Uniformity of the splicing pattern of the E6/E7 transcripts in human papillomavirus type 16-transformed human fibroblasts, human cervical premalignant lesions and carcinomas

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We utilized the RNA polymerase chain reaction (PCR) to analyse the transcripts of the E6/E7 open reading frames of human papillomavirus type 16 (HPV-16). Total RNA was isolated from 14 cervical squamous carcinomas, nine cervical intraepithelial neoplasias and from human fibroblasts transformed with different HPV-16 constructs. In all specimens two spliced transcripts were detected. Sequence analysis of the cloned PCR products showed that both transcripts were generated by splicing out an intron in E6, from nucleotides (nt) 226 to 409 in one transcript and from nt 226 to 526 in the other. The major transcript present in all RNA specimens had the smallest intron in E6. The RNA PCR described here is the method of choice for analysing splice and donor sites in tissue specimens where a limited amount of RNA is available. Results obtained with transformed cells revealed no difference in splicing whether HPV-16 was controlled by its homologous promoter or by a heterologous promoter, the Rous sarcoma virus long terminal repeat.

The human papillomavirus (HPV) types 16 and 18 probably play a role in the development of cervical carcinomas (zur Hausen, 1987; Boshart et al., 1984). The DNAs of these viruses have been detected in invasive cervical carcinomas (Dürst et al., 1983, 1985), premalignant lesions (Crum et al., 1988) and in cell lines derived from cervical carcinomas, such as CaSki (Yee et al., 1985; Baker et al., 1987) and HeLa (Schwarz et al., 1985; Schneider-Gädicke & Schwarz, 1986). Viral transcripts have been detected in carcinomas (Lehn et al., 1985; Shirasawa et al., 1988), premalignant lesions (Crum et al., 1988; Shirasawa et al., 1988) and HPV-positive cell lines (Schwarz et al., 1985; Smotkin & Wettstein, 1986). Almost all transcripts contain E6/E7 sequences (Smotkin & Wettstein, 1986; Shirasawa et al., 1988) and evidence has been presented that the E6 and E7 open reading frames (ORFs) are involved in the transforming activity of the virus (Tanaka et al., 1989; Storey et al., 1988). In in vitro HPV-transformed cells also, most transcripts contain E6 and/or E7 sequences. The E6 and E7 transcripts have been extensively studied in RNA isolated from CaSki cells and it has been shown that transcription of the E6 and E7 ORFs gives rise to at least three transcripts. The major transcript has an intron within E6 and probably represents the mRNA encoding the E7 protein. The splice donor and acceptor sites of the mRNAs in CaSki cells were mapped by S1 and ExoVII nuclease digestion (Smotkin et al., 1989). E7 protein is present in CaSki cells, as shown by immunoprecipitation and Western blotting (Smotkin & Wettstein, 1986, 1987; Seedorf et al., 1987).

The study of HPV transcripts in patients' specimens has been hampered by the difficulty of recovering sufficient RNA from the often very small lesions. The polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis & Faloona, 1987), now commonly used as a very sensitive method for the detection of small quantities of DNA, can also be used for the detection of specific transcripts after conversion of the RNA to DNA by reverse transcriptase (RT) (Yee et al., 1985). Cloning and sequencing of the RNA PCR amplification products then allows the precise analysis of transcripts in very small tissue samples.

We applied the RNA PCR to study the splice pattern of the E6/E7 transcripts in premalignant and malignant cervical lesions and in in vitro HPV-16-transformed human fibroblasts. RNA isolated from CaSki cells was used to allow comparison with the published splice pattern. Total RNA was isolated from the cultured cells by centrifugation of a guanidinium thiocyanate (GTC) lysate through a CsCl cushion (Chirgwin et al., 1979). Approximately 1 to 5 µg of ethanol-precipitated RNA was directly used in the RNA PCR. Tumour and premalignant tissues were stored at −80 °C until use.
E6/E7 primers

\[ \begin{array}{c|c|c|c}
\text{E7} & \text{E1} \\
544 & 855 & 859 \\
\hline
\text{E6} & 65 & 556 & 1000
\end{array} \]

Primer 1: \text{5' NNNAAGCTTCTGCAATGTTTCAGGACCC 3'}

Primer 2: \text{5' NNNGGATCCCCATTGGTACCTGCAGGATC 3'}

Primer 3: \text{5' NNNAAGCTTCGCGACGTGAG/GTGTATTAAC 3'}

Primer 4: \text{5' NNNAAGCTTCGCGACGTGAG/ATCATCAAGA 3'}

The underlined sequence is the linker sequence used to facilitate the cloning of the specific amplification products. The 5' primers (primers 1, 3 and 4) were made with a HindIII site and the 3' primer (primer 2) contained a BamHI site. N could be any nucleotide.

For the preparation of RNA from patients' material, 30 frozen 10 μm sections, cut with a microtome, were dissolved directly in GTC.

Primer 2 was selected (see Fig. 1) to initiate the RT reaction. One of the other primers (1, 3 and 4) was used together with primer 2 in the PCR. The RT reaction was performed in a 20 μl reaction mix containing 75 mM-KCl, 50 mM-Tris-HCl pH 8.3, 3 mM-MgCl₂, 10 mM-DTT, 1 mM-dNTPs, 150 ng primer 2, 20 units RNase inhibitor and denatured total RNA. The reaction was started with 5 units avian myeloblastosis virus RT and incubated for 30 min at 42 °C, then the RT was inactivated at 95 °C for 5 min. After addition of 80 μl of PCR reaction mixture containing buffer (50 mM-KCl, 10 mM-Tris-HCl pH 8.9, 3.6 mM-MgCl₂ and 100 μg bovine serum albumin/ml), 150 ng primer 1 (or 3 or 4) and 2 units Taq polymerase (Cetus Perkin Elmer) the mixture was subjected to 35 PCR amplification cycles. One-tenth of the reaction mixture was examined on a 2% agarose gel. The specificity was confirmed by hybridization of the blotted agarose gel with an end-labelled HPV-16-specific probe.

The RNA PCR applied to RNA isolated from CaSki cells resulted in three bands when primer 2, located just upstream of the E7 termination codon, and primer 1, located 2 nucleotides (nt) upstream of the cap site (P97), were used. The size of the 608 bp and 491 bp bands corresponded with the expected size for the E6* and E6** transcripts, respectively, as described (Smotkin & Wettstein, 1986; Smotkin et al., 1989). Although the size of the upper band corresponds roughly with the full-length transcript described by Smotkin et al. (1989), it is possible that this PCR product is due to DNA present in the RNA specimens, as suggested by the presence of a strong 791 bp band in the RT-minus reaction.

Identical RNA-specific amplification products were detected in nine HPV-16 DNA-positive invasive carcinomas, four of which are shown in Fig. 2, together with...
the amplification products obtained from CaSki cell RNA. In addition five other squamous carcinomas containing HPV-18 or HPV-33 or an unknown HPV type were analysed. The HPV-16 RNA-specific amplification products were absent when these HPV-16-negative tumours were analysed, showing the specificity of the reaction (Fig. 2a, lanes 12 and 13).

Identical HPV-16 RNA-specific products were obtained in four out of nine randomly selected cervical intraepithelial neoplasia (CIN) lesions in which the presence of HPV-16 DNA had not previously been confirmed. Four of them are shown (Fig. 2b). The absence of transcripts in some CIN lesions was due to the absence of HPV-16 DNA from these lesions, as determined by a DNA PCR (not shown). The possibility that the isolated RNA was of low quality could be excluded by a control RNA PCR on the ribosomal protein S14 messenger (Rhoads et al., 1986) (data not shown).

As well as patients' RNA specimens, human fibroblasts transformed by different HPV-16 constructs containing either the homologous or a heterologous promoter (Rous sarcoma virus long control region) (Smits et al., 1988, 1989) were analysed with the RNA PCR. Again the same two spliced transcripts were detected in each of the transformed cells. No difference in splicing pattern was found if HPV-16 RNA was transcribed either from the homologous HPV-16 promoter or from the heterologous promoter.

The PCR products obtained from the two spliced E6/E7 transcripts were isolated from the agarose gel, cloned and sequenced (Sanger, 1977). From the determined sequence the splice acceptor and donor sites could be located exactly. The E6* amplification product of 608 bp was obtained from an RNA spliced between nt 226 and 409, the E6** 491 bp amplification product from an RNA spliced between nt 226 and 526. The RNA PCR is clearly the best method of determining the location of splice donor and acceptor sites of transcripts. It is less time-consuming and requires less material than constructing a cDNA library and, in contrast to S1 mapping of the splice donor and acceptor sites, the RNA PCR allows the determination of their exact position.

Primers were selected specifically to amplify each of the two splice products. These primers (Fig. 1) consisted of 10 nt identical to the splice donor site at 226 nt and 10 nt identical to the acceptor site at 409 nt (primer 3) or at 526 nt (primer 4), resulting in one unique RNA PCR product after amplification. All specimens positive in the RNA PCR using primer 1 and primer 2 were also positive using primers unique for the two splice sites. All specimens negative in the RNA PCR described above were also negative in this analysis.

Our observation that the E6 splicing pattern is uniform in HPV-16-transformed cells as well as in premalignant and malignant cervical lesions shows that alterations in the E6 splicing may not play a role in the development of a malignant cell from a premalignant (transformed) cell. Other factors might be involved in this transition, e.g. a mutation in a cellular tumour suppressor gene (zur Hausen, 1987), or activation of an oncogene (Ocadiz et al., 1987). Also integration of the HPV DNA into the host genome might be important, leading to an alteration in the structure of the early transcripts by readthrough into the host genome (Shirasawa et al., 1988). We are currently analysing the 5' and 3' ends of the early transcripts in HPV-16-transformed human cells and in premalignant and malignant cervical lesions to test this assumption.

It is of interest to note that so far we have not observed HPV-16 DNA-positive carcinomas that are transcriptionally inactive, although some early reports (Lehn et al., 1985; Schwarz et al., 1985) stated that HPV-16 DNA is not always expressed in cervical carcinomas. This point clearly requires a more extensive study.

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