Evidence for a promoter-like activity in the short non-coding region of human papillomaviruses

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A short non-coding region (SNR) commonly exists between the E5 and L2 open reading frames of human papillomaviruses (HPVs). Except for the poly(A) signal for early gene transcripts, no biological functions have been discovered for the SNR. To test a possible promoter-like activity of the SNR, we carried out CAT reporter assays using constructs containing the SNRs from HPV-16, -18 and -33 linked to a promoterless CAT gene. We reproducibly observed enhanced expression of CAT gene by the SNRs. Co-expression of a transcriptional activator (LAP/NF-IL6) or deletion of the poly(A) signal augmented the promoter-like activity of the SNRs. RNase protection assays revealed a LAP-inducible CAT mRNA properly initiated from the HPV-16 SNR. These results may suggest that the SNR has a promoter activity that is regulated by keratinocyte differentiation.

Introduction

Certain types of human papillomaviruses (HPVs) such as -16, -18 and -33 are frequently associated with anogenital cancers worldwide (Pfister, 1987; Galloway & McDougall, 1989; zur Hausen, 1989; Maki et al., 1991). HPVs naturally infect mucosal or cutaneous squamous epithelium, where episomal replication of viral DNA is seen in the basal layers and vegetative replication takes place only in the upper layers of terminally differentiating keratinocytes (Pfister, 1987; Galloway & McDougall, 1989; zur Hausen, 1989). Inability to propagate HPVs in conventional tissue culture makes it difficult to study the complete life cycle of HPVs including the regulation of late gene expression. The HPV genome has two non-coding regions, the long control region (LCR) upstream of the E6 open reading frame (ORF) and a short non-coding region (SNR) between the E5 and L2 ORFs. The LCR contains the origin of DNA replication, the promoters for early and late genes and a number of cis-acting regulatory elements (Galloway & McDougall, 1989). On the other hand, no biological function has been discovered for the SNR except for the poly(A) signal for the transcripts of early genes (Galloway & McDougall, 1989).

Chow et al. (1987a, b), using the electron microscopic R-loop technique, demonstrated rare transcripts starting just upstream of the L2 ORF and ending after the L1 ORF in wart tissues infected with HPV-1, -6 and -11. Notably, only these transcripts appeared to encode L2. Baker & Howley (1987) examined viral cDNA derived from bovine fibropapilloma productively infected with bovine papillomavirus type 1 (BPV-1). They found that cDNA species encoding L2 were the rare ones starting just upstream of the L2 ORF. Rohlf et al. (1991) also reported rare cDNA species starting just upstream of the L2 ORF and encompassing the L2 and L1 ORFs among the viral cDNAs derived from HPV-16-immortalized human keratinocyte cell lines. Interestingly, no other mRNA species encoding L2 have been described so far. One possibility is that these mRNAs are derived from splicing or truncation of longer transcripts initiated from a late promoter in the LCR or from one of the other promoters found in the early gene region (Galloway & McDougall, 1989). Another possibility is that the SNR carries a weak promoter activity that is tightly regulated by keratinocyte differentiation and used only for transcripts encoding the minor capsid protein L2. In the present study, we have obtained evidence supporting the latter possibility.

Methods

Plasmids. The SNR regions were cloned from HPV-16 (Seedorf et al., 1985), HPV-18 (Cole & Danos, 1987) and HPV-33 (Cole & Streeck, 1986) by using a strategy based on polymerase chain reaction (PCR) as described previously (Fujikawa et al., 1994) and linked to the promoterless pCAT-Basic (Promega).

Transfection of cells and CAT assay. Cells were cultured routinely as described previously (Fujikawa et al., 1994). Primary human epidermal keratinocytes were purchased from Kurabo (Tokyo, Japan). Subconfluent cells were co-transfected with 5 μg of one of the CAT plasmids and 2 μg of pSRα bowel (Fujikawa et al., 1994) by using Lipofectin (Gibco BRL). Some cultures were also co-transfected with 2 μg of pCMV-LAP (Descombes et al., 1990). The CAT assay was
performed after normalization of transfection efficiency by β-galactosidase assay as described previously (Sambrook et al., 1989).

RNase protection assay. To create a probe for RNase protection assays (Zinn et al., 1983), the HindIII–EcoRI fragment from pSNR16(3982/4236)CAT was subcloned into pBluescript II SK(−) using a strategy based on PCR as described previously (Fujikawa et al., 1994). The uniformly labelled antisense probe was prepared using MAXIscript kit (Ambion, Austin, Texas). Total RNA samples were prepared from transfected HeLa cells as described previously (Sambrook et al., 1989). RNase protection assays were performed using RPA II Kit (Ambion). Protected fragments were fractionated on a 6% denaturing polyacrylamide gel and exposed to X-ray film.

Computer analysis. Computer analysis was carried out using MacPattern 2.0.1 with tfd Database (Ghosh, 1990).

Results and Discussion

To test a possible promoter-like activity of SNRs, we carried out CAT reporter assays (Gorman et al., 1982). The SNRs from HPV-16 (nt 3982–4236; Seedorf et al., 1985), HPV-18 (nt 4158–4243; Cole & Danos, 1987) and HPV-33 (nt 4082–4209; Cole & Streeck, 1986) were subcloned into a promoterless CAT reporter gene vector, pCAT-Basic (Fig. 1 a). A human cervical cell line HeLa (HPV-18+) was transfected with these constructs and transient expression of the CAT reporter gene was determined. The SNRs of the three types of HPV reproducibly enhanced CAT gene expression (Fig. 1 b). The promoter-like activity of HPV-33 SNR was relatively strong whereas that of HPV-16 SNR was intermediate. The activity of HPV-18 SNR was weak but still reproducible. We further tested the promoter-like activity of HPV-16 SNR in three cervical carcinoma cell lines and normal human epidermal keratinocytes (NHEK). We found that HPV-16 SNR was capable of inducing expression of the CAT gene to higher than background levels in all the cell lines, regardless of the presence of endogenous HPV genomes (Fig. 1 c). No such promoter-like activity was seen when the same HPV-16 SNR

![Fig. 1. CAT reporter assay for HPV SNRs. (a) Schematic representation of the CAT plasmids used in the present study. (b) Induction of the CAT gene by SNRs of HPV-16, -18 and -33. HeLa cells were co-transfected with 5 μg of pSNR16(3982/4236)CAT, pSNR18(4158/4243)CAT or pSNR33(4082/4209)CAT and 2 μg of pSRαβ-gal. The experiments were repeated four times with reproducible results. (c) Induction of the CAT reporter gene by HPV-16 SNR in different cell lines. Indicated cell lines were co-transfected with 5 μg of one of the CAT plasmids and 2 μg of pSRαβ-gal. The experiments were repeated twice with reproducible results. (d) Effects of LAP co-expression and deletion of poly(A) signal on promoter-like activity of HPV SNRs. HeLa cells were co-transfected with 5 μg of one of the CAT plasmids and 2 μg of pSRαβ-gal. Some cultures were also co-transfected with 2 μg of pCMV-LAP (indicated as LAP+). Lanes 1 and 2, pSNR16(3982/4236)CAT; lane 3, pSNR16(3982/4211)CAT; lanes 4 and 5, pSNR18(4158/4243); lane 6, pSNR18(4158/4234); lanes 7 and 8, pSNR33(4082/4209); lane 9, pSNR33(4082/4175); lanes 10 and 11, pCAT-Basic. Lanes 2, 5, 8 and 11, also co-transfected with pCMV-LAP (LAP+). The experiments were repeated four times with reproducible results.}
Table 1. Promoter-like activity of HPV-16 SNR

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>CAT activity (%)*</th>
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<tr>
<td>pCAT-Basic</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>pSNR16(3982/4236)CAT</td>
<td>4.6±0.4</td>
</tr>
<tr>
<td>pSNR16(4236/3982)CAT†</td>
<td>1.3±0.1</td>
</tr>
</tbody>
</table>

* HeLa cells were transfected with the indicated plasmids and CAT assay was done. Average ± SD of three separate experiments is shown.
† The SNR region of HPV-16 (nt 3982 to 4236) was linked to the promoterless CAT gene in the reverse orientation.

region was connected to the promoterless CAT gene in the reverse orientation (Table 1). Collectively, these results suggested that the SNRs have a promoter-like activity.

Computer analysis showed that the sequences of SNRs from HPV-16, -18 and -33 were not well conserved, except for high contents of T and the common presence of a poly(A) signal (AATAAA) in their 3'-terminal sides (Fig. 2a). Several potential TATA-box sequences were found in each SNR, but a classical one (TATAA) was only seen in HPV-16 SNR (Fig. 2a). The most downstream TATA-box-like sequence was located upstream of the poly(A) signal in the case of HPV-16 and HPV-18, and downstream of the poly(A) signal in the case of HPV-33. A poly(A) signal is known to be repressive for transcription starting from upstream and occlusion mechanisms of downstream poly(A) signals have been described or postulated for various viruses (Proudfoot, 1991). The promoter-like activity of HPV-33 SNR may thus be relatively strong because of the presence of a potential TATA-binding protein (TBP) site downstream of the poly(A) signal. We also noted presence of potential binding sites for NF-IL6/LAP [5'-T(T/G)NNGNAAT(T/G)- 3'] (Ghosh, 1990), which occurs several times in each SNR (Fig. 2a). A downstream one was overlapping or very close to the poly(A) signal (Fig. 2b). LAP, a liver-activating protein, was originally discovered as a major binding protein of the albumin promoter in rats (Descombes et al., 1990). NF-IL6, the human homologue of LAP, was first identified as a factor binding to the interleukin 1 (IL-1) -responsive element of the IL-6 gene (Akira et al., 1990). It is now known that NF-IL6/LAP plays important roles in

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![Fig. 2. Computer analysis of the SNRs from HPV-16, -18 and -33. (a) Schematic representation of the sites of potential regulatory elements in the SNRs. Only the most downstream TBP site is shown. (b) The nucleotide sequences of the SNRs from HPV-16, -18 and -33 in the vicinity of poly(A) signal. The potential binding site for LAP/NF-IL6 is indicated by bold letters and the poly(A) signal is underlined.](image-url)
Table 2. Effect of LAP co-expression or deletion of the poly(A) signal on the promoter-like activity of SNRs

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>CAT activity (%)†</th>
</tr>
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<tbody>
<tr>
<td>pCAT-Basic</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>pCAT-Basic + pCMV-LAP</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>pSNR16(3982/4236)CAT</td>
<td>5.1 ± 1.8</td>
</tr>
<tr>
<td>pSNR16(3982/4236)CAT + pCMV-LAP</td>
<td>24.9 ± 9.6</td>
</tr>
<tr>
<td>pSNR16(3982/421I)CAT</td>
<td>55 ± 20</td>
</tr>
<tr>
<td>pSNR18(4158/4243)CAT</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>pSNR18(4158/4243)CAT + pCMV-LAP</td>
<td>5.2 ± 1.9</td>
</tr>
<tr>
<td>pSNR18(4158/4234)CAT</td>
<td>11 ± 2.7</td>
</tr>
<tr>
<td>pSNR33(4082/4209)CAT</td>
<td>26 ± 8.9</td>
</tr>
<tr>
<td>pSNR33(4082/4209)CAT + pCMV-LAP</td>
<td>73 ± 14</td>
</tr>
<tr>
<td>pSNR33(4082/4175)CAT</td>
<td>12 ± 3.1</td>
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* See Fig. 1(a).
† HeLa cells were transfected with indicated plasmids and CAT assay was done. Average ± so of three separate experiments was shown.

To further examine CAT transcripts promoted by HPV-16 SNR, we carried out a highly sensitive RNase protection analysis (Zinn et al., 1983). Total RNA samples were prepared from HeLa cells that were transfected with pSNR16(3982/4236)CAT only, pSNR16(3982/4236)CAT + pCMV-LAP, pCAT-Basic only, or pCAT-Basic + pCMV-LAP, and were annealed with an antisense RNA probe covering the HPV-16 sequence from nt 3982 to 4236 (Fig. 3a). After treatment with RNase A and RNase T1, the protected fragments were separated by electrophoresis and visualized by autoradiography. As shown in Fig. 3(b), numerous bands were found to be protected in samples from HeLa cells transfected with pSNR16(3982/4236)CAT (lane 4) or pSNR16(3982/4236)CAT + pCMV-LAP (lane 3), suggesting transcriptional initiation from a number of aberrant sites. A single band of about 280 bp was, however, specifically enhanced by co-expression of LAP. From the length of the band, this species of transcripts...
was estimated to start about 20 bp downstream of a putative TBP site and about 40 bp upstream of the L2 ORF. The probable start site was thus very close and even identical to the nucleotide position 4199 mapped as the 5′ terminus of the cDNA species encoding HPV-16 L2 (Rohlf et al., 1991). No such protected bands were seen in samples of HeLa cells transfected with pCAT-Basic alone (lane 5) or pCAT-Basic + pCMV-LAP (not shown). Our repeated trials to determine exact transcriptional initiation sites by primer extension analysis (McKnight & Kingsbury, 1982) have not been successful so far, probably because of low abundance of specific mRNA.

The presence of an SNR between the E5 and L2 ORFs is a common feature of HPVs (Galloway & McDougall, 1989). The biological functions of the SNR are not well understood, except that it physically separates the early genes from the late genes and carries a poly(A) signal that is used for the termination of transcripts from the early genes (Galloway & McDougall, 1989). In the present study, we have provided evidence that the SNRs have a weak promoter activity. This promoter may be responsible for transcription of a minor species of mRNA encoding L2 which was repeatedly observed previously (Chow et al., 1987a, b; Baker & Howley, 1987; Rohlf et al., 1991).

The SNRs from different HPVs are not well conserved in size or sequence but are commonly rich in T residues. Each SNR also contains several potential binding sites for NF-IL6/LAP. It is now known that NF-IL6/LAP and other members of the C/EBP family are important regulators of gene expression. For example, it is postulated that the ratio of LAP and LIP (a liver-inhibitory protein which shares with LAP the 152 amino acids of the C-terminal DNA-binding domain but lacks the activation domain) is important for regulation of the differentiation-dependent gene expression of hepatocytes (Descombes & Schibler, 1991). LAP itself was also found to be a repressor factor binding to a silencer element of the gene for glutathione transferase P, which is silent in normal liver but strongly expressed during hepatocarcinogenesis (Imagawa et al., 1991). Furthermore, NF-IL6 was shown to repress the transcriptional activity of the LCR of HPV-16 (Kyo et al., 1993). Similarly, C/EBPβ, another synonym of LAP/NF-IL6, was detected as a cellular factor binding to the negative regulator element-2 (NR2) of the LCR of BPV-4 (McCaflery & Jackson, 1994). Collectively, NF-IL6/LAP as well as other members of the C/EBP family may play important roles in regulation of the early and late gene expression of HPVs during keratinocyte differentiation.

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