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APM-1, a novel human gene, identified by aberrant co-transcription with papillomavirus oncogenes in a cervical carcinoma cell line, encodes a BTB/POZ-zinc finger protein with growth inhibitory activity

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Integration of human papillomavirus (HPV) DNA into the host cell genome is an important step in cervical carcinogenesis. In tumour cells with integrated HPV DNA, transcription of viral oncogenes *E6* and *E7* continues into the flanking cellular sequences thereby producing viral–cellular fusion transcripts. Analysis of cellular sequences flanking the integrated HPV68 DNA in the cervical carcinoma cell line ME180 revealed homozygosity of the mutant allele in ME180 cells. We speculated that this could indicate the existence of a cellular tumour suppressor gene in the integration region. We report here the identification of a novel human gene, named *APM-1*, which is co-transcribed with the HPV68 *E6* and *E7* genes and is present in the 3′-cellular part of the ME180 viral–cellular fusion transcripts. The *APM-1* gene encodes a protein with a BTB/POZ domain and four zinc fingers, and is located at chromosome 18q21. *APM-1* transcripts are detected in normal cervical keratinocytes, but not in the majority of cervical carcinoma cell lines analysed. The *APM-1* gene caused a reduction of clonal cell growth *in vitro* of HeLa and CaSki tumour cells. These characteristics make *APM-1*, the first novel human gene identified in a HPV integration region, a likely candidate for the postulated tumour suppressor gene.

Keywords: BTB/POZ domain/chromosome 18q21/HPV/integration/tumour suppressor

Introduction

Cancer development is a multifactorial and multistep process in which sequential genetic changes accumulate causing the activation of cellular proto-oncogenes and the loss or inactivation of tumour suppressor genes (Vogelstein and Kinzler, 1993; Lanfrancone *et al.*, 1994). In addition, viruses play an essential role as initiators and drivers of the tumorigenic process in some types of human tumours (zur Hausen, 1996). Human papillomaviruses (HPV) are causally involved in the genesis of cervical carcinomas (zur Hausen, 1994). Types HPV16 and HPV18 are the prototypes of the cancer-associated papillomaviruses, but other related HPV types have also been detected in malignant tumours and cell lines.

The oncogenic potential of HPV16 and HPV18 has been attributed primarily to the two early genes, *E6* and *E7*. Their products are multifunctional immortalizing and growth-promoting proteins. Major pathways by which they interfere with normal cell regulation are the inactivation of two key tumour suppressors, the p53 protein (by *E6*) and the retinoblastoma gene product pRB (by *E7*) (Hoppe-Seyler and Scheffner, 1997; Tommasino and Jansen-Duerr, 1997).

In addition to HPV infection and viral oncogene expression, mutations of host cell genes have to accumulate for a fully malignant phenotype of cervical epithelial cells. The detection of chromosome losses and aberrations and of losses of allele heterozygosity reflect the requirement for multiple genetic changes in cervical carcinogenesis (Mullokandov *et al.*, 1996). However, the human genes involved have still to be identified.

The HPV DNA genome persists as an extrachromosomal episome in the nucleus of the infected non-malignant cells. In cancer cells and cell lines, however, the viral DNA is found integrated into the host cell genome. Integration can occur into different chromosomal locations with a preference for fragile sites and near oncogenes such as the *myc* genes (Cannizzaro *et al.*, 1988; Couturier *et al.*, 1991).

Integration of HPV DNA is considered to be an important step in cervical carcinogenesis. Recent studies supporting this view have shown that integration leads to increased stability of the mRNAs encoding the viral *E6* and *E7* oncogenes (Jeon and Lambert, 1995), to increased levels of viral oncogene expression and selective growth advantage of the cells (Jeon *et al.*, 1995). Conversely, expression of the *E6* and *E7* oncogenes has been shown to result in an increased frequency of DNA integration (Kessis *et al.*, 1996).

In the integrated HPV genomes, the early region which harbours the *E6* and *E7* oncogenes is usually truncated and lacks the 3′-terminal part with the poly(A) signal (Schwarz *et al.*, 1985). Therefore, transcription initiated at the viral early promoter continues into the flanking cellular sequences, and RNA processing results in multiply spliced fusion transcripts composed of 5′-terminal viral sequences with the *E6* and *E7* oncogenes and 3′-terminal co-transcribed cellular sequences (Schneider-Gädicke and Schwarz, 1986).

Besides affecting the expression of the viral oncogenes, the integration event may also cause alterations of cellular genes within the target region. For example, integration of HPV DNA near *myc* genes has been reported to enhance the proto-oncogene expression (Couturier *et al.*, 1991).

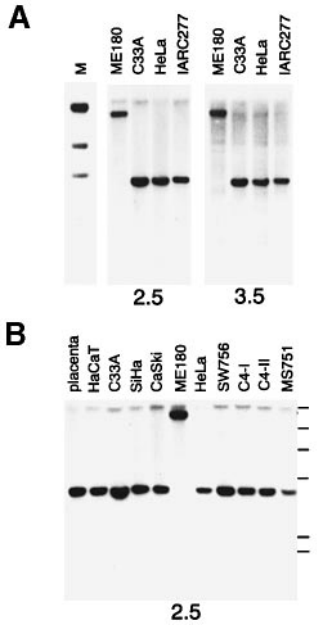

Fig. 1. Southern blot analysis of different cervical carcinoma cell lines with hybridization probes representing the left and right flanking cellular sequences of the integrated HPV68 DNA in ME180 cells. (**A**) The genomic DNA was cleaved with *Sac*I, and the filters were hybridized either with probe 2.5, a 2.5 kb *Sac*I–*Bam*HI fragment containing the left flanking cellular sequences, or with probe 3.5, a 3.5 kb *Bam*HI–*Sac*I fragment containing the right flanking cellular sequences. (**B**) The genomic DNA was cleaved with *Hin*dIII, and the filter was hybridized with probe 2.5. Lane M in (A) and the bars in (B) indicate the positions of the λ *Hin*dIII size marker fragments. The names of the cell lines are given above the lanes. DNA isolated from a biopsy of normal human placenta was also included.

Since the integrated HPV DNA is a tag for the chromosomal target region, it may also provide a useful tool for the identification of novel cancer-relevant cellular genes (Kahn *et al.*, 1996). In the present study, we have analysed in the human cervical carcinoma cell line ME180 the cellular target region of the integrated HPV DNA (Reuter *et al.*, 1991) which has meanwhile been classified as an HPV68 subtype (Longuet *et al.*, 1996). We report here the identification of a novel human gene, *APM-1*, which is present in the viral–cellular fusion transcripts due to co-transcription with the HPV68 *E6* and *E7* genes. The *APM-1* gene encodes a protein with zinc fingers and a BTB/POZ domain that exerts the activities of a cell growth inhibitor.

Results

Homozygosity of the mutant allele carrying the integrated HPV68 DNA in the cell line ME180

From a genomic DNA library of the cell line ME180, the integrated HPV68 DNA had previously been isolated together with flanking cellular sequences at both sides (Reuter *et al.*, 1991). To examine the cellular target region of HPV68 DNA integration, the cloned flanking cellular sequences were used as radiolabelled probes in genomic Southern blot hybridizations (Figure 1). Remarkably, the hybridization patterns of ME180 DNA revealed homozygosity of the mutant allele carrying the integrated HPV68 DNA, a result suggesting that loss of heterozygosity has occurred after the HPV DNA integration event. Since allele loss is characteristic for loci harbouring tumour suppressor genes (Knudson, 1993), we speculated that the cellular target region of HPV68 DNA integration might harbour a tumour suppressor gene.

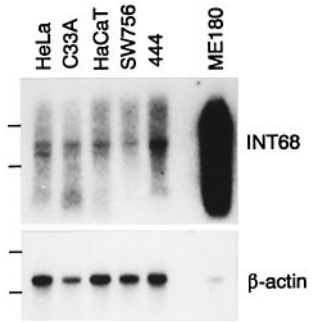

Fig. 2. Identification of transcripts originating from the cosmid INT68 region which harbours the integration site of HPV68 DNA in the cell line ME180. A filter with poly(A)$^+$-RNA from different cervical carcinoma cell lines was hybridized with the complete cosmid DNA as radiolabelled probe. Hybridization of repetitive sequences was blocked with Cot-1 DNA. The filter was rehybridized with a human β-actin probe. The horizontal bars indicate the positions of the 28S and 18S rRNAs.

Transcribed human sequences in the target region of HPV68 integration

To search for the suspected gene, we first examined by Northern blot analysis whether the cloned cellular sequences flanking the integrated HPV68 DNA hybridize with human mRNAs. Since this was not the case (data not shown), we isolated the genomic region encompassing the ME180-specific site of HPV68 DNA integration from a cosmid library of normal human leukocyte DNA. One cosmid clone, designated INT68 (because it contains the chromosomal target site of HPV68 DNA integration), was used for the further analyses.

To examine whether the cosmid insert harbours transcribed sequences, Northern hybridizations with the radiolabelled INT68 DNA probe were performed (Figure 2). In ME180 poly(A)$^+$-RNA, the cosmid probe produced very strong hybridization signals which were indistinguishable from the signals produced with an HPV68 *E6*/*E7* probe (not shown). In the poly(A)$^+$-RNAs of the other cell lines tested, two transcripts of ~4 kb and ~3 kb length were detected. These results indicated first, that the cosmid INT68 includes sequences that are co-transcribed with the HPV68 genes *E6* and *E7* to produce the viral–cellular fusion transcripts in ME180 cells, and second, that the cosmid INT68 harbours sequences that are transcribed in other cell lines in the absence of integrated HPV68 DNA and thus should be integral parts of authentic cellular mRNAs.

To localize the transcribed sequences within the cosmid insert, the INT68 DNA was dissected into *Sac*I fragments which were used as individual probes in Northern hybridizations. The viral–cellular fusion transcripts of ME180 cells hybridized with a *Sac*I fragment of 5.5 kb (not shown) which was subsequently cloned and partially sequenced (see Figure 4B for the *Sac*I map of cosmid INT68). Comparison of the partial sequences with the database-stored sequences revealed that the fragment contains sequence motifs with the potential to encode

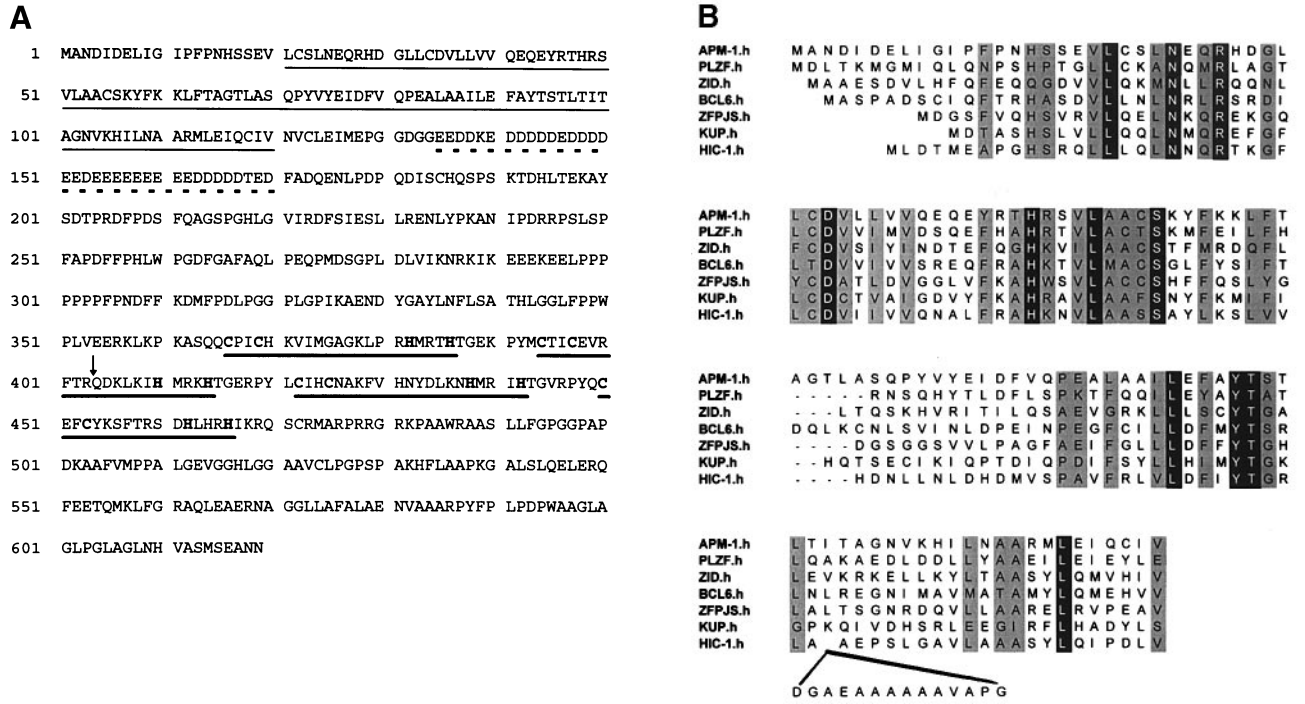

Fig. 3. Amino acid sequence of the APM-1 protein. (**A**) Complete amino acid sequence of APM-1 as deduced from the nucleotide sequence of the viral–cellular fusion transcript cDNA of cell line ME180. The complete fusion transcript cDNA sequence is available under DDBJ/EMBL/GenBank accession number Y14591. The N-terminal BTB/POZ domain is indicated by underlining, the acidic region by a broken line, and the four C_2H_2 zinc fingers by heavy underlining. The vertical arrow marks the cellular exon–exon junction. (**B**) Comparison of the N-terminal 120 amino acid residues of APM-1 with the N-terminal BTB/POZ domains of other zinc finger proteins of human origin (indicated by h after the gene name). White letters in black columns denote the positions of amino acid residues which are identical in all seven proteins. Grey shading marks positions with identical amino acids in at least four of the seven proteins compared. For the other human proteins, the sequence accession numbers and positions shown are: Q05516, pos. 1–115 (PLZF), X 82018, pos. 1–110 (ZID), P41182, pos. 1–118 (BCL-6), L16896, pos. 1–108 (ZFPJS), P24278, pos. 1–108 (KUP), and L41919, pos. 1–123 (HIC-1).

Cys_2-His_2 zinc fingers of the Krueppel type. The INT68 zinc finger sequences were not identical with any of the already known human zinc finger-encoding genes. Furthermore, a 0.5 kb subfragment containing the zinc finger motifs also hybridized with the fusion transcripts of ME180 cells (not shown). These results indicated that a novel human gene encoding a zinc finger protein is located in the INT68 region and is co-transcribed with the HPV68 *E6* and *E7* genes in ME180 cells. We named the human gene *APM-1* to indicate that its transcription is affected by papillomavirus DNA integration in ME180 cells.

Fusion transcript structure and exon–intron composition of the human APM-1 gene

To characterize the ME180-specific fusion transcripts, more than 20 positive clones were isolated from a ME180 cDNA library and were structurally characterized by PCR and hybridization analysis. The results indicated that the co-transcribed cellular sequences of all cDNA clones are identical, and that the transition from the 5′-viral into the 3′-co-transcribed cellular sequences occurs in all cDNA clones at a splice donor located at the beginning of HPV68 ORF E1.

One representative cDNA clone, Mefust-4, was selected for complete sequence analysis. According to the nucleotide sequence, the fusion transcript contains a 5′-terminal HPV68 segment of 839 bp with the early genes *E6* and *E7* followed by a 3′-terminal cellular segment of 3779 bp. The cellular part includes an open reading frame with the coding potential for a protein of 619 amino acid residues (Figure 3A). The deduced protein contains four Krueppel-type Cys_2-His_2 zinc fingers, of which two-and-a-half are encoded in the 0.5 kb subfragment of the 5.5 kb *Sac*I fragment of cosmid INT68. These results show that the ORF in the co-transcribed cellular part of the fusion transcripts harbours the *APM-1* gene.

Computer-based alignment analysis revealed that the N-terminus of the deduced APM-1 protein shows strong similarity to a conserved motif called the BTB or POZ domain (Figure 3B). The BTB/POZ domain is a newly characterized motif of ~120 amino acid residues which is evolutionary conserved, primarily found at the N-terminus of zinc finger proteins, and involved in protein–protein interactions (Bardwell and Treisman, 1994; Albagli *et al.*, 1995; Chen *et al.*, 1995). The ATG triplet located 17 bp downstream of the viral–cellular exon junction can be assigned as the potential translational start codon of the *APM-1* gene based on the sequence alignment and because it is in a good sequence context for translational initiation according to the Kozak rules (Kozak, 1996). The BTB/POZ domain is followed by a highly acidic region (Figure 3A).

Comparison of the cDNA sequence with the partial and complete nucleotide sequences of the genomic *Sac*I fragments 5.5 kb and 4.5 kb (4.5a) and of the adjacent terminus of the 3.3 kb fragment allowed identification of the exon junctions in the fusion transcript cDNA and the exon locations within cosmid INT68. The results are summarized in Figure 4. The cDNA clone Mefust-4 includes a 3′-terminal A_{20} stretch. Fifteen of these

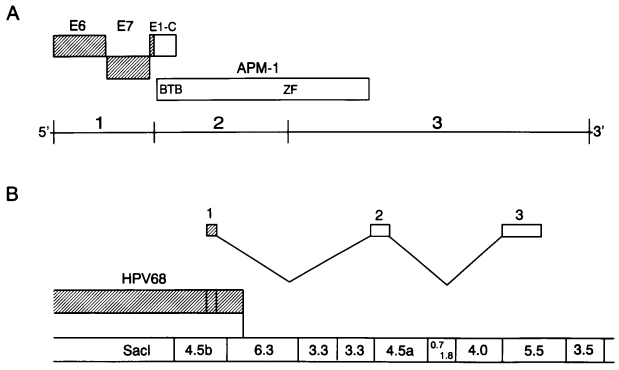

Fig. 4. Structural organization of the viral–cellular fusion transcripts of ME180 cells and of the human genomic region harbouring the *APM-1* gene and the HPV68 integration site. (**A**) The ORFs present in the 4618 bp sequence of the fusion transcript cDNA (DDBJ/EMBL/GenBank accession number Y14591) are shown by boxes (5′-end: first ATG codon, 3′-end: termination codon). Viral sequences are indicated by diagonal hatching. The cDNA is composed of three exons. Exon 1 (pos. 1–839) is of viral origin, exon 2 (pos. 840–2063) and exon 3 (pos. 2064–4618) are of human origin. The HPV68 *E6* gene (pos. 1–477) encodes a protein of 158 amino acids, and the HPV68 *E7* gene (pos. 485–817) a protein of 110 amino acids. The two viral ORFs are followed by a small fusion ORF E1-C (pos. 824–1060) potentially encoding a protein composed of six amino acid residues derived from the HPV68 *E1* gene, and 72 amino acid residues of cellular origin. The *APM-1* coding region (pos. 856–2715) extends over two exons. The stop codon which defines the *APM-1* ORF at the 5′-side is located in the viral exon 5 bp upstream of the viral–cellular exon junction. The first ATG codon providing the putative translational start site (see Figure 3) is located in the cellular part 17 bp downstream of the exon junction. The positions of the encoded BTB and zinc finger domains are indicated. The ATG codon of the HPV68 *E6* gene is set as starting point for numbering. The cDNA clone Mefust-4 used for sequence analysis does not include the nucleotides from positions 1–12 which have been taken from the HPV68 DNA sequence (Reuter *et al.*, 1991; accession number M73258). The EST sequences with DDBJ/EMBL/GenBank accession numbers H44758 and H44759 of the *Homo sapiens* cDNA clone 187988 are nearly identical to positions 1366–1688 and 1887–2215 of the fusion transcript cDNA sequence indicating that cDNA clone 187988 is derived from an *APM-1* transcript. (**B**) *Sac*I restriction map of the cosmid INT68. The numbers indicate the fragment sizes in kb. The integration site of HPV68 DNA in ME180 cells is located in the *Sac*I fragment 6.3. The positions of the fusion transcript exons within the INT68 region (exons 2 and 3 containing the *APM-1* coding region) and within the integrated HPV68 DNA (exon 1) are given on top. The introns are shown by the zig-zag lines.

A-residues are found in the respective genomic sequence, followed by a potential polyadenylation signal AATAAA and a potential splice donor sequence. These data indicate that the fusion transcripts may either be terminated at this polyadenylation signal or, alternatively, may contain an additional 3′-untranslated exon.

APM-1 transcript patterns

To analyse expression of the *APM-1* gene, Northern analyses with poly(A)$^+$-RNA from cervical carcinoma cell lines and cultured normal cervical epithelial cells were performed. Figure 5 gives a synopsis of the results. Using various subfragments of the INT68 genomic segment and of the Mefust-4 cDNA as radiolabelled probes (depicted in Figure 5, panels k and l), altogether three cellular mRNA species with sizes of ~5 kb, 4 kb and 3 kb, respectively, were detected in the cell lines that do not harbour the integrated HPV68 DNA. The different hybridization profiles indicate that the 5 kb transcript is

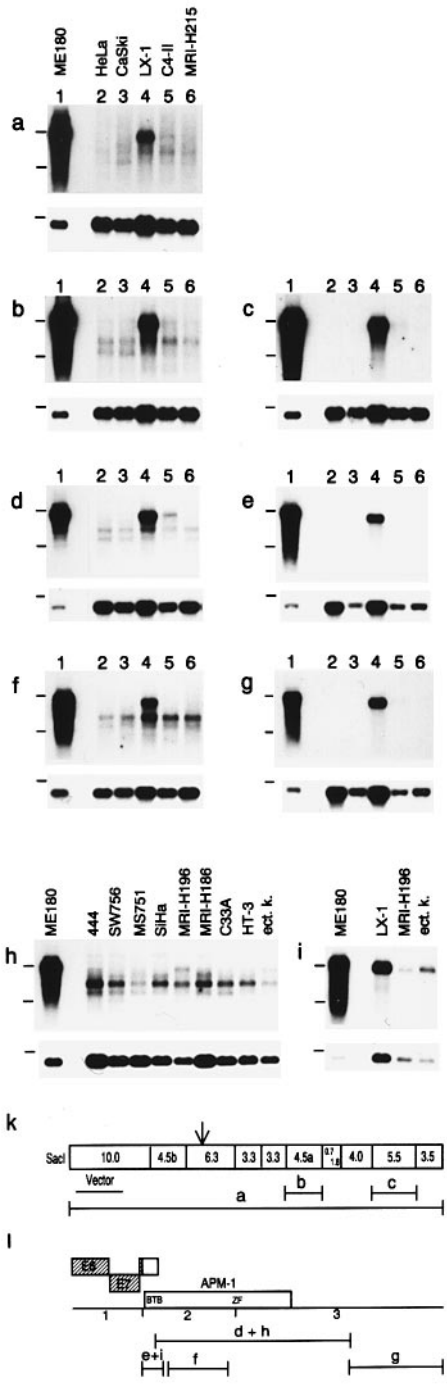

Fig. 5. Synopsis of Northern blot hybridizations. Poly(A)$^+$-RNA was isolated from different cervical carcinoma cell lines and from cultured normal human ectocervical keratinocytes (ect.k.). The RNA filters were hybridized with probes derived either from cosmid INT68 (panels a–c) or from the fusion transcript cDNA (panels d–i). The results of rehybridization of the filters with a GAPDH probe are shown at the bottom part of each panel. The horizontal bars indicate the positions of the 28S and 18S rRNA (top part) and of the 18S rRNA (bottom part). The small letters a to i also indicate the locations of the fragments used as hybridization probes within the *Sac*I map of cosmid INT68 (panel k) and within the cDNA (panel l). For a description of the genes and exons see legend to Figure 4.

the most relevant of these three mRNA species with regard to the *APM-1* gene. Poly(A)$^+$-RNA of the lung carcinoma cell line LX-1 was included in the Northern experiments as a positive control, since the 5 kb mRNA was discernible

in this cell line with a strong hybridization signal. One important feature of the 5 kb mRNA is that it hybridized with all the different cDNA probes derived from the *APM-1* coding region and the 3′-untranslated part (Figure 5, panels d–i). These hybridization properties indicate that the exons 2 and 3 of the fusion transcript are present in identical form in the cellular 5 kb mRNA. We concluded that the 5 kb transcript is the authentic cellular mRNA for the *APM-1* gene. Another feature of the 5 kb transcript is the restricted presence in the cervical carcinoma cell lines. The 5 kb mRNA was detectable in the cultured normal cervical keratinocytes (Figure 5, panel i), but in only two out of the 11 cell lines tested (C4-II and MRI-H196; Figure 5, panels d, h and i). The levels of the 5 kb mRNA in the normal cervical cells and the two carcinoma cell lines are much lower than in the LX-1 cell line.

The two other mRNA species, with sizes of ~3 kb and 4 kb, differ in two important aspects from the 5 kb *APM-1* mRNA. First, they seem to be ubiquitously present, in particular the 4 kb species. Second, they did not hybridize with the second *APM-1* coding exon (probes c and g) or the BTB/POZ domain segment (probe e), but only with a probe containing the 3′-part of the first *APM-1* coding exon (probe f). These hybridization properties suggest that the 3 kb and 4 kb transcripts do not harbour the complete coding region of the *APM-1* gene and the 3′-untranslated part. It should be mentioned here that the cellular mRNAs detected with the INT68 cosmid probe in the initial Northern analysis (see Figure 2) are the 4 kb and 3 kb transcripts.

For additional analysis of the cellular *APM-1* mRNAs, we used cosmid INT68 to employ a strategy for cosmid-specific selection of cDNA clones (Korn *et al.*, 1992, 1997). Sixteen cDNA clones with insert sizes ranging between 150 bp and 924 bp were isolated and sequenced (data not shown). Six cDNAs were derived from human fetal liver, 10 from fetal brain, but none from adult skeletal muscle. In most cases, the nucleotide sequences of the cDNA inserts were identical to the respective parts of the *APM-1* sequences present in the ME180 fusion transcripts. Interestingly, in one liver cDNA clone the first *APM-1* coding exon (i.e. exon 2 of the fusion transcript) is preceded by a 5′-terminal sequence of 127 bp which probably constitutes part of a 5′-non-coding exon of the cellular *APM-1* mRNA (not shown). The 127 bp segment did not hybridize with the INT68 cosmid DNA (not shown).

Chromosomal localization of the HPV68 integration site and of the APM-1 gene

To determine the chromosomal localization of the HPV68 integration site and of the *APM-1* gene, fluorescence *in situ* hybridizations to metaphase chromosomes of normal human cells and ME180 cells were performed. Hybridization of the biotin-labelled INT68 cosmid probe to normal human metaphase chromosomes and banding of the chromosomes by DAPI revealed the specific localization of the target sequences on the long arm of chromosome 18 within band 18q21 (Figure 6).

The copy number of the viral integration locus in ME180 cells was assessed by dual colour fluorescence *in situ* hybridization with the biotin-labelled INT68 cosmid and a digoxigenin-labelled HPV68 restriction fragment.

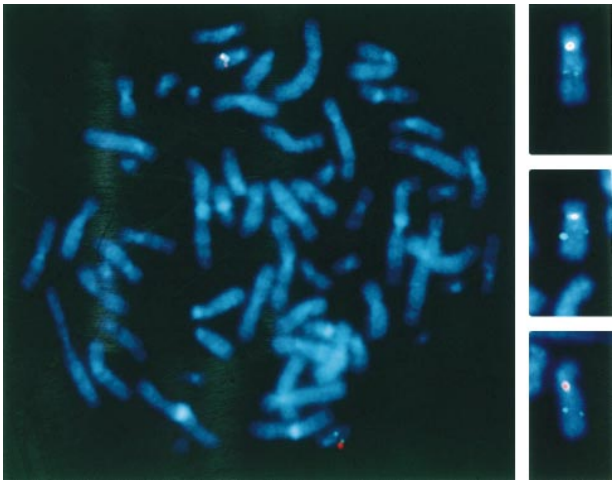

Fig. 6. Chromosomal localization of the HPV68 integration site and the human *APM-1* gene by fluorescence *in situ* hybridization. The left panel shows a complete metaphase cell of the cell line ME180 after *in situ* hybridization with the INT68 cosmid probe (green) and the HPV68 probe (red). The right panels show chromosome 18 homologues from normal human metaphase cells after hybridization with INT68 (green) and with the probe L1.84 specific for chromosome 18 centromeres (red). All panels show digitized images resulting from electronic overlay of two or three fluorescence images obtained from the hybridization signal and the DAPI stain, respectively.

In the analysed metaphase cells, two copies of chromosome 18 were found which contain the human genomic DNA of the integration site and the viral sequences. The highly specific co-localization of both sequences is illustrated in Figure 6. No INT68 sequences without co-localization with HPV68 DNA were found. These data demonstrate that ME180 cells contain two copies of the mutant chromosome 18 harbouring the integrated HPV68 DNA, and lack copies of the normal (unoccupied) chromosome 18.

Effect of APM-1 on cell growth

The present study was initiated by the speculation that a cellular tumour suppressor gene might be located in the genomic target region of HPV68 DNA integration in the cell line ME180. We tested this assumption for the identified *APM-1* gene using a colony formation assay. After inserting the *APM-1* gene and the complete fusion transcript cDNA, Mefust, into the eukaryotic expression vector pSG5, each of the expression constructs was co-transfected with the G418 selection plasmid pSV2-neo into cells of the HeLa and CaSki cervical carcinoma cell lines, both of which do not contain detectable amounts of the endogenous *APM-1* transcript (see Figure 5). The pSG5 vector alone and the cDNA antisense construct were used as controls. The results of the colony formation assays are shown in Figure 7. The clonal growth of both HeLa and CaSki cells was strongly reduced by the transfected *APM-1* expression plasmid. Compared with the pSG5 control, the Mefust fusion transcript cDNA also exerted a growth inhibitory effect which, however, was weaker than that of the *APM-1* construct and was comparable with the effect of the antisense construct. The results indicate that the *APM-1* gene encodes a protein with growth inhibitory activity for cervical carcinoma cell lines.

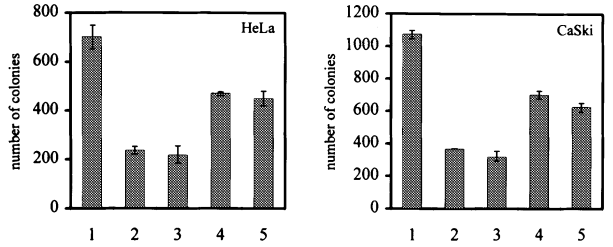

Fig. 7. Colony formation assay to examine the influence of the *APM-1* gene on tumour cell growth. HeLa or CaSki cells were transfected with either pSG5 vector alone (1), the expression plasmid pSG5–APM-1 using two different DNA preparations (2, 3), the antisense construct pSG5–APM-1-as (4), or the expression plasmid pSG5–Mefust (5). Two additional independent experiments were performed with HeLa cells and one additional experiment with CaSki cells. All experiments yielded comparable results. Transfection efficiency was controlled by co-transfection of the pCMV-gal plasmid and measurement of β-galactosidase activity.

Discussion

In this study we report the identification of a novel human gene, *APM-1*, that is aberrantly co-transcribed with HPV68 *E6* and *E7* genes in the cervical carcinoma cell line ME180 due to integration of the papillomavirus DNA into the gene locus. The *APM-1* gene is localized in region q21 of chromosome 18 and encodes a zinc finger protein with an N-terminal BTB/POZ domain. In transfection assays, the *APM-1* gene product showed the potential to reduce tumour cell proliferation *in vitro*.

From the structures of the cDNAs and the genomic DNA, we deduce that the HPV68 DNA is integrated into an intron that separates the 5′-untranslated exon(s) from the first coding exon of the *APM-1* gene. As a consequence, the protein-encoding part of the *APM-1* gene is separated from the promoter region, and transcription is placed under control of the early promoter of the integrated HPV68 DNA. A hallmark of the mature fusion transcripts is their polycistronic structure with the viral *E6* and *E7* genes and the cellular *APM-1* gene being present as separate cistrons (Figure 4A). Eukaryotic mRNAs are usually monocistronic, and initiation of translation is generally restricted to the first AUG start codon downstream of the 5′-cap (Kozak, 1996). In the fusion transcripts the AUG codon of the *APM-1* gene is preceded by the AUG codons of the three viral genes *E6*, *E7* and *E1* which are all in a good or adequate sequence context for initiation of translation according to the Kozak rules (Kozak, 1996). These structural features suggest that translation of the *APM-1* gene from the fusion transcripts may be inefficient or even impossible. With regard to expression of the *APM-1* gene in the ME180 cell line, integration of HPV68 DNA might thus have created an apparently paradoxical situation in which efficient transcription from the viral promoter and high fusion transcript levels actually create underexpression.

The human *APM-1* gene as present in the fusion transcripts encodes a protein with an amino-terminal BTB/POZ domain and four C_2H_2 zinc fingers of the Krueppel type. These two structural features define the *APM-1* gene as a novel member of the subfamily of genes encoding Krueppel-like zinc finger proteins with a BTB/POZ domain. The BTB (for *Broad Complex, tramtrack* and *bric à brac*) or POZ (for poxvirus and zinc finger) domain has originally been defined in products of *Drosophila* genes involved in developmental regulation and in poxvirus proteins (Bardwell and Treisman, 1994; Zollman *et al.*, 1994; Albagli *et al.*, 1995). It has been characterized as a domain that mediates homomeric or heteromeric protein–protein interactions (Bardwell and Treisman, 1994; Chen *et al.*, 1995), that targets the protein to distinct nuclear substructures (Dhordain *et al.*, 1995), and that is involved in transcriptional regulation or chromatin modelling (Albagli *et al.*, 1995; Chang *et al.*, 1996; Seyfert *et al.*, 1996).

Three human members of the BTB/POZ subfamily of zinc finger-encoding genes are known to be involved in carcinogenesis. Expression of the *bcl-6* proto-oncogene is deregulated by chromosomal translocations in diffuse large-cell lymphoma (Ye *et al.*, 1995). The *PLZF* gene is fused to the retinoic acid receptor α-encoding gene in a small subset of acute promyleocytic leukaemias (Dong *et al.*, 1996). The *HIC-1* gene has been identified recently by its underexpression in tumour cells due to hypermethylation and is viewed as a candidate tumour suppressor gene (Makos Wales *et al.*, 1995).

The homozygosity of the mutant allele which carries the integrated HPV68 DNA in ME180 cells, as revealed by genomic Southern analysis (Figure 1), was initially taken as a hint for the existence of a tumour suppressor gene within the integration region. The ability of the *APM-1* gene to reduce the clonal growth of HeLa and CaSki cervical carcinoma cells *in vitro* (Figure 7) argues in favour of the assumption that the *APM-1* gene might be the postulated tumour suppressor gene. Furthermore, fluorescence *in situ* hybridization on metaphase chromosomes revealed that ME180 cells contain two copies of the mutant chromosome 18 harbouring the integrated HPV68 DNA and completely lack the normal chromosome 18. This situation probably resulted after the HPV integration event from the loss of the remaining normal copy and duplication of the mutant copy. Due to these genetic changes, the *APM-1* gene is present in ME180 cells only in the mutated form.

An ~5 kb transcript could be identified as the authentic cellular *APM-1* mRNA (Figure 5). The Northern blot analyses indicate that the *APM-1* gene is expressed in normal cervical epithelial cells, but that it is not expressed or underexpressed in the majority of the cervical carcinoma cell lines analysed. Southern blot analysis gave no indication for a loss of the *APM-1* gene in any of the cell lines with undetectable 5 kb mRNA (see Figure 1). Altogether, these data suggest that loss or reduction of *APM-1* gene expression might occur during cervical carcinogenesis and further support the assumption of a tumour suppressor role of the *APM-1* gene. It will be of interest to determine whether alterations in *APM-1* gene expression can also be detected in other types of human carcinomas.

Besides the 5 kb *APM-1* mRNA, two other transcripts of ~3 kb and 4 kb are produced from the INT68 genomic region (Figures 2 and 5). In contrast to the restricted presence of the *APM-1* mRNA, these two transcripts—and in particular the 4 kb species—are almost ubiquitously detected. The two transcripts hybridize only with the 3′-part of the first *APM-1* coding exon. Their exact structures and coding informations have yet to be determined, and the question whether they have the same or the opposite

orientation with regard to the *APM-1* mRNA remains to be answered.

We have mapped the *APM-1* gene to chromosome 18q21 (Figure 6). This genomic region seems to be involved in human carcinogenesis in multiple ways. For example, it harbours the tumour suppressor genes *DCC* and *DPC4* which are deleted in colorectal and pancreatic cancers, respectively (Cho and Fearon, 1995; Hahn *et al.*, 1996). Allele losses at 18q21 have also been reported for an HPV16-positive oral carcinoma and its derived cell line (Steenbergen *et al.*, 1995) and for an HPV18-immortalized keratinocyte line (Klingelhutz *et al.*, 1993). It is evident from genetic studies that genes other than the already identified candidate tumour suppressor genes are involved in allele losses at 18q21 (Hahn *et al.*, 1996). Here the *APM-1* gene provides a novel candidate and marker gene.

In this study we provide direct evidence that the integration of HPV DNA can alter the integrity and expression of cellular genes located in the chromosomal target region. Furthermore, we show that the integrated HPV DNA can serve as a marker for the identification of novel human genes that are themselves possibly involved in carcinogenesis. Future studies will clarify the possible functions of the APM-1 protein in the regulation of transcription or chromatin folding, and the importance of *APM-1* gene alterations in human cancer.

Materials and methods

Cell lines, primary cells and cell culture conditions

The human cervical carcinoma cell lines CaSki, C4-I, C4-II, C33A, HeLa, HT-3, ME180, MS751 and SiHa were obtained from the American Type Culture Collection, the non-tumorigenic HeLa×normal human fibroblast hybrid cell line 444 from E.Stanbridge (Irvine, USA), the immortalized keratinocyte line HaCaT from P.Boukamp (DKFZ, Heidelberg), the lymphoblastoid cell line IARC277 from M.Pawlita (DKFZ), and the cervical carcinoma cell lines MRI-H186, MRI-H196 and MRI-H215 as well as the lung carcinoma cell line LX-1 from H.Löhrke (Tumorbank, DKFZ). The primary ectocervical keratinocytes were derived from a cervical biopsy obtained from hysterectomy. The established cells and the primary cells were cultivated as described previously (Geisen *et al.*, 1997).

Recombinant plasmids

Plasmid pCMV-Gal contains the *Escherichia coli* β-galactosidase gene driven by the CMV promoter. The eukaryotic expression vector pSG5 containing the SV40 early promoter was obtained from Stratagene (Heidelberg, Germany). Plasmid pSV2-neo harbours the *neo* gene conferring resistance to the antibiotic G418. The expression plasmid pSG5–Mefust was constructed by inserting the complete fusion transcript cDNA of clone Mefust-4 into the *Eco*RI-digested pSG5 vector. The *APM-1* gene, together with the 3′-untranslated region, was prepared from Mefust-4 cDNA by cleavage with *Mun*I and *Eco*RI. The fragment was inserted into pSG5 in sense and antisense orientation to yield the expression plasmid pSG5–APM-1 and the antisense construct pSG5–APM-1-as, respectively.

DNA and RNA analysis

Total high-molecular weight DNA was isolated from cells by proteinase K digestion and phenol extraction. DNAs digested with restriction enzymes were separated electrophoretically on 0.8% agarose gels, and transferred to Hybond N^+ membranes (Amersham Buchler, Braunschweig, Germany). Total RNA was isolated from cells by the acidic guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Poly(A)$^+$-RNA was selected using dynabeads oligo(dT)$_{25}$ (Dynal, Hamburg, Germany). RNAs were denatured, fractionated in 1% agarose gels and transferred to Hybond N^+ membranes using 10× SSC-buffer. The DNA probes were labelled with ^{32}P by random priming (Stratagene, Heidelberg, Germany). Filter hybridization and washing were done essentially as described (Geisen *et al.*, 1997). In Southern and Northern hybridizations with genomic probes, preincubations of the probes with human Cot-1 DNA (Gibco-BRL, Eggenstein, Germany) were performed to block hybridization of the repetitive sequences.

Isolation of cosmid clones

To isolate the human genome region corresponding to the integration region of HPV68 DNA in the cell line ME180, we used a cosmid library of normal human leukocyte DNA (Poustka *et al.*, 1984) distributed on seven plates containing 2–3×10^5 clones each. In a first screening, a Southern filter with DNA of the seven fractions was hybridized with the flanking cellular sequences of the integrated HPV68 DNA as labelled probe. The filters of the positive fractions were then hybridized, and positive clones were isolated and purified.

cDNA cloning

A fusion transcript cDNA product of 1338 bp was amplified by reverse transcription of ME180 poly(A)$^+$-RNA and hot start PCR using a primer in sense orientation from the HPV68 E1 region (5′-CAATGGCCAA-TTGTGAAG-3′) in combination with an antisense primer from the cellular zinc finger-encoding region of the genomic 5.5 kb *Sac*I fragment (5′-TCGTAGTTGTGCACGAACTTGG-3′). A ME180 cDNA library in phage λNM1149 was kindly prepared by H.Sommer (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany). Positive clones were identified by plaque hybridization with the 1338 bp RT–PCR product as radiolabelled probe.

Cosmid-specific selection of cDNA clones

Cosmid INT68 was used to enrich cDNA clones derived from this region of the human genome. The selection was performed as described (Korn *et al.*, 1992, 1997). In brief, the biotinylated cosmid DNA was hybridized to cDNA inserts which had been amplified by PCR from three cDNA libraries derived from fetal human brain, fetal human liver and adult skeletal muscle, respectively. Filters carrying the enriched cDNA collection were hybridized with the 1338 bp RT–PCR product as probe. The presence of identification markers in the terminal adapter sequences of the enriched cDNAs allowed identification of the origin of each cDNA insert.

Determination and computer analysis of genomic and cDNA sequences

DNA sequencing was performed by the dideoxy chain termination method using a primer walking approach. Dideoxynucleotide incorporation into the sequencing products was improved by the use of a manganese-containing buffer (Tabor and Richardson, 1989). Computer analysis of the nucleotide sequences and derived amino acid sequences were done using self-written software and the program package of the Heidelberg Unix Sequence Analysis Resources (HUSAR) program library.

The nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession numbers Y14591 (cDNA sequence), Y14592 (4.5 kb *Sac*I fragment of cosmid INT68) and Y14593 (partial sequence within the 5.5 kb *Sac*I fragment of cosmid INT68).

Fluorescent in situ hybridization of chromosomes

Chromosomes were prepared from peripheral blood of a normal individual or from the cell line ME180 following standard protocols. Cosmid INT68 was labelled with biotin, whereas a 4 kb *Spe*I fragment of HPV68 DNA and the chromosome 18-specific alphoid DNA probe L1.84 (Devilee *et al.*, 1986) were labelled with digoxigenin by nick translation before hybridizing to metaphase chromosomes as described (Lichter *et al.*, 1990). Briefly, labelled probe (60 ng) was combined with human Cot-1 DNA (3 μg) and salmon sperm DNA (7 μg) and was hybridized at 37°C overnight. Following post-hybridization washes, the biotin-labelled probe was detected via avidin conjugated to FITC and the digoxigenin-labelled probes via anti-digoxigenin antibodies conjugated to rhodamine. Chromosomes were banded with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Digitized images of FITC, rhodamine or DAPI fluorescence were recorded separately with a CCD camera (Photometrics, Tucson, Arizona, USA), electronically overlayed, carefully aligned and pseudocoloured. Photographs were taken from the monitor.

Colony formation assay

Cells were seeded into 10 cm dishes at a density of 2.5×10^5 cells per dish 24–48 h before transfection. The transfection was performed with a mixture of 19 μg effector or control DNA and 1 μg pSV2-neo DNA per dish. Medium containing G418 at a concentration of 800 μg/ml (CaSki cells) or 1000 μg/ml (HeLa cells) was added 1 day after

transfection to select for G418-resistant cells. G418-containing medium was changed every third day. After 2 weeks of selection, the cells were stained and colonies (>8 cells) were counted to determine clonal cell growth. For each plasmid DNA, transfections were done in triplicate.

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