Sense and antisense transcripts of human papillomavirus type 16 in cervical cancers

Vera Vormwald-Dogan, Bertram Fischer, Hubertus Bludau, Ulrich Karl Freese, Lutz Gissmann, Dagmar Glitz, Elisabeth Schwarz and Matthias Dürst

Human papillomavirus type 16 (HPV-16) transcription was analysed in one squamous cervical carcinoma by cDNA cloning and DNA sequencing, and in eight additional squamous cervical carcinomas and 11 precancerous lesions by RNA–RNA in situ hybridization. The nucleotide sequences of the cDNA clones revealed structures of early HPV-16 mRNAs (E6*-E7-E1 E4-E5) in agreement with data reported for other premalignant and malignant tumours. cDNA clones possibly representing viral RNA of antisense orientation were also detected. These RNAs included sequences of the upstream regulatory region, part of the early and the late region of the genome. In three of eight squamous cervical carcinomas examined by in situ hybridization, signals specific for viral antisense RNA were also found. The antisense RNAs had a predominantly nuclear localization. Viral antisense RNA could not be detected in any of 11 HPV-16-positive premalignant lesions. The expression of HPV antisense RNA is likely to be linked to viral integration into the host genome. The possible effects of viral antisense transcription with regard to tumour progression remain to be determined.

Human papillomavirus type 16 (HPV-16) together with a subset of other genital types are thought to play a key role in the genesis of cervical cancer. Evidence in support of this is largely of an experimental nature (for review see zur Hausen, 1989). The DNA of HPV types associated with anogenital carcinomas, particularly that of HPV-16 and HPV-18, but also that of some other cancer-linked HPV types, is able to immortalize normal human foreskin and cervical keratinocytes, and to extend the lifespan of fibroblasts (Dürst et al., 1987; Pecoraro et al., 1989; Pirisi et al., 1987; Schlegel et al., 1988; Woodworth et al., 1989). The viral genes E6 and E7 have been specifically identified as viral oncogenes responsible for an altered proliferation of the target cell (Barbosa et al., 1991; Hawley-Nelson et al., 1989; Münger et al., 1989a; Watanabe et al., 1989). Recent experiments suggest that an interaction of these viral proteins with cellular regulatory proteins could at least in part be responsible for these phenotypic alterations (Barbosa et al., 1990; Dyson et al., 1989; Gage et al., 1990; Münger et al., 1989b; Werness et al., 1990). Maps of HPV-16-specific transcripts in cell lines derived from premalignant and malignant lesions have been produced but differences which might correlate with progression of cervical neoplasia were not apparent (Baker et al., 1987; Doorbar et al., 1990; Schneider-Maunoury et al., 1990; Schwarz et al., 1985). Irrespective of the malignant potential of the cell line involved, the majority of transcripts encoding E6 and E7 were initiated from the viral promoter, P97, just downstream of the upstream regulatory region (URR). Three types of E6-E7 mRNAs were identified. Of these, two mRNA species (E6*I and E6*II) are spliced within the E6 open reading frame (ORF) and are presumed to encode the E7 protein which is abundantly expressed in HPV-16-positive cell lines (Smotkin & Wettstein, 1986; Smotkin et al., 1989). As HPV transcription is strongly dependent on epithelial cell differentiation (Bedell et al., 1991; Crum et al., 1988, 1990; Dürst et al., 1991; Stoler et al., 1990) some investigators have also analysed the viral transcriptional pattern in precursor lesions and cervical carcinomas (Cornelissen et al., 1990; Dilts et al., 1990; Johnson et al., 1990; Sherman et al., 1992; Shirasawa et al., 1988). However most of these studies have concentrated on the early region of the viral genome and were performed by reverse transcription and polymerase chain reaction.

We have studied a cervical carcinoma which harboured both episomal and integrated HPV-16 DNA viral transcript structures by cDNA cloning and sequence analysis. For this purpose a cDNA library was constructed in λ NM1149 using poly(A)+ RNA according to standard procedures (Sambrook et al., 1989). The library
was screened for HPV-16-positive clones by plaque hybridization using the entire cloned genome of HPV-16 as a hybridization probe. Fourteen clones were isolated and were mapped by restriction enzyme digestion and hybridization using subgenomic HPV-16 DNA fragments. Each cDNA clone was sequenced in both directions, using the dideoxynucleotide chain termination method of Sanger et al. (1977).

In comparison to the HPV-16 prototype DNA sequence (Seedorf et al., 1985) numerous sequence alterations were found among the cDNA clones which are summarized in Table 1. Some sequence alterations were confined to individual cDNA clones and were not found in another clone spanning the same genomic region. This may be explained on the basis of two different HPV-16 populations within the same cancer or may be attributed to cloning artefacts. No DNA sequence mutations were found for the E6 and E7 ORF which is suggestive of selective pressure on the integrity of these genes. The locations of 5' and 3' ends and the splicing patterns of the cDNAs are shown in Fig. 1. For unknown reasons only one of the cDNA inserts contained an oligo(dA) sequence at its 3' end, although oligo(dT) had been used to prime reverse transcription. In contrast, all but three cDNAs had oligo(dC) at their 5' terminus. Since first-strand synthesis products had been tailed with dG, the oligo(dG) tail was used to determine the polarity of mRNAs from which the cDNAs were derived. cDNA clones 1, 3a, 9, 16 and 21 represent early viral mRNAs of sense orientation. Note the position of oligo(dC) tails at the 5' ends of cDNA clones in Fig. 1(a) containing a spliced E6*I ORF and an unspliced E6
and non-viral sequences and thus very likely represent virus–cell fusion transcripts. As deduced from the position of the oligo(dC) tails they are probably derived from antisense RNA (compare the positions of oligo(dC) tails of cDNA clones in (a) and (b) of Fig.1). This RNA species appears to be initiated in the E1 ORF at position 2364 and presumably terminates within the flanking cellular region. As there are no apparent consensus DNA sequences for splicing it is likely that nt 1482 actually reflects the break point within the viral genome at which integration into the host chromosome had taken place.

Of the seven cDNA clones which map to the late region, four are clearly of antisense orientation (clones 4, 5, 6 and 18 in Fig. 1b). For the three remaining clones the orientation could not be determined with certainty (Fig. 1c). The 5' ends of the putative antisense clones are heterogeneous. If they were to represent the authentic sites of transcriptional initiation, two of them would be located in the URR (clones 5 and 18) and the other two in the 5' region of the L1 ORF (clones 4 and 6). However we cannot rule out the possibility that the 5' ends are artificial due to premature termination of first-strand cDNA synthesis which may occur as a result of secondary structures within the RNA template. Alternatively, activation of cryptic viral promoters due to conformational changes and/or cis-acting cellular elements as a consequence of viral integration are also plausible. The 3' ends of cDNA clones 4 and 6 are nearly identical (positions 4228 and 4224) and map to the 5' region of the L2 ORF. This particular region of the HPV-16 genome contains poly(A)-rich stretches which probably permitted initiation of reverse transcription by oligo(dT), thus giving rise to cDNA clones with truncated 3' ends. This explanation may also apply to clone 7. Alternatively, although unlikely, the two virus–cell hybrid cDNAs and the cDNAs with L1/L2 sequences could be derived from single- or double-stranded genomic DNA fragments which might have contaminated the poly(A)+ RNA.

To examine whether synthesis of viral antisense RNA might be a common phenomenon in HPV-16-induced lesions, 11 premalignant lesions of the cervix (low grade and high grade cervical intraepithelial neoplasia) and eight additional squamous cervical cancers were analysed by RNA–RNA in situ hybridization for the presence of sense and antisense RNA using strand-specific probes which correspond to parts of the URR, the E6–E7 and the L1 region of the HPV-16 genome (for a detailed protocol, see Dürst et al., 1991). Tissue sections were examined and photographed under a Zeiss Axio phot microscope equipped with a rotatable lightfield and darkfield condenser to reveal tissue histology and to enable good signal visualization respectively. The following observations were made. (i) E6–E7 mRNA could be

<table>
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<th>ORF</th>
<th>Nucleotide position*</th>
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<tr>
<td>E1</td>
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<td>del GCA</td>
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<td></td>
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</tr>
<tr>
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<td>4155</td>
<td>ins T</td>
<td>3a</td>
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<tr>
<td></td>
<td>4162</td>
<td>C→T</td>
<td>16</td>
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<tr>
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<td>4163</td>
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<tr>
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<td>7861‡</td>
<td>del A</td>
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* Nucleotide positions and the nature of each mutation (del, deletion; →, change; ins, insertion) based on the HPV-16 prototype sequence (Seedorf et al., 1985) are listed.
† Mutations detected in more than one cDNA clone which probably reflect natural sequence divergence among different HPV-16 isolates.
‡ Mutations reflecting errors in the published HPV-176 DNA sequence. An additional thymidine at nt 3903 gave rise to a full-length E5 ORF as described by Halbert & Galloway (1988) and Bulb et al. (1988). A deletion of adenine at nt 7861 (Romanczuk et al., 1990) generated another E2-binding site ACC(N6)GGT (Spalholz et al., 1987) in the URR whereas an insertion of CAT at nt 6900 and a deletion of CAT at nt 6951 led to a slight modification within the L1 ORF (Parton, 1990).
detected in each biopsy (the term mRNA is used for HPV transcripts of sense orientation only) (see also Fig. 2d). No mRNAs encoding structural (late) proteins were evident in high grade lesions and in the majority of cancers (see also Fig. 2e). (ii) Antisense RNA could be detected in each of three cancer biopsies with hybridization probes spanning, in part, the URR, and the early and the late regions of the viral genome. Hybridization results of serial sections of one of the tumours are shown in Fig. 2 (f, g and h). (iii) Viral antisense RNA signals were predominantly located in the nuclei of cells whereas in serial sections of the same tumour viral mRNA was detected in both cytoplasm and nucleus (Fig. 2 and data not shown). (iv) Cells expressing viral antisense RNA invariably expressed viral mRNA. However in one of the biopsies there were also clusters of cancer cells which express mRNA only, indicative of clonal variability within a tumour (data not shown).

A possible false interpretation of the in situ hybridization data due to partially denatured HPV DNA could be excluded since partially denatured DNA would give rise to hybridization signals with probes representing different parts of the genome both in sense and antisense orientation. This was not the case as demonstrated in Fig. 2. Moreover in serial sections of the same tumours RNase A digestion prior to hybridization prevented a positive hybridization signal. In the same experiment the integrity of the probe (residual RNase A could degrade the probe) was also monitored by treating serial sections with RNase A followed by heat denaturation prior to hybridization which permits the detection of viral DNA only (data not shown). The detection of HPV-16 antisense RNAs is in agreement with a recent report by Higgins et al. (1991). We have not detected viral antisense RNA in HPV-16-positive premalignant lesions which should predominantly harbour extrachromosomal viral DNA (Cullen et al., 1991; Dürst et al., 1985).

Expression of viral antisense RNA therefore does not seem to be an intrinsic property of the episomal viral genome. Integration of viral DNA occurs frequently in cervical cancer (Dürst et al., 1985) and is known to result in the production of virus–cell fusion mRNAs encoding viral genes and 3′ cellular sequences (Schneider-Gädicke & Schwarz, 1986). Likewise, integration of viral DNA into a transcriptionally active region of the host chromosome could account for fusion transcripts which are initiated from a cellular promoter and which extend across the whole or part of the integrated viral genome thereby giving rise to viral sense and/or antisense transcripts. Indeed, such fusion transcripts were identified in a keratinocyte cell line immortalized by HPV-16 in vitro (Rohlf et al., 1991). In this respect viral antisense RNA-containing tumours may turn out to be of value for the identification of cellular genes which have become inactivated or activated due to viral integration.

It is not yet clear whether the synthesis of HPV antisense RNA in cervical cancers interferes with gene expression in analogy to other systems (for review see Hélène & Toulmé, 1990; Takayama & Inouye, 1990). Under experimental conditions, inducible synthesis of RNA complementary to the HPV-18 E6–E7 ORFs in the HPV-18-positive cervical carcinoma cell line C4-I has been shown to correlate with a decrease in viral E7 protein and growth inhibition in vitro (von Knebel-Doeberitz et al., 1988). The same correlation, i.e. growth retardation concomitant with the expression of viral antisense RNA, was found when these cell lines were...
tested for tumorigenicity in nude mice (von Knebel-Doeberitz et al., 1992). This creates an apparent dilemma in trying to understand why antisense transcription, particularly of the E6 and E7 genes, is possible at all in cervical carcinomas. One likely explanation may be that in contrast to the naturally occurring antisense RNA in the cancers, the artificially expressed antisense RNA was correctly processed and transported to the cytoplasm. This may be a prerequisite for effective inhibition which may predominantly take place at the level of translation, rather than inhibition of RNA splicing and/or interference with mRNA transport to the cytoplasm. Clearly, despite high levels of viral antisense RNA in the nuclei of some cancers, viral mRNA is still transported to the cytoplasm, thereby ensuring the level of E6 and E7 expression necessary to maintain the transformed phenotype. Nevertheless, the possibility that viral antisense RNA might influence some biological properties of the carcinomas, such as tumour progression, requires further examination.

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References


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