Point mutations in Sp1 motifs in the upstream regulatory region of human papillomavirus type 18 isolates from cervical cancers increase promoter activity

Barbara Rose,1 Gertrud Steger,2 Xiao-Ping Dong,2 Carol Thompson,1 Yvonne Cossart,1 Martin Tattersall3 and Herbert Pfister2

1, 3Departments of Infectious Diseases1 and Cancer Medicine3, Blackburn Building (DO6), The University of Sydney, NSW 2006, Australia
2Institut für Virologie, Universität zu Köln, Germany

Evidence of the functional significance of two naturally occurring mutations at nt 40 or 41 in the Sp1 motif in the promoter proximal segment of the upstream regulatory region (URR) of human papillomavirus (HPV) type 18 is presented. In electrophoretic mobility shift assays, Sp1 protein bound more efficiently to the Sp1 mutant motifs than to the prototype; while in both HeLa and HT3 cells, luciferase activity controlled by the mutant URRs was upregulated 2- and 3-fold, or 4- and 6-fold, in comparison with the prototype URR or HeLa cell-derived URR respectively. The HeLa URR represents a more appropriate baseline for promoter activity, containing a series of point mutations representative of most HPV-18 cancer isolates, including one in the Yin Yang 1 (YY1) site at the P105 promoter. The effect of the Sp1 mutations was found to be largely maintained in the context of the HeLa URR containing the prototype YY1 site.

Specific types of human papillomavirus (HPV), notably types 16 and 18, are now accepted as aetiological agents of cervical cancer (reviewed by Pfister, 1996). E6 and E7 have been identified as viral oncoproteins, and exert their effects through interactions with tumour suppressor proteins such as p53 and pRB (Werness, 1990; Dyson et al., 1989). HPV-18 shows immortalizing and transformation properties superior to HPV-16 in vitro, suggesting that HPV-18 may exhibit a more aggressive phenotype. This is supported by reports associating HPV-18 with more rapidly progressing dysplasia and increased tumour aggression (Walker et al., 1989; Burnett et al., 1992; Rose et al., 1995; Burger et al., 1996); but is not yet universally accepted (Ip et al., 1992; Chen et al., 1994). Earlier reports localized differences in the immortalizing and transforming potentials of these viruses to the 3' segment of the viral upstream regulatory region (URR) (Romanczuk et al., 1991; Villa et al., 1991), and it has been shown that this region contains clusters of motifs interacting with cellular transcription factors which are key modulators of E6/E7 expression (reviewed by Hoppe-Seyler & Butz, 1994). Organizational differences between the HPV-16 and -18 URRs affecting the level of E6/E7 expression may help explain the observed differences in their biological potential.

We have been interested in the biological significance of natural mutations occurring in URR enhancer or promoter motifs critically influencing E6/E7 expression levels. A recent survey of 28 HPV-18 cancer isolates (and that from HeLa cells), using the prototype strain (Cole & Danos, 1987) as reference, identified 19 point mutations, eight impacting transcription factor binding sites (Rose et al., 1997). In an extension of this work presented here, the functional significance of two naturally occurring mutations in the Sp1 site proximal to the HPV-18 E6/E7 promoter was assessed. Sp1 is a key activator of RNA polymerase II dependent promoters in HPV (Hoppe-Seyler & Butz, 1992) as in other systems (Kadonga et al., 1986), and has been recently identified as an accessory element in papillomavirus replication (Demeret et al., 1995).

The two mutations investigated, a G for T at nt 40, and a G for A at nt 41, fall within the Sp1 motif at nt 34–43 (Hoppe-Seyler & Butz, 1992). The former occurred in two of the 28 isolates (one of two Austrian and the single strain from the United States) while the latter was found in three isolates (one of 22 Australian and two of three Newmean). Initially, electrophoretic gel mobility shift assays (EMSAs) were performed to determine the effect of each of the Sp1 mutations on DNA–protein binding. Assays were carried out using 32P-labelled and unlabelled double-stranded oligonucleotides representing bp 34–53 (aagcttAGGGAG(T/G)(A/G)ACCG-AAAACCGGT) of the HPV-18 prototype (nt 40 = T, nt 41 = A ‘18P40/41’) or variant (nt 40 = G, ‘18M40’ and nt 41 = G ‘18M41’) genomes. Lower-case letters refer to nucleotides
added to create a *Hind*III overhang for unrelated purposes. In competition experiments, binding of 15 ng Sp1 protein (Promega) to 20 000 c.p.m. of the 18M40 oligonucleotide was competed with 12.5-, 25-, 50-, 100- and 500-fold excess of unlabelled 18P40/41 and 18M40 essentially as described (Steger & Corbach, 1997). Similarly, binding of Sp1 to labelled 18M41 was competed with unlabelled 18P40/41 and 18M41.

Dried gels were exposed for 3 h to Kodak X-Omat film at −70 °C, and signals were also resolved using a phospho-imager (Molecular Dynamics). Sp1 protein bound to both wild-type and mutant elements, but unlabelled oligonucleotides containing the mutation at nt 40 or 41 competed for binding more efficiently than the prototype. The highest concentration of unlabelled mutant competitor abolished binding of Sp1 protein to the labelled mutant oligonucleotide 18M40 completely, and reduced binding of Sp1 to labelled
Six µpALuc (de Wet et al., 1987) was subcloned into the luciferase gene reporter first series of experiments, the HPV-18 prototype URR involving known transcription factor binding sites. The HPV-18 prototype URR (pALuc18P) was assessed in the context of the HPV-18 prototype URR (pALuc18P) with/without mutations in the Sp1 site at nt 40 or 41 (pALuc18P40/41M); in the URR derived from HeLa cell HPV-18 isolate (pALucHeLa) containing a mutation at nt 40 or 41 (pALucHeLa40/41M); in pALucHeLa with/without a C to T mutation in the YY1 site at nt 104 (pALucHeLa104P) with/without the Sp1 mutations at nt 40 or 41 (pALucHeLa40/41P, pALucHeLa41P104P and pALuc18P) with a T to C mutation at nt 104 (pALuc18P104M).

In HeLa cells, luciferase activity under the control of HPV-18 URRs with/without mutations in the promoter proximal Sp1 site in HeLa cells and HT3 cells. The URR derived from the HeLa cell HPV-18 isolate (pALucHeLa) served as reference and is denoted as 1. Promoter activity was assessed in the context of the HPV-18 prototype URR (pALuc18P) with/without mutations in the Sp1 site at nt 40 or 41 (pALuc18P40/41M); in the URR derived from HeLa cell HPV-18 isolate (pALucHeLa) containing a mutation at nt 40 or 41 (pALucHeLa40/41M); in pALucHeLa with/without a C to T mutation in the YY1 site at nt 104 (pALucHeLa104P) with/without the Sp1 mutations at nt 40 or 41 (pALucHeLa40/41P, pALucHeLa41P104P and pALuc18P) with a T to C mutation at nt 104 (pALuc18P104M).

These findings were consistent with the observation that the changes – to G in each case – brought this aberrant Sp1 motif closer to the high affinity-binding consensus Sp1 sequence (Kadonga et al., 1986).

Investigations were then undertaken to assess the effect of the increased binding on homologous promoter activity. In the first series of experiments, the HPV-18 prototype URR (nt 6928–118) was subcloned into the luciferase gene reporter pALuc18P (de Wet et al., 1987) as described (Dong et al., 1994) to form pALuc18P. PCR-based site-directed mutagenesis (Ausubel, 1992) was used to induce a G for T at nt 40 and a G for A at nt 41 into pALuc18P to form pALuc18P40M and pALuc18P41M respectively. Transient transfections were performed in triplicate on 80% confluent cultures of human cervical carcinoma-derived HeLa (ATCC: CCL2) and HT3 (ATCC: MTB32) cells by the calcium phosphate co-pre- cipitation method as previously described (Dong et al., 1994). Six µg of luciferase gene reporter plasmid and 1 µg pCMV-β-galactosidase expression vector (used as internal control for transfection efficiency) were used per 6 cm dish. Luciferase assays were performed on harvested cell lysates as published (de Wet et al., 1987). Standard amounts of protein were used in assays to determine β-galactosidase activity and HPV-18 promoter activity was determined by calculating the luciferase:protein ratios adjusted to account for the variation in transfection efficiencies. Results were based on at least four independent experiments.

In HeLa cells, luciferase activity under the control of the HPV-18 URR carrying a mutation at nt 40 (pALuc18P40M) or nt 41 (pALuc18P41M) was found to be upregulated at least 2-fold and 3-fold respectively in comparison with the prototype (pALuc18P) (Fig. 2). The effect in both instances was marginally less in HT3 cells. While the use of the HPV-18 prototype as baseline for promoter activity conformed with many previous studies dissecting the role of various transcription factor binding sites in HPV E6/E7 expression, none of the 28 HPV-18 cancer isolates tested in the recent survey (Rose et al., 1997) was identical in sequence to the prototype, which had been cloned originally from a Brazilian tumour. The majority, including all with the Sp1 mutations, contained a T to C change at nt 104 proximal to the E6/E7 promoter impacting a Yin Yang 1 (YY1) site, as well as a series of point mutations not involving known transcription factor binding sites. The HPV-18 URR derived from HeLa cells had been previously found to carry a spectrum of base changes representative of our natural isolates and was thus considered to provide a more appropriate baseline for this study than the prototype. The HPV-18 URR from HeLa cells was cloned into pALuc to produce pALucHeLa, and the nt 40 and nt 41 mutations were subsequently generated in this construct to form pALucHeLa40M and pALucHeLa41M respectively. The elevated promoter activity associated with the Sp1 mutations in the context of the prototype strain was sustained at approximately the same level in the context of pALucHeLa. Interestingly, however, luciferase activity using pALucHeLa was only half that of the prototype (pALuc18P). In contrast to the prototype, the HeLa URR contains the T to C mutation in the YY1 site at nt 104. We had showed earlier that YY1 binding occurred with either the T or C at nt 104, but with twice the affinity with the T (Rose et al., 1997). YY1 can play a major role in regulating promoter activity (depending on location and cell context), and is known to interact with Sp1 in the adeno-associated virus P5 promoter (Seto et al., 1993). In order to investigate the effect of this mutation within the YY1 site on promoter activity, a C mutation was generated at nt 104 in pALuc18P (pALuc18P104M). Surprisingly, promoter activity in the context of pALuc18P was found to be slightly higher with the C at nt 104 than with a T, but the difference was not significant in either HeLa or HT3 cells (Fig. 2). These findings indicated
that this particular YY1 site played only a minimal role in promoter regulation. Luciferase assays carried out following replacement of the C with T at nt 104 in the pALucHeLa construct (pALucHeLa104P) supported the minor involvement of the YY1 mutation in promoter activity. The Sp1 mutations continued to upregulate promoter activity in the context of the T at nt 104 in the pLucHeLa construct (constructs pALucHeLa40M104P and pALucHeLa41M104P respectively), but the effect was not as pronounced as in the context of pALucHeLa, that is with a C at nt 104 (Fig. 2).

Across all experiments, the mutation at nt 41 had a greater effect on promoter activity than that at nt 40 (4- to 5-fold compared with more than 4- to over 6-fold in relation to the pALucHeLa baseline). This is consistent with suggestions that the mutation at nt 41 had a greater effect on protein DNA binding than that at nt 40 (Fig. 1a–d). The effect of the Sp1 mutations on promoter activity was essentially the same in HT3 as in HeLa cells.

Overall, the findings provide evidence that naturally occurring point mutations in this Sp1 motif result in higher affinity protein binding and stronger E6/E7 promoter activity. However, it is not completely clear how the in vitro effects of these Sp1 mutations translate to the situation in vivo. Sp1 is required for the activity of the P105 promoter, reportedly coordinating the signal from the viral enhancer, and was recently shown to contribute to the efficiency of virus replication. The precise mechanisms involved are not yet clear, but evidence in other systems suggests that the primary function of Sp1 may be to facilitate the binding of components to the pre-initiation complex by competing with the nucleosome assembly upstream of the TATA box (Workman & Buchman, 1993). Alternatively, it has been suggested that Sp1 may promote melting of the strands by unwinding or bending the DNA molecule thus facilitating the binding of the TATA box-binding protein to the promoter (Demeret et al., 1994). Additionally, the DNA binding domains of Sp1 (and E2) may promote the formation of the initiation complex.

In productive HPV infection, E6/E7 expression is maintained at a level which allows virus replication, but does not lead to malignant progression. The viral E2 protein plays an important role in promoter repression, through binding to its two recognition sites (BS1 and BS2) in the promoter proximal region of the HPV-18 URR (Steger & Corbach, 1997). The binding of E2 protein to these sites has been shown in vitro to interfere with the binding of Sp1 to its cognate sequence lying nearby (Demeret et al., 1995). This suggests that stronger binding of Sp1 may decrease the ability of the E2 protein to repress promoter activity. The repression of the E6/E7 promoter seems of critical importance, since E2-encoding sequences are preferentially deleted as the virus integrates into the host chromosome on malignant conversion (Schwarz et al., 1986), resulting in elevated E6/E7 expression. Stronger Sp1 binding may also have an effect on virus replication, although this has not yet been established.

This study strengthens earlier evidence of the biological significance of some naturally occurring mutations in the HPV genome, including that associating YY1 deletions in the HPV-16 URR with malignant conversion in the absence of integration (Dong et al., 1994). However, further studies in a large clinically well-defined series of cancers, as well as information on the prevalence of these mutations in benign and premalignant cervical lesions, are needed to confirm the biological significance of the Sp1 mutations. Additionally, since our findings indicate that the YY1 site at P105 has only a minor role in promoter activity, further investigations are needed to explain the observed difference in baseline promoter activity associated with the HPV-18 prototype and pALucHeLa URRs. Current evidence suggests the involvement of an as-yet unidentified transcription factor binding site.

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References


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