molecular cloning of the HPV-18-related papillomavirus DNA, genomic MS751 DNA was cleaved with *Eco*RI. Digestion with this restriction enzyme produces a single fragment of about 9·5 kb in length that hybridized to the HPV-18 probe (Fig. 1*b*). To enrich for virus-specific DNA, *Eco*RI-cleaved MS751 DNA was fractionated in a 0·7% agarose gel and DNA fragments of about 9·5 kb were eluted. For construction of a phage library, *Eco*RI-cleaved, dephosphorylated LambdaGEM-12 arms (Promega) were used. Lambda arms and genomic inserts were ligated using equal molar ratios. *In vitro* packaging of the DNA was done with Packagene extracts from Promega. Recombinant phages were amplified in *E. coli* strain LE392. Plaque hybridization of replica filters was performed under conditions of reduced stringency (T_m − 36 °C) with two HPV-18 subgenomic probes (1·8 kb and 3·4 kb). This approach was chosen to distinguish more easily between positive and false-positive clones. The 1·8 kb fragment comprises part of the E7-ORF and E1-ORF (nucleotide positions 658–2472), the 3·4 kb fragment comprises part

Author for correspondence: Matthias Dürst.
Fax +49 6221 424932. e-mail m.duerst@dkfz-heidelberg.de

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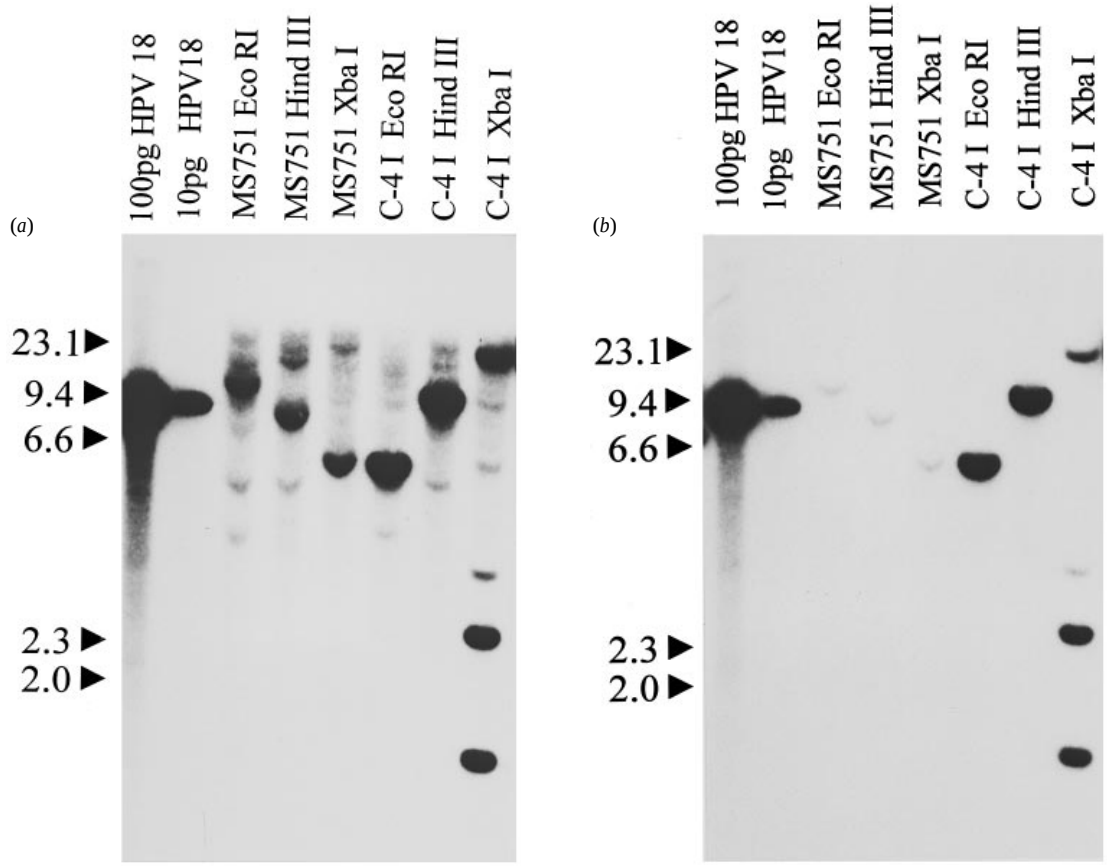

Fig. 1. Southern blot analysis of MS751 and C-4 I DNA with HPV-18 as hybridization probe. (*a*) Hybridization was performed under low-stringency conditions (T_m — 36 °C). (*b*) Same filter as in (*a*) washed under stringent conditions (T_m — 18 °C) and re-exposed under identical conditions. The arrowheads indicate the positions (bp) of the lambda *Hin*dIII size-marker fragments. As sensitivity markers 100 pg and 10 pg of cloned HPV-18 DNA were used.

Table 1. Summary of all nucleotide exchanges and amino acid substitutions after sequence alignment of the E6- and E7-ORF of HPV-45 from MS751 and that of the reference clone

ORF	Nucleotide position	Base exchange MS751 ↔ reference	Amino acid substitution MS751 ↔ reference
E6	259	A ↔ G	Tyrosine ↔ cysteine
E6	497	G ↔ A	Glycine
E7	590	T ↔ C	Tyrosine ↔ histidine
E7	600	A ↔ G	Glutamine ↔ arginine
E7	603	C ↔ A	Alanine ↔ glutamic acid
E7	670	C ↔ T	Cysteine
E7	769	C ↔ A	Asparagine ↔ lysine
E7	832	A ↔ G	Glutamic acid

of the L2-ORF, the entire L1-ORF, the non-coding region and the E6-ORF (nucleotide positions 5144–658) of HPV-18. Screening of 10^5 recombinant phages led to the identification of a single positive recombinant clone that carried an insert of about 9·5 kb. This insert was subcloned into the plasmid vector Bluescribe (Stratagene) for further analysis. DNA sequence analysis was performed by the dideoxy method (Sanger *et al.*, 1977) by using the oligonucleotide primers M13 reverse and M13 universal, which flank the multiple cloning site of the vector. The first 50–200 nucleotides at each end of the 9·5 kb insert were determined, and the sequence data were analysed by use of the HUSAR (Heidelberg Unix Sequence Analysis Resources) program package. DNA sequence comparison revealed no identities to any HPV DNA. Instead, identities to human Alu-repeat sequences were found (Batzer *et al.*, 1996). In order to determine the identity of the HPV sequences that form part of the 9·5 kb fragment, it was decided to amplify a region within the putative viral E6-ORF. This was achieved by using a set of consensus primers specific for the E6-ORF of high-risk HPV types (Lungu *et al.*, 1995). A fragment of 200 bp was amplified and cloned into a plasmid vector (TA-Cloning Kit, Invitrogen). The complete 200 bp fragment was sequenced and was shown to share 99·5% identity with HPV-45 DNA (Naghashfar *et al.*, 1987). A single nucleotide exchange,

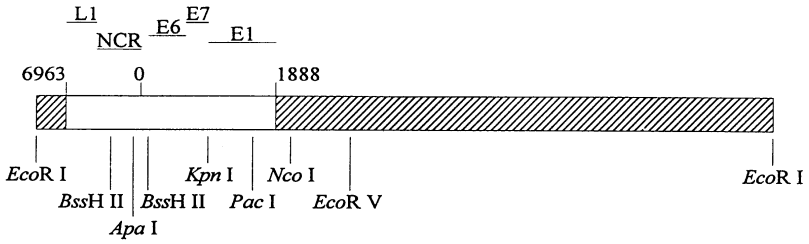

Fig. 2. Physical map of the 9·5 kb *Eco*RI restriction fragment cloned from MS751 genomic DNA and location of viral and cellular sequences. The positions of cleavage sites for restriction enzymes are shown. Cellular sequences are represented by regions with diagonal hatching. HPV sequences are given as an open bar and the open reading frames are indicated on top.

whereby guanine replaces adenine, was noted at position 259 (numbering is based on the HPV-45 sequence available under Genbank accession number X74479). Subsequently, the entire E6-ORF and E7-ORF contained within the cloned 9·5 kb fragment were sequenced by using a set of HPV-45-specific oligonucleotide primers. By alignment of this sequence to HPV-45, a nucleotide sequence identity of 98% was determined. A cluster of nucleotide exchanges is evident at the 5′ end of the E7-ORF. The positions of all nucleotide exchanges and their effect at the amino acid level are summarized in Table 1.

To further characterize the 9·5 kb fragment, in particular to define more precisely which region of the viral genome had integrated into the host DNA, a Southern blot with several different restriction digests of HPV-45 DNA was hybridized with the cloned 9·5 kb fragment as probe. From the hybridization pattern it could be inferred that part of the L1-ORF, the entire non-coding region, the E6-ORF and E7-ORF and part of the E1-ORF are contained within the 9·5 kb fragment. The remaining parts of the viral genome (3′E1, E2, E4, E5, L2 and 5′L1) are deleted (data not shown). On the basis of this information we used HPV-45-specific oligonucleotides to sequence the junctions between the integrated HPV sequences and the 5′- and 3′-flanking cellular sequences. The breakpoints in the viral genome for integration were found to be at nucleotide positions 1888 and 6963. An exact physical map of the 9·5 kb fragment and the location of the viral and cellular sequences are shown in Fig. 2. Similar to the situation of most other HPV-positive cervical carcinoma cell lines, the E1-ORF of the viral genome cells is disrupted in MS751 as a consequence of integration (Schwarz *et al.*, 1985). This uncouples the upstream regulatory region from several downstream early genes including the E2 gene. At least for HPV-16 and HPV-18 the E2 gene product can act as a negative modulator of E6–E7 gene expression (Bernard *et al.*, 1989; Ham *et al.*, 1991; Romanczuk *et al.*, 1990). Lack of E2 gene expression, either by deletion or uncoupling from its promoter, may therefore result in an upregulation of the viral oncoproteins E6 and E7.

Expression of viral DNA was examined by Northern blot hybridization of MS751 poly(A)+ RNA with radiolabelled subgenomic HPV-45 fragments. One major (1·2 kb) and one minor (3 kb) mRNA species were detected with a probe that covers the E6-ORF and E7-ORF. No hybridization signals were observed with probes containing E1-E2-E4 and L1-L2-ncr sequences, respectively (data not shown). These data indicate that expression is confined to ORFs E6 and E7. It is likely, that in analogy to HPV-18-positive cervical carcinoma cell lines, both mRNA species comprise virus–cell fusion transcripts in which the 3′-terminal host sequences are spliced to 5′-terminal exon sequences from the viral E6-E7-E1 region (Schneider-Gädicke & Schwarz, 1986).

This study has shown that cells of the human cervical carcinoma cell line MS751 contain DNA of HPV type 45. On a phylogenetic basis, HPV-45 is most closely related to HPV-18 (73·9%), followed by HPV-39 (59·2%) (Bernard *et al.*, 1994; Delius & Hofmann, 1994). The previous assumption that MS751 cells harbour HPV-18 DNA (Pater & Pater, 1985; Yee *et al.*, 1985) can be explained by cross-hybridization between both HPV types because of the close sequence relationship (Naghashfar *et al.*, 1987). It should be noted that in the early eighties, when cervical carcinoma cell lines were screened for the presence of HPV-specific sequences, HPV-16 and HPV-18 were the only oncogenic HPV types available as molecularly cloned probes (Boshart *et al.*, 1984; Dürst *et al.*, 1983).

HPV-45 is a cancer-associated type first cloned from a recurrent cervical lesion displaying mild to moderate dysplasia with koilocytosis (Naghashfar *et al.*, 1987). Its prevalence in genital tract infections is rather low (Naghashfar *et al.*, 1987; Williamson *et al.*, 1994). However, there seems to be significant geographical variation. A clustering of HPV-45 was noted for Western Africa (Bosch *et al.*, 1995). Sequence comparison of HPV-45 from the cervical carcinoma cell line MS751 with the originally cloned isolate (Naghashfar *et al.*, 1987) revealed some interesting differences at the amino acid level of the E7 gene. Three hydrophilic amino acids at the amino-terminal end of the protein which are characteristic for the original HPV-45 isolate are replaced by hydrophobic amino acids (Table 1). It is likely that these alterations have an effect on the functional properties of the protein. It will now have to be investigated whether these differences relate in any way to the transforming potential of HPV-45. Moreover, the existence of two HPV-45 strains with different biological potential should be considered.

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