Analysis of human papillomavirus type 16 E6–E7 transcription in cervical carcinomas and normal cervical epithelium using the polymerase chain reaction

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Cervical biopsies were collected from Birmingham women having cervical intraepithelial neoplasia or invasive cervical carcinoma and normal controls, and examined for the presence of human papillomavirus type 16 (HPV-16) E6–E7 DNA and mRNA using an adaptation of the polymerase chain reaction. HPV-16 E6–E7 sequences were detected in all abnormal biopsies and in 90% of the normal biopsies examined, confirming previous studies describing the high prevalence of cervical HPV-16 infection. While we were unable to identify any qualitative differences in RNA transcripts from the p97 promoter, substantial quantitative differences in HPV-16-specific early region transcripts between normal and cytologically abnormal cervixes were observed. These results suggest that although the level of E6–E7 transcription may contribute to the malignant phenotype, additional factors are likely to be important in the development of cervical neoplasia.

Introduction

An aetiological role for human papillomavirus (HPV) infection in the development of cervical neoplasia is widely postulated, although not universally accepted (Reid et al., 1982; Walker et al., 1983; Howley, 1986; Munoz et al., 1988). However, the recent development of the polymerase chain reaction (PCR) (Saiki et al., 1988) and its application to the detection of the so-called oncogenic HPV types associated with cervical cancer (predominantly types 16 and 18) in DNA extracted from cervical smear samples have revealed that the prevalence of HPV-16 in both cytologically abnormal and normal cervical smears is far higher than previously reported (Young et al., 1989; Bevan et al., 1989; Tidy et al., 1989). However, this apparent inconsistency might be explained either by quantitative differences in the level of HPV-16 infection in normal compared to abnormal cervical tissue, or by alterations in the transcriptional activity of the viral genome during the development of neoplasia.

Several analyses of the transcription of HPV-16 in cervical cancer-derived cell lines and in fresh cervical carcinoma biopsies (Pater & Pater, 1986; Baker et al., 1987; Tsunokawa et al., 1986; Smotkin & Wettstein, 1986), and also pre-invasive cervical lesions (Shirasawa et al., 1988) have been carried out. Together they show that transcription predominantly of the E6–E7 region of the HPV-16 genome is selectively retained in the neoplastic tissue. E6 and E7 have been identified as the transforming genes of HPV-16 by a number of studies and it appears that both proteins are necessary for full transformation of primary cells (Seedorf et al., 1987; Tanaka et al., 1989; Watanabe et al., 1989; Munger et al., 1989). The detailed analysis of HPV-16 E6–E7 region transcription in both cervical cancer-derived cell lines (CaSki, SiHa) and fresh carcinoma biopsies (Smotkin & Wettstein, 1986; Smotkin et al., 1989) has revealed the existence of three alternative transcripts originating from the p97 promoter. First, the full-length collinear transcript is capable of encoding the full E6 and E7 protein products. Second, two mRNA species are generated by alternative splicing within the E6 exon. Both are identical to the collinear transcript until the splice donor site at nucleotide position 226, the first transcript utilizes a splice acceptor site at nucleotide 408, and the second an acceptor at nucleotide 525. Both of these mRNA species are capable of encoding a truncated E6 protein, known as E6*, the function of which is as yet unknown, and are known as E6*I and E6*II (Smotkin et al., 1989). Both transcripts contain the full coding sequence for the E7 protein, although since the splicing in E6*II positions the termination codon of E6 close (36 nucleotides) to the initiation codon for E7 it is unclear whether this mRNA could function efficiently for the translation of E7 (Smotkin et al., 1989).
The structure of both the E6 and E7 of the so-called oncogenic HPV types (16 and 18) has been studied in some detail. It appears that both proteins consist of Cys-X-X-Cys repeats and have been shown to mediate zinc binding (Barbosa et al., 1989), which in eukaryotic cells is involved in the binding of protein to DNA and in protein dimerization. Furthermore, the product of the E7 open reading frame has been shown in common with adenovirus E1A and simian virus 40 large T, to bind to the product of the retinoblastoma (Rb1) gene. Rb1 appears to play a role in limiting cellular proliferation. The loss of Rb1, by mutation of the gene or by its binding to a protein such as HPV-16 E7, is correlated with increased cellular proliferation (Dyson et al., 1989). In the light of these observations it is appropriate to investigate the presence of mRNAs encoding the E6 and E7 gene products in normal and neoplastic cervical epithelium, in an attempt to evaluate the role of these viral proteins in the transformation process.

Methods

Clinical specimens. Cervical biopsies were collected as follows. (i) From women known to have no history of cytological abnormality undergoing hysterectomy. Immediately following removal, half of the cervix was separated from the remainder of the uterus, immediately snap-frozen and stored in liquid nitrogen. (ii) From women referred to a colposcopy clinic with cytological abnormality. Cervical biopsies were collected, snap-frozen and stored in liquid nitrogen. Samples were graded according to the level of cervical intraepithelial neoplasia (CIN) present. (iii) From previously untreated patients with invasive cervical carcinoma at the time of first section insertion. Again, biopsies were immediately snap-frozen and subsequently stored in liquid nitrogen.

Extraction of DNA and RNA. DNA and RNA were extracted from cervical material using a modification of the guanidinium thiocyanate method (Shirasawa et al., 1988). RNA was treated with RNase-free DNase, the absence of DNA being confirmed by PCR with primers A and B (Fig. 1).

Detection of HPV-16 DNA and RNA by PCR. Reverse transcription/polymerase chain reaction (RT/PCR) was carried out in a one-tube reaction consisting of 10 mM-Tris-HCl pH 8.3, 50 mM-KCl, 1.7 mM-MgCl2, 0.005% gelatin (300 Bloom), 200 μM-dATP, dTTP, dCTP, 50 μM-deazaGTP, 150 μM-deazaGTP, 2 μM each primer and 200 ng of DNA or whole RNA. For detection of DNA, samples were subjected to 40 rounds of amplification as described by Young et al. (1989) in a DNA thermal cycler (Perkin-Elmer Cetus). Amplification conditions were: denaturation 30 s at 94 °C, primer annealing 90 s at 45 °C, extension 240 s at 70 °C. For reactions containing RNA, samples were heated for 2 min at 90 °C followed by rapid cooling on ice. RNAguard (Pharmacia) (0.5 units/μl) and 5 units of avian myeloblastosis virus reverse transcriptase (Pharmacia) were added and incubation was continued for 60 min at 42 °C. Samples were then treated in exactly the same way as DNA samples. Negative control reactions were a reaction containing all components except target DNA/RNA, and reactions containing HeLa cell (HPV-18-containing), C15 (Epstein–Barr virus-positive nude mouse-passaged nasopharyngeal carcinoma) or B95.8 (transformed marmoset B cell line) RNA or DNA. Positive controls used CaSki DNA and RNA or purified plasmid DNA as template.

Detection of amplified products. Amplified samples were analysed by electrophoresis through 3% NuSieve agarose gels followed by Southern transfer onto Hybond-N+ nylon membrane. Detection was achieved by hybridization to a 32P-labelled oligonucleotide probe, as shown in Fig. 1. Use of primers R and B with probe P in RNA analysis allowed the detection of the full-length transcript and E6*I; primers R and C for PCR followed by hybridization to primer B (used now as a probe) allowed detection of all three alternative mRNA species (Fig. 1).

Results

Detection of HPV-16 E6–E7 spliced transcripts by PCR

Fig. 2 illustrates the results obtained when RNA extracted from cervical biopsies was subjected to RT/PCR analysis. Using primers R and B (Fig. 2a), amplified products of the predicted size were visible following Southern blotting. A band of 340 bp represents the full-length transcript from p97, and that at 158 bp represents a spliced mRNA using the splice acceptor site at position 408 (E6*I). Fig. 2(b) shows the results obtained when primers R and C are used; three bands are visible, representing the full-length transcript (420 bp), the spliced mRNA using the splice acceptor site at position 408 (E6*I; 238 bp) and the spliced mRNA using the second site at position 525 (E6*II; 124 bp). The examples shown in Fig. 2 are representative of all those examined. Using primers R and B, both transcripts (full-length and E6*I) are detectable in all the samples shown. However, using primers R and C, only the full-length transcript was identified in the normal samples illustrated and spliced transcripts were detected in only three of the six CIN biopsies. All the transcripts appear to be present in the three carcinoma samples illustrated. No amplified products were detected in control PCRs containing only primers or HPV-16-negative cell DNA or RNA. In addition, DNA from the carcinoma biopsies yielded only amplified products generated from the full-length sequence; no shorter fragments were identified (Fig. 2).

Table 1 summarizes the presence of HPV-16 E6–E7
transcripts in the total samples examined. HPV-16 DNA was detected in all biopsies and HPV-16 RNA was detected in 90% of the normal samples and in 100% of the abnormal samples. Using primers R and B, there was no detectable difference between the normal and abnormal cervical biopsies. In all but one normal sample both the full-length and the E6*I transcription products were evident. When results obtained using primers R and C are considered, the rate of detection of all three classes of transcript is decreased. In particular, E6*I is detected less frequently with these primers than R and B (at 33% in CIN1 samples, 56% in CIN2 and CIN3, and 73% in carcinomas). The product of splicing at the second acceptor site, E6*II, is detected infrequently in normal biopsies (10%) and CIN1 (0%), but was detected in 45% of CIN2 and CIN3. However, E6*II and E6*I were detected at equal frequency in the carcinoma biopsies. Since the rate of detection of
the full-length transcript in these samples using primers R and C was also decreased it became apparent that the two sets of primers were not equally efficient in amplifying RNA.

Differential efficiency of the two primer pairs

Fig. 3 shows the results of PCR using decreasing amounts of CaSki RNA with either set of primers. It is apparent that primers R and B are 10 times more sensitive than primers R and C. The spliced RNA transcript (E6*I) is still clearly visible in 5 ng of CaSki RNA with R and B (Fig. 3a, lane 4), whereas 50 ng of the same RNA is required for clear visualization of E6*I and E6*II using primers R and C (Fig. 3b, lane 6). The reason for this difference in efficiency of primers B and C is unclear. Both have the same $T_m$ (58°C) and the difference in efficiency lies in the PCR step of the procedure rather than the reverse transcription, since the relative detection thresholds are the same whether reverse transcription is performed using random primers or the HPV-16-specific oligonucleotides (data not shown).

HPV-16 in cervical carcinoma biopsies is more transcriptionally active than in normal biopsies

The levels of HPV-16 DNA and RNA in several of the normal and carcinoma biopsies were then compared by a similar set of titrations using primers R and B. It was noted that all samples contained approximately equivalent quantities of HPV-16 E6 DNA but that the levels of RNA differed significantly between these two sets of specimens. Titrations of carcinoma and normal RNA samples were carried out using primers R and B and primers specific for human keratin K14 (50000 Mr RNA) (Hanukoglu & Fuchs, 1982). This keratin is a marker for stratified squamous epithelial tissue being expressed in less-differentiated cell layers (Nelson & Sun, 1983). PCR amplification across the third intron of K14 RNA gave the predicted 185 bp product in control epithelial cells (CaSki, HeLa, C15). By comparing both sets of titrations it was possible to arrive at an estimate of the relative abundance of the two sets of transcripts. Fig. 4 shows the results of such an analysis. It is apparent from Fig. 4 that the cervical carcinoma contains in excess of 500 times more HPV-16 RNA than the normal epithelium, and 10 times more of the keratin transcript.
Fig. 4. Levels of HPV-16 E6, E6*I and keratin transcripts in carcinoma biopsy and normal cervical epithelium. RNA from normal and carcinomatous cervix was titrated using the HPV-16 primers R and B (a) and the 50000 Mr epidermal keratin primers (b) in RT/PCR. Amplified products of 340 bp and 158 bp (HPV-16) and 185 bp (keratin) are indicated. Quantities of whole RNA used for carcinoma: lane 1, 1 ng; lane 2, 10 ng; lane 3, 50 ng; lane 4, 100 ng; lane 5, 200 ng; lane 6, 500 ng. For normal cervix the quantities were: lane 7, 1 ng; lane 8, 10 ng; lane 9, 50 ng; lane 10, 100 ng; lane 11, 200 ng; lane 12, 500 ng.

The net effect is that per unit of cellular RNA the carcinoma produces over 50 times more HPV-16 p97 transcripts than the normal epithelium. Similar analysis of other samples has revealed that there is at least a 10-fold excess in HPV-16 p97 transcripts in carcinomas when compared with normal cervix. This last point has been confirmed in a number of cases where it has been possible to increase the input of normal RNA in the reactions by 50-fold (10 μg instead of 200 ng). Under these conditions all three transcripts became visible using primers R and C. Using the analysis described, no qualitative differences in transcription from p97 have yet been identified.

Discussion

We describe here a method for the sensitive detection of low abundance transcripts in RNA extracted from clinical specimens. Hitherto, analysis of viral mRNA production in cervical carcinoma cell lines and biopsies has relied on the technique of Northern blotting (Shirasawa et al., 1988; Schwarz et al., 1985; Tsunokawa et al., 1986) and subsequent hybridization to subgenomic probes. It has not been possible using these methods to characterize any spliced mRNA molecules in cervical biopsies. Smotkin & Wettstein (1986) originally identified the E6*I transcript by S1 and exonuclease VII digestion and primer extension in cell lines, but were unable to confirm their results on a carcinoma biopsy owing to the limited amount of RNA available. Using the present method, once splice donor and acceptor sites are identified in RNA extracted from cultured cell lines their importance in clinical specimens may be easily evaluated. This method can be used in the study of any clinically significant microorganism providing that the sequence of the relevant gene(s) is available.

It appears from the data presented that there is no gross qualitative difference in HPV-16 transcription between normal cervical epithelium and cervical carcinoma specimens. However, it is clear that the transcripts encoding the transforming proteins of HPV-16 are present in much greater amounts in the carcinoma biopsies even after correction for the relative amount of epithelium-specific RNA. Assuming that the level of E6 and E7 mRNA accurately represents the amount of the relative protein in these samples, and that E7 is an important effector protein in cellular transformation, then the net effect of this increased transcription may be increased cellular proliferation (Dyson et al., 1989). However, it is impossible using the method described to evaluate the relative proportion of HPV-16-infected cells in normal compared to cancerous tissue. Due to the clonal nature of cervical carcinoma (Lehn et al., 1985) it is likely that every tumour cell contains similar amounts of HPV-16 DNA together with E6–E7 RNA. However, in normal tissue, HPV-16 infection is probably confined to a smaller proportion of cells with a concomitant reduction in the level of E6–E7 transcription.
The reason for the elevated transcription of the E6–E7 region in the carcinomas examined may be a deregulation of p97 transcription as a result of integration of the HPV-16 DNA into the cellular genome, with a disruption of the E2 open reading frame. Meanwell et al. (1987) examined 31 cervical carcinoma specimens containing HPV-16 and found evidence of integration in seven, but none in the nine normal samples examined. Tidy et al. (1989) report that of 23 cervical carcinomas, 16 contained integrated HPV-16 (seven contained only integrated DNA, nine contained both integrated and episomal DNA) and that in 13 of these the integration event had interrupted the E1–E2 region while leaving the upstream regulatory region E6–E7 region intact.

An additional role for intracellular surveillance mechanisms in controlling HPV infections has been proposed (zur Hausen, 1986, 1989). Thus, HPV early region gene transcription may be selectively down-regulated in normal tissue in vivo by activation of controlling cellular genes by a putative humoral factor (Rosl et al., 1988). Thus, a loss of sensitivity to this surveillance mechanism, perhaps induced by other risk factors, would lead to uncontrolled HPV gene transcription and the subsequent development of the malignant phenotype. Our results on HPV-16 E6–E7 transcription in normal, CIN and carcinoma tissue is consistent with this hypothesis and stresses the potential importance of cellular genes in the development of cervical neoplasia.

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References


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