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The promoter of a novel human papillomavirus (HPV77) associated with skin cancer displays UV responsiveness, which is mediated through a consensus p53 binding sequence

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An aetiological role has been proposed for human papillomavirus (HPV) in skin carcinogenesis within the immunosuppressed patient population. To examine this possibility, we have focused on an HPV type that, to date, has been identified only in the cutaneous lesions of renal transplant recipients despite a high degree of sequence homology with other HPVs commonly found in warts in the general population. We report that the non-coding region of this virus, HPV type 77, contains a consensus binding site for the tumour suppressor protein p53, and we show by gel-retardation analysis that this sequence does indeed bind p53. Furthermore, using reporter gene assays, we demonstrate that HPV77 promoter activity is stimulated by UV radiation and that this response is mediated through the p53 binding site. This is the first report of a p53-dependent positive response element within a viral genome. Our results suggest a possible novel mechanism by which specific types of HPV might act as cofactors with UV radiation in cutaneous transformation.

Keywords: HPV/immunosuppression/p53/skin cancer/UV

Introduction

Inactivation of the p53 tumour suppressor gene is a common event in human carcinogenesis, implying that the p53 protein plays a fundamental role in the regulation of cell growth. In non-transformed cells, levels of p53 protein rise after exposure to DNA damaging agents, leading in turn to the activation of the growth arrest or apoptosis pathways. These effects are mediated, at least in part, by the ability of p53 to function as a sequence-specific transcriptional activator (reviewed in Levine, 1997). Evidence suggests that for efficient DNA binding, p53 requires two copies of a 10 bp motif 5′-PuPuPuC-(A/T)(T/A)GPyPyPy-3′ separated by 0–13 bp (El-Deiry *et al*., 1992). Two of the more notable p53 target genes identified to date are *WAF1*/*CIP1*/*p21*, which encodes a cyclin-dependent kinase inhibitor required for p53-dependent cell cycle arrest (El-Deiry *et al*., 1993), and *bax* (Miyashita and Reed, 1995), whose product is a powerful promoter of apoptosis. A number of other cellular genes are known to be directly transactivated by p53, these include: *IGF-BP3*, *mdm2*, *cyclin G*, *PCNA* and *GADD45* (reviewed in Ko and Prives, 1996). In addition, Bourdon *et al.* (1997) have identified p53 binding sites in the promoter and/or intronic regions of a further 30 human genes. Most recently, Polyak *et al.* (1997) employed SAGE (serial analysis of gene expression) methodology in an attempt to discover novel cellular genes involved in p53-dependent apoptosis. Using this approach, a total of 40 genes, which included only two known targets of p53, were found to be expressed at greater levels in p53-expressing than in p53-null cells. Although some of these may not actually be induced by p53 under physiological conditions, these data strongly suggest that the current list of p53 target genes is far from complete.

Human papillomaviruses (HPVs) are small DNA viruses with a strict tropism for stratified squamous epithelia. Although the majority of HPV types are associated with benign wart-like lesions, there is considerable evidence to support an aetiological role for HPVs 16 and 18 in anogenital carcinogenesis (zur Hausen, 1989, 1994). One possible mechanism by which HPVs contribute to cell transformation is suggested by the well documented ability of the HPV16 and HPV18 E6 proteins to associate with and promote the rapid degradation of p53 via the ubiquitin-dependent proteolytic pathway (Scheffner *et al*., 1990, 1993). In addition, HPV types 5 and 8 are strongly associated with non-melanoma skin cancer (NMSC) in patients with the inherited disease epidermodysplasia verruciformis (EV), which is characterized by the widespread development of warts containing a number of HPV types not found in the normal population. In contrast to the E6 proteins of HPVs 16 and 18, those of HPVs 5 and 8 lack the ability to degrade p53 (Steger and Pfister, 1992). It is conceivable therefore, that these proteins may have a different cellular target. This possibility is consistent with data from *in vitro* assays indicating that the E6 open reading frame (ORF) of HPVs 5 and 8 encodes a major transforming activity (Iftner *et al*., 1988). NMSC is also a major cause of morbidity in renal transplant recipients (RTRs) and other patients subjected to immunosuppressive therapy. As in EV patients, tumours arise predominantly on sun-exposed skin and frequently harbour HPV DNAs. These combined data point to a role for UV radiation (UVR) and HPV as cofactors in skin carcinogenesis in both patient populations. Although it is probable that any mechanism would include the well documented DNA-damaging and immunosuppressive effects of UVR, additional roles for UVR as a co-carcinogen with HPV cannot be excluded. For instance, in view of earlier reports of a

UV-associated transcriptional upregulation in polyoma virus (Rutberg *et al*., 1992), one possibility is that UVR might stimulate HPV promoter activity and thereby increase the expression levels of the E6 and E7 oncoproteins.

Although EV-specific HPV DNAs have been identified in RTR squamous cell carcinomas (SCCs), evidence suggests that the spectrum of HPVs associated with these tumours also includes many previously unidentified types (Shamanin *et al*., 1996; de Villiers *et al*., 1997). One type that has been identified in a number of RTR tumours and warts, but not in lesions from immunocompetent individuals or EV patients, is HPV77 (Shamanin *et al*., 1994; Delius *et al*., 1998). HPV77 is most closely related to HPV29, a type that appears to be restricted to benign skin lesions (Favre *et al*., 1989). HPV77 also shares a high degree of DNA sequence homology with HPVs 3, 10 and 28, which are commonly detected in benign plane warts in the general population (de Villiers, 1989). We were therefore interested in how HPV77 might differ from these other closely related types. Specifically, we have investigated whether these observations could be explained by differences between the non-coding sequences of HPV77 and those of other HPVs. We have identified a consensus p53 binding site within the HPV77 non-coding region (NCR). Although similar motifs are present in the corresponding regions of the genomes of HPV29 and other closely related types, these sequences are considerably more degenerate. We have demonstrated that p53 does indeed bind specifically to the HPV77 sequence. Functional studies show that the activation of p53 under physiological conditions results in the stimulation of HPV77 promoter activity. This is the first report of a p53-dependent positive response element within a viral genome. In addition, these data suggest a novel mechanism by which HPV77 and UVR might act as cofactors in cutaneous carcinogenesis.

Results

The non-coding region of HPV77 contains a sequence homologous to the consensus p53 binding site

As a first step towards understanding how HPV77 transcription may be regulated, we sequenced the NCR and used computer-assisted analysis to identify potential binding sites for cellular transcription factors. In common with the regulatory regions of other HPV types from different subgroups of the HPV superfamily, the HPV77 NCR contains putative recognition sequences for the cellular transcription factors AP-1 and NF-1 (Figure 1A). Interestingly, the HPV77 NCR also contains an element with close homology to the consensus binding site for p53. Alignment of the HPV77 NCR with the NCRs of other closely related viruses revealed that the corresponding motifs contained more than three mismatches with the El-Deiry consensus sequence (Figure 1A) and consequently did not meet the criteria proposed by Bourdon *et al*. (1997) for a functional p53-responsive element. The HPV77 sequence possesses several interesting features that distinguish it from all previously identified p53-responsive elements (Figure 1B). Specifically, the first decamer is A/T-rich and a perfect palindrome, while the second decamer lacks both the conserved cytosine and guanine at positions 4 and 7, respectively. Two additional decamers bearing a lesser degree of homology to the El-Deiry decamer were identified in the HPV77 NCR, 12 bp upstream and 4 bp downstream of the potential p53 binding site (Figure 1A). This is consistent with the recent observation that the majority of published sequences of p53-responsive elements contain four or more El-Deiry decamers (Bourdon *et al*., 1997). These combined data suggested that the HPV77 enhancer region might be subject to regulation by p53.

The p53 protein binds specifically to the consensus binding site in the HPV77 NCR

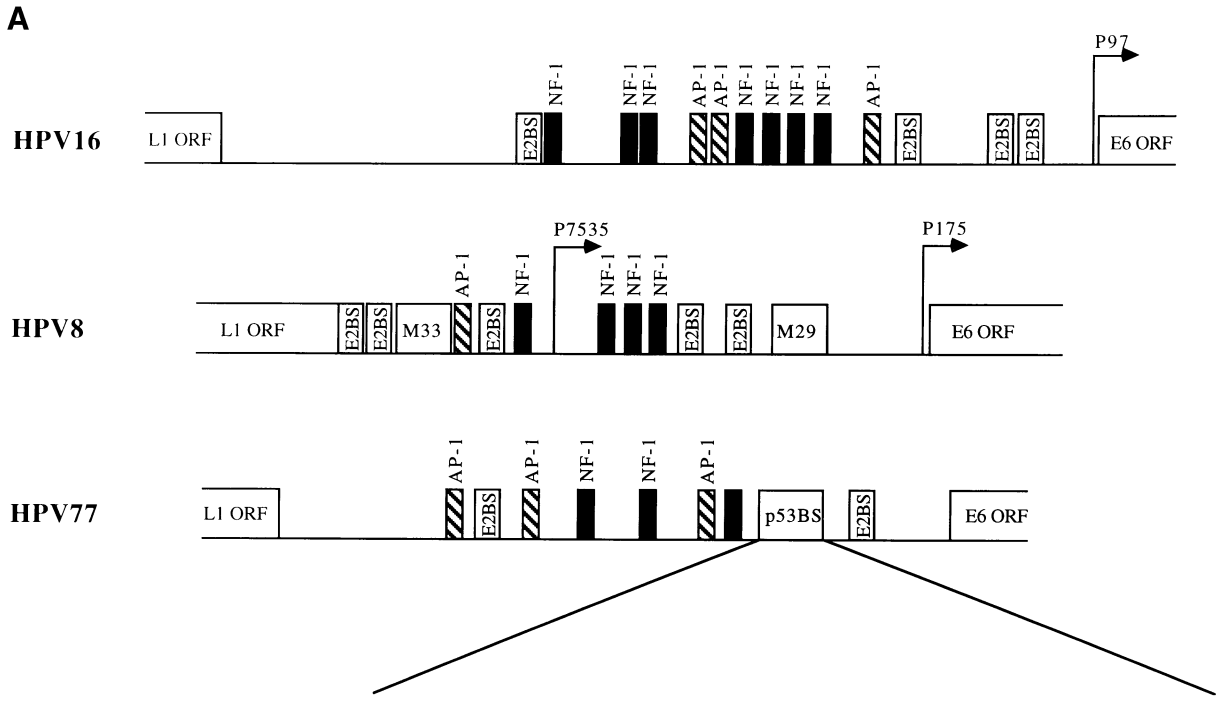
Gel-retardation assays were performed to determine whether p53 could bind to the putative p53-responsive element in the HPV77 NCR. For these experiments, *in vitro* translated p53 was incubated with ^{32}P-labelled oligonucleotides corresponding to either the 20 bp HPV77 sequence or a mutant version containing three nucleotide substitutions in each decamer, which by analogy with other known p53 binding sites would be predicted to disrupt binding. Reactions were performed in the presence of pAb421, an antibody previously shown to bind within the C-terminus of p53 and activate sequence-specific DNA binding (Hupp *et al*., 1992), or in the presence of the DO-1 or pAb1801 antibodies, which bind epitopes in the N-terminus of p53 but do not activate p53 DNA binding effectively (Hupp *et al*., 1992; Thomas *et al*., 1995). For comparative purposes, oligonucleotides corresponding to the p53 consensus sequences previously identified in the *WAF1* (El-Deiry *et al*., 1993) and *bax* (Miyashita and Reed, 1995) gene promoters were included in this analysis.

In the presence of pAb421, complexes were detected between p53 and wild-type HPV77 and the *WAF1* and *bax* oligonucleotides (Figure 2A), but not between p53 and the mutant probe (Figure 2B). As expected, p53 DNA binding was not activated effectively in the presence of the pAb1801 or DO-1 antibodies. In accordance with previous data (Friedlander *et al*., 1996), p53 appeared to display a far higher affinity for the *WAF1* oligonucleotide than the *bax* oligonucleotide. The level of p53 binding to the consensus site in the HPV77 NCR was comparable to that seen with the *bax* sequence. Competition experiments (Figure 2B) revealed that the p53–HPV77 DNA complex could be disrupted by the addition of excess unlabelled wild-type, but not mutant, HPV77 oligonucleotide. Similar results were obtained using *bax* or *WAF1* competitor oligonucleotides. All three wild-type oligonucleotides also efficiently disrupted the association between p53 and the *bax* oligonucleotide (Figure 2C). In contrast, p53–*WAF1* DNA complexes were effectively competed only by the homologous oligonucleotide. Taken together, these data strongly suggest that p53 associates specifically with the HPV77 motif.

Considering the phylogenetic relationship between HPV77 and other cutaneous HPV types, we were then interested to determine whether the homologous regions of the NCR of closely related viruses such as HPV10 and HPV29 were also able to bind p53. As shown in Figure 1A, the corresponding regions of HPVs 10 and 29 contain more bases that differ from the p53 consensus binding sequence than HPV77. When these were tested

in gel-shift assays, unlike the HPV77 NCR sequence, these sequences were unable to bind p53 (Figure 2D), indicating that any potential p53 effects on HPV gene transcription are restricted to specific HPV types.

A notable finding was that the p53 protein formed two distinct complexes with the different oligonucleotides (see Figure 2B and D). The primary p53–HPV77 DNA complex appeared to correspond to the faster migrating of the p53–*WAF1* and *bax* DNA complexes. To determine the relative contributions of the first and second palindromes, we synthesized a 20 bp hybrid oligonucleotide comprising the first decamer from the HPV77 motif and the second decamer from the WAF1 motif, or vice versa. Gel-retardation analysis with these oligonucleotides (Figure 2E, lanes 3 and 4) revealed that the pattern of complexes formed with the *in vitro* translated p53 was determined by the composition of the second decamer of the p53 binding site. Since the second decamer of the HPV77



B

p53 CON		RRRCWWGYYYRRRCWWGYYY
HPV77		AAACATGTTTGcAaATcCCC
WAF1		GAACATGTCCcAACATGTTg
Bax		AGACAAGCCTGGGCgTGggC
Mdm2		GGtCAAGTTgGGACAcGTCC
IGFBP3	box A	AAACAAGCCa*cAACATGCTT
	box B	GGGCAAGaCCtGcCAAGCCT
CYCLIN G		AGACcTGCCCGGGCAAGCCT
GADD45		GAACATGTCTAAGCATGCTg
RGC		GGACTTGCCTGGcCTTGCCT
MCK		tGGCAAGCCT*tGACATGgCC

R = A or G, W = A or T, Y = C or T

Fig. 1. (**A**) A schematic comparison of the HPV77 NCR, which comprises the sequences between the L1 and E6 ORFs, with the NCRs of high-risk mucosal HPV type 16 and EV-specific HPV type 8. The positions of binding sites for the virally encoded E2 transcription factor and cellular transcription factors NF-1 and AP-1 are shown, as are the locations of viral promoters where known. In the case of the HPV77 NCR, these sites were identified by computer-assisted analysis and are therefore only putative. The M33 and M29 sequences located within the HPV8 genome are conserved EV-specific regulatory elements shown to be involved in protein binding. The putative p53 binding site identified in the HPV77 NCR consists of two contiguous decamers with strong homology to the p53 consensus sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′. Two additional imperfect motifs are located 12 bp upstream and 4 bp downstream of this element. These sequences are aligned with the corresponding non-coding sequences of closely related HPVs. Motifs homologous to the p53 consensus decamer are underlined with bases matching the consensus sequence indicated in bold type. (**B**) Comparison of the putative HPV77 p53 binding site with p53 recognition sequences in the promoter and/or intronic regions of eight known cellular p53 target genes. Bases matching the consensus sequence are shown in uppercase letters. An asterisk indicates a 1 bp separation between the two decamers of the consensus sequence.

motif differs from that of all known cellular-derived sequences in its lack of a conserved cytosine and guanine at positions 4 and 7, respectively, we next synthesized mutant oligonucleotides where the base at position 4 or 7 of the second decamer was exchanged between the WAF1 and HPV77 sequences in order to investigate the contribution of these residues in complex formation. Gel-retardation analysis (Figure 2E, lanes 5–8) suggested that the base at position 4 was influential in determining the extent to which these differently migrating complexes bound.

We were next interested in investigating whether activated cellular-derived p53 was also able to bind to the

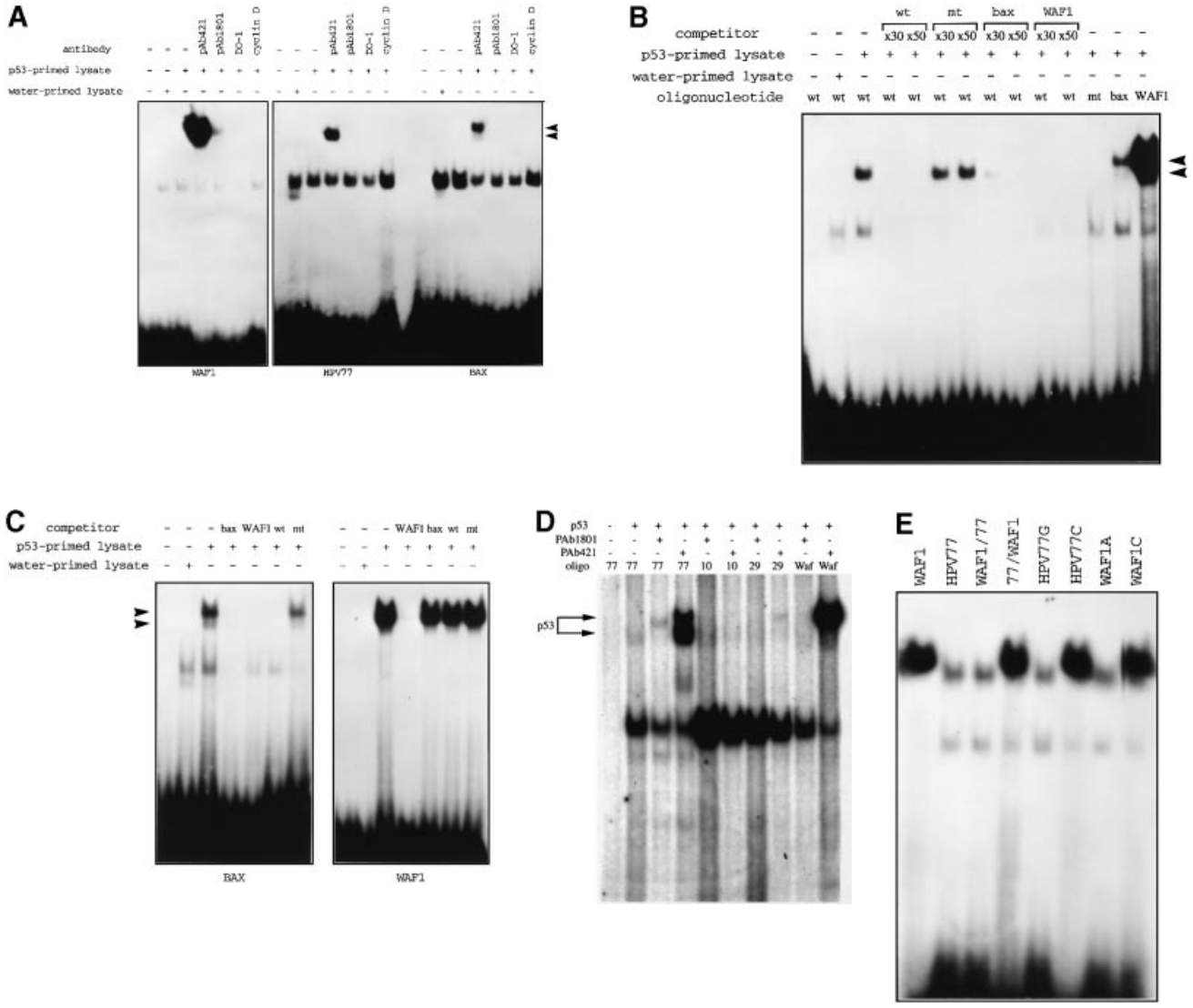


Fig. 2. (**A**) *In vitro* translated p53 binds to the HPV77 p53 consensus sequence in the presence of pAb421 but not pAb1801 or DO-1. ^{32}P-end-labelled oligonucleotides corresponding to the p53 consensus sequence in the HPV77 NCR or to the p53 consensus sequences from the *bax* or *WAF1* gene promoters were incubated with *in vitro* translated p53 in the presence of pAb421, pAb1801 or DO-1 anti-p53 antibody or non-specific anti-cyclin D antibody. DNA–protein complexes were separated by non-denaturing polyacrylamide gel electrophoresis. The positions of the p53-specific bands are indicated with arrowheads. (**B**) The binding of *in vitro* translated p53 to the HPV77 p53 consensus sequence is specific. Gel-shift assays were performed as above using end-labelled oligonucleotides corresponding to the p53 consensus sequence in the HPV77 NCR (wt), to a mutant version (mt) with three nucleotide substitutions in each decamer or to the p53 consensus sequences from the *bax* or *WAF1* gene promoters. All reactions were carried out in the presence of pAb421. Where indicated, experiments were performed in the presence of a 30- or 50-fold excess of unlabelled competitor oligonucleotide. (**C**) The HPV77 wild-type oligonucleotide can efficiently compete complexes formed between p53 and the *bax*, but not *WAF1*, oligonucleotide. Gel-shift assays were performed as before using end-labelled *bax* or *WAF1* oligonucleotides in the presence of a 50-fold excess of unlabelled competitor oligonucleotide. The positions of the p53-specific bands are indicated with arrowheads. (**D**) p53 does not form complexes with sequences from the HPV77-related viruses HPV10 or HPV29. Gel-shift assays were performed to determine whether p53 was able to bind to the equivalent region of either HPV10 or HPV29. Specific binding of p53, indicated by arrowheads, was only seen for the HPV77 and *WAF1* oligos. (**E**) The identity of the base at position 4 of the second decamer of the p53 consensus sequence plays a role in determining the pattern of complex formation with *in vitro* translated p53. Gel-shift assays were performed as above in the presence of pAb421 using wild-type HPV77 or *WAF1* oligonucleotides, or hybrid or mutant variants thereof. The hybrid oligonucleotides comprised the first decamer of the HPV77 motif and the second decamer of the *WAF1* motif, or vice versa, and were designated 77/*WAF1* or *WAF1*/77, respectively. The HPV77 mutant oligonucleotides, designated 77C or 77G, were generated by replacing the base at position 4 or 7, respectively, of the second decamer with the corresponding cytosine or guanine from the *WAF1* sequence. Conversely, the cytosine or guanine at position 4 or 7 in the second decamer of the *WAF1* oligonucleotide was replaced with the adenine or cytosine from the corresponding position in the HPV77 motif to yield the *WAF1* mutant oligonucleotides, designated *WAF1*A and *WAF1*C, respectively.

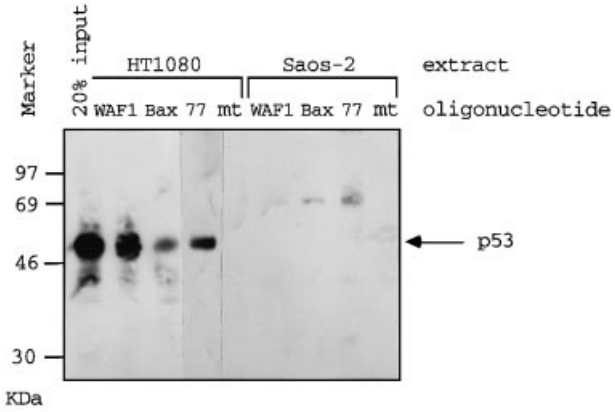


Fig. 3. The HPV77 p53 consensus sequence binds activated cellular-derived p53 protein. Biotinylated oligonucleotides corresponding to the wild-type HPV77 p53 consensus sequence (77), the mutant version described above (mt) or to the consensus sequences from the *bax* or *WAF1* gene promoters were bound to streptavidin-coated Sepharose beads prior to incubation with nuclear extracts from UV-irradiated cultures of the p53 wild-type human osteosarcoma line HT1080 or the p53-null human osteosarcoma line Saos-2. Bound proteins were eluted, separated by 10% SDS–PAGE and analysed by Western blotting using the DO-1 antibody. As a measure of the efficiency of p53 capture, an aliquot of HT1080 extract corresponding to 20% of the input protein was included in the immunodetection.

HPV77 sequence. In these experiments, biotinylated oligonucleotides corresponding to the wild-type or previously described mutant version of the HPV77 p53 binding site or to the p53-responsive elements in the *WAF1* and *bax* promoters were mixed with streptavidin-coated Sepharose beads. The immobilized oligonucleotides were incubated with nuclear extracts prepared from UV-irradiated cultures of the p53 wild-type human osteosarcoma line HT1080 or the p53-null osteosarcoma line Saos-2. Bound proteins were then eluted and analysed by immunoblotting using the DO-1 antibody. As shown in Figure 3, cellular-derived p53 was captured by the wild-type HPV77 and the *WAF1* and *bax* oligonucleotides, but not by the mutant HPV77 sequence. These data strongly suggest that UV-activated cellular p53 protein binds specifically to the consensus binding site in the HPV77 NCR.

The p53 consensus sequence from the HPV77 NCR confers p53 responsiveness on a heterologous promoter

Reporter gene assays were performed to determine whether the core p53 consensus sequence from the HPV77 NCR is sufficient to confer p53 responsiveness on a heterologous promoter. Previous investigators have reported that four El-Deiry consensus decamers are required for efficient p53-mediated transcription from a heterologous promoter (Kern *et al*., 1992); hence a reporter construct HPV77(2)CAT containing two copies of the 20 bp HPV77 motif cloned upstream of a polyoma virus promoter and CAT gene was generated for these experiments. For comparative purposes, two copies of the p53 consensus sequence from either the *bax* or *WAF1* promoters were likewise cloned into the parent plasmid to generate the bax(2)CAT and WAF(2)CAT constructs, respectively. Reporter gene plasmids were co-transfected with either a p53 expression construct or an expression vector lacking the p53 insert into the p53-null osteosarcoma line Saos-2. CAT activity from the HPV77(2)CAT construct increased 5-fold in the presence of p53 (Figure 4A) compared with 11- and 13-fold increases from the bax(2)CAT and WAF(2)CAT constructs, respectively (Figure 4B). To confirm that these increases were mediated by p53, cultures were additionally co-transfected with a construct encoding HPV18 E6 that targets p53 for degradation. As can be seen in lanes 5–6 of Figure 4A, activation of the reporter gene was effectively abolished by the expression of HPV18 E6. In marked contrast, expression of the HPV77 E6 protein had no noticeable effect on the p53-mediated transactivation of any of the three reporter gene constructs (Figure 4A, lanes 7–8).

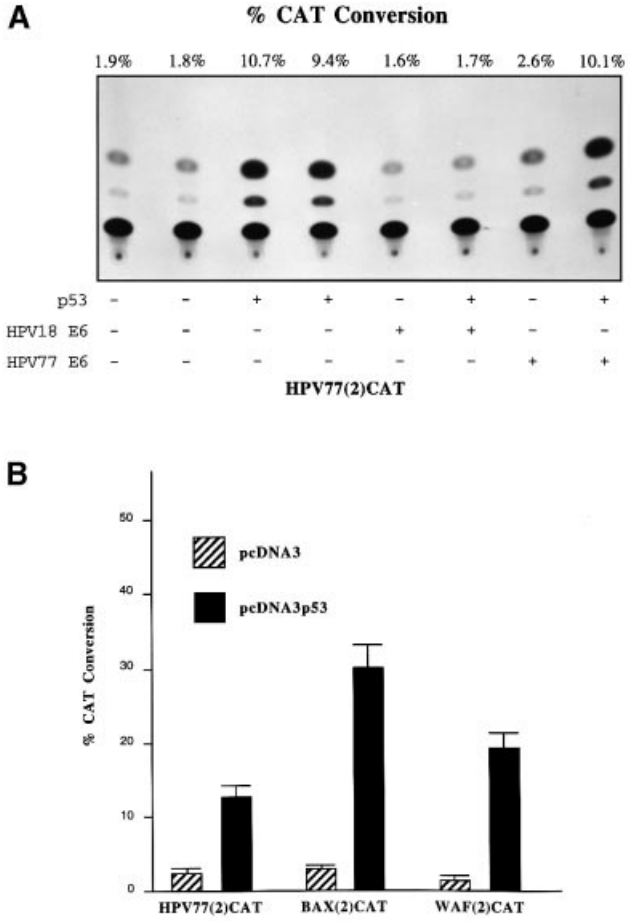


Fig. 4. The HPV77 p53 consensus sequence confers p53 responsiveness on a heterologous promoter. (**A**) Saos-2 cultures were co-transfected with the HPV77(2)CAT reporter construct and either a p53 expression construct or an expression vector lacking the p53 insert. Where indicated, cultures were also co-transfected with an HPV18 or HPV77 E6-expressing construct. Cultures were harvested after 48 h and CAT assays performed using 10 μg of each protein extract. Percentage CAT conversion was defined as the percentage of [^{14}C]chloramphenicol substrate converted to acetylated [^{14}C]chloramphenicol. (**B**) Cultures were transfected with CAT reporter and p53 expression constructs as indicated and reporter assays performed as above. The data presented in the bar graph represent the mean of two separate experiments with error bars depicting the difference between the mean and the higher of the two values.

The HPV77 p53 consensus sequence confers p53 responsiveness on its homologous HPV promoter

We next examined whether the presence of a functional p53 response element in the HPV77 NCR had any signi-

ficance for HPV77 promoter activity under physiological conditions. In view of the occurrence of HPV77-associated lesions at sun-exposed sites, we used UV irradiation as the mechanism of p53 activation. A reporter gene construct designated HPV77NCR–CAT was generated for these experiments by cloning a 0.8 kb fragment encompassing the entire HPV77 NCR upstream of a promoterless CAT gene. In addition, to distinguish between p53-dependent and p53-independent changes in promoter activity, we generated the HPV77mtNCR–CAT construct in which the wild-type HPV77 p53 binding site was replaced by the non-binding mutant sequence used in bandshift analysis. The previously described pWWP-CAT construct (El-Deiry *et al*., 1993) containing the *WAF1* gene promoter was also included in reporter gene analysis as a positive control. Considering the likely cell specificity of the HPV77 promoter, experiments were performed in primary skin keratinocytes as these are the natural host cells of HPV77. Cultures were irradiated with UVB light 24 h after transfection with the appropriate reporter gene construct. Under these conditions, HPV77 promoter activity increased ~5-fold after UV irradiation (Figure 5A). This increase was comparable to the UV-induced stimulation of *WAF1* promoter activity (Figure 5B). Irradiation had no effect on HPV77 promoter activity in cultures co-transfected with the HPV18E6 expression vector, implying that UV responsiveness is dependent upon the activation and stabilization of p53 (Figure 5A, lanes 5–6). The pivotal role of p53 in this response was confirmed by the finding that mutation of the p53 consensus site abrogated the UV-inducibility of the HPV77 promoter. Mutation of the p53 consensus sequence within the HPV77NCR–CAT construct also reduced basal HPV77 promoter activity (Figure 5A, lanes 9 and 10). To investigate this observation further, p53 levels in control and irradiated cells were examined by Western blot analysis 12 h after treatment. Protein extracts from untransfected cultures were also included in the analysis as a negative control. It is clear from the results shown in Figure 5C that the transfection methodology used to introduce reporter gene constructs into the cells led to a marked increase in p53 expression levels in the absence of UV irradiation, consistent with the earlier results of Renzing and Lane (1995). As expected, irradiation of cultures resulted in a further increase in p53 expression levels. These results strongly suggest that p53-mediated transactivation contributed to HPV77 promoter activity in the control mock-irradiated cultures. It is likely, therefore, that the 5-fold difference in reporter gene activation observed between control and irradiated cultures underestimates the extent of the p53-dependent increase in HPV77 promoter activity.

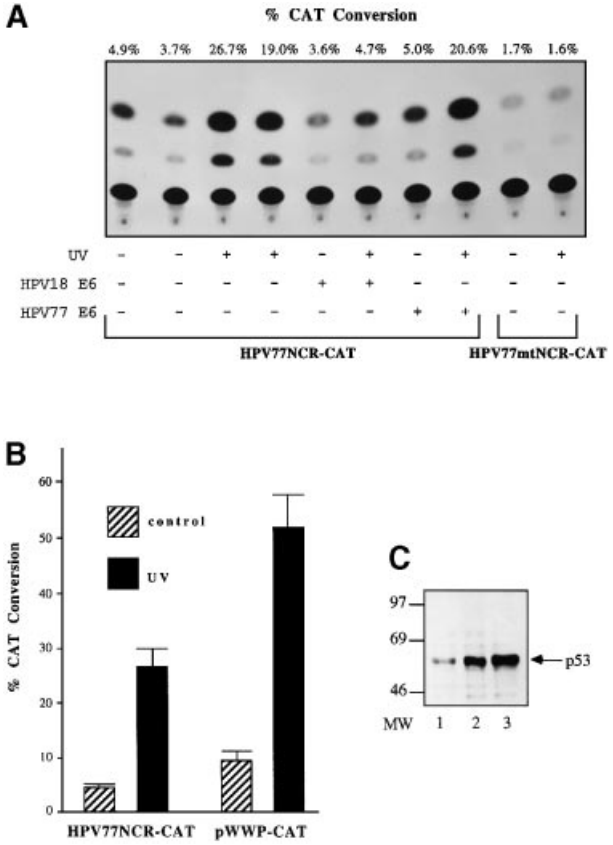


Fig. 5. (**A**) and (**B**) UV irradiation leads to an increase in HPV77 promoter activity that is dependent upon the presence of a wild-type p53 consensus sequence. Primary skin keratinocytes were transfected with CAT reporter and HPVE6 expression constructs as indicated. Where required, cultures were irradiated with 15 mJ/cm^2 of UVB light 24 h after the removal of transfection solutions. Cultures were harvested for analysis after a further 48 h. The data presented in the bar graph represent the mean of two separate experiments with error bars depicting the difference between the mean and the higher of the two values. (**C**) Western blot analysis of p53 expression in cultured primary skin keratinocytes after irradiation or mock irradiation. Protein extracts were prepared from cultured cells 12 h after treatment and separated by 10% SDS–PAGE. p53 protein levels were analysed by immunoblotting using the DO-1 antibody. Lane 1, untransfected cells; lane 2, control transiently transfected cells; lane 3, irradiated transiently transfected cells.

Functional consequences of p53 activation of HPV77 transcription

The activation of HPV77 gene transcription following UV-induced activation of p53 is likely to have significant effects upon the response of the infected cell to UV damage. We have shown recently that the HPV77 E7 protein shares certain biochemical properties with oncogenic anogenital HPV16, including the ability to co-transform primary cells in culture (J.Pennington, S.Clayson, E.-M.de Villiers, C.Proby, I.M.Leigh and A.Storey, manuscript submitted). We tested whether p53 was able to upregulate E7 expression directly in the p53-null keratinocyte cell line RTS3b (Rapp *et al*., 1997) from constructs containing wild-type or p53 binding site mutant HPV77 NCRs, p77NCRE6E7 and p77NCRmtE6E7, respectively. In these mini genomic constructs, the viral oncogenes are in their natural configuration with respect to the promoter/enhancer. Transfection of these cells followed by RT–PCR amplification of HPV77 E7 mRNA consistently showed that E7 was upregulated in response to p53 in cells containing the wild-type NCR construct (p77NCRE6E7), whilst E7 mRNA levels remained unchanged in cells containing the p53-binding-site-defective NCR construct p77NCRmtE6E7 (Figure 6). These experiments suggest that a direct increase in E7 as a result of p53 activation may contribute towards deregulated cell growth in early stages of the development of HPV-associated lesions.

In contrast to HPV18 E6, expression of the HPV77 E6 protein failed to abrogate the UV-induced stimulation of HPV77 promoter activity (Figure 5A, lanes 7 and 8). This

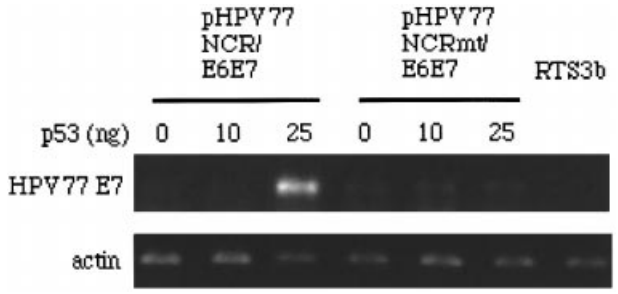


Fig. 6. Activation of HPV77 E7 expression by p53 is dependent upon the HPV77 p53 response element. RTS3b (p53-null) keratinocytes were transfected with either 1 μg pHPV77NCRE6E7 or the p53 binding site mutant pHPV77NCRmtE6E7 plasmid together with increasing amounts of a p53 expression plasmid (Storey *et al*., 1998). In these HPV plasmids, the E6 and E7 genes are in their natural configuration with respect to the NCR. Total RNA was harvested for analysis of E7 message by RT–PCR. E7 expression was only detected when p53 was co-transfected with the wild-type NCR construct.

result was in agreement with the data obtained using the heterologous promoter constructs. To explore this observation further, we investigated whether the HPV77 E6 protein shared the ability of the HPV16 and 18 E6 proteins to bind p53. In these experiments, ^{35}S-labelled *in vitro* translated p53 was mixed with equal amounts of either HPV77, HPV16 or HPV18 E6–glutathione *S*-transferase fusion (GST) proteins immobilized on glutathione-linked Sepharose resin. Experiments were performed at 4°C to minimize the degradation of p53. The HPV77 E6 protein retained ~4-fold less p53 than the HPV16 and 18 E6 proteins (Figure 7A). Nevertheless, the amount of p53 bound by HPV77 E6 was markedly higher than that associated with GST protein alone. These data were consistent with the recent results of Li and Coffino (1996) who showed that both high- and low-risk HPV E6 proteins bind to a site within the C-terminus of p53. These investigators also reported that the E6 proteins of HPVs 16, 18 and other types associated with anogenital cancer, but not low-risk HPV types, bind to a second site within the core structure of p53. Only the latter interaction was found to be involved in mediating the degradation of p53. To investigate whether HPV77 E6 was able to target p53 for degradation, *in vitro* translated HPV77 and HPV16 E6 proteins were incubated with *in vitro* translated p53 at 30°C. Negligible amounts of p53 remained after incubation for 2 h with HPV16 E6, whereas incubation with HPV77 E6 had no detectable effect on the level of p53 in this assay (Figure 7B).

To explore whether this was also the case for the HPV77 E6 protein under *in vivo* conditions, we next investigated the ability of the HPV77 E6 protein to degrade p53 in a cellular based assay. In this system, a p53 expression construct was co-transfected with an HPV77 or HPV16 E6 expression construct or an expression vector lacking an E6 insert into the p53-null osteosarcoma line Saos-2. After incubation for 36 h, the cells were fixed and p53 protein detected by immunocytochemistry using DO-1. The numbers of p53-positive cells were then counted and expressed as a proportion of the total number of cells. The percentage of p53-positive cells in cultures transfected with the HPV77 E6 expression construct was comparable to that observed in cultures transfected with the parent expression vector (Table I). These combined data clearly

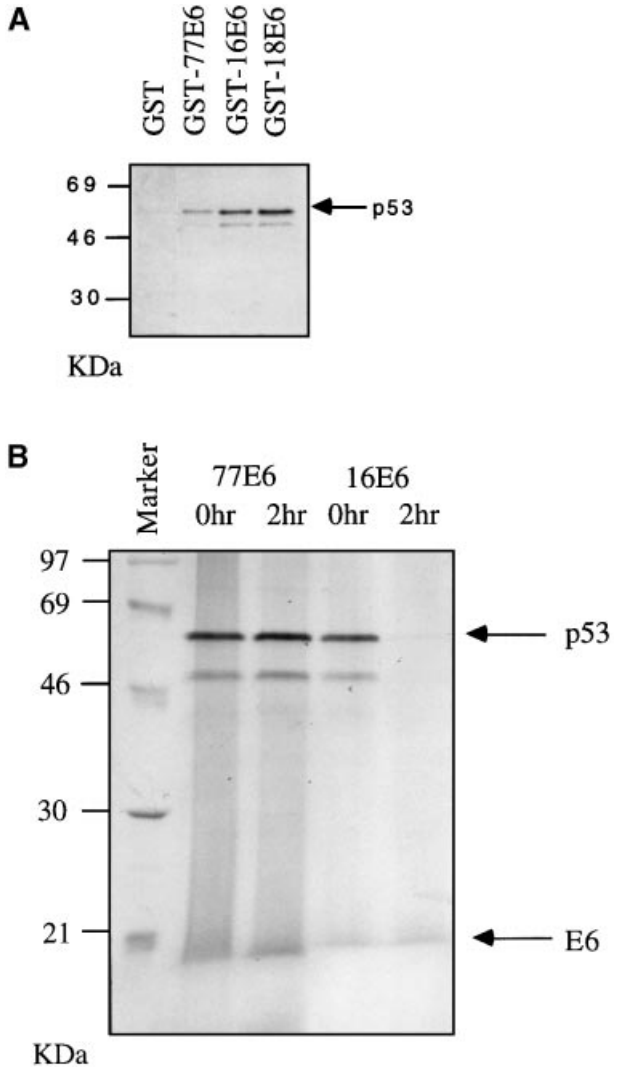


Fig. 7. The HPV77 E6 protein binds p53 but does not enhance its degradation. (**A**) Radiolabelled *in vitro* translated p53 was incubated with GST–E6 fusion proteins or GST alone immobilized on glutathione-linked Sepharose. Bound protein was eluted after 1 h and visualized by SDS–PAGE and autoradiography. Results were quantified by densitometry and expressed as a percentage of HPV18 E6 p53 binding. (**B**) Radiolabelled *in vitro* translated p53 and E6 proteins were incubated at 30°C with aliquots removed at the 0 and 2 h time points for analysis by SDS–PAGE and autoradiography.

Table I. HPV77 E6 does not enhance the degradation of p53 *in vivo*

Plasmid	Percentage positive cells
pRSVp53	7.1 (2.6)
pcDNA3	0 (0)
pRSVp53 + pcDNA3	6.5 (1.6)
pRSVp53 + pcDNA3 16E6	0 (0)
pRSVp53 + pcDNA3 77E6	7.3 (2.1)

P53-null Saos2 cells were seeded onto coverslips and co-transfected with pRSVp53 and either pcDNA3 control or the relevant pcDNA3E6 plasmid. Cells were fixed after 36 h and stained for p53 using the DO1 antibody. For each set of coverslips, p53-positive stained cells were counted in five separate fields chosen randomly from three slides and values expressed as a percentage of total cell numbers. The figure listed in this table represents the mean of the five values with standard deviations in parentheses.

show that the HPV77 E6 protein is unable to enhance the degradation of p53.

Discussion

P53 is the most frequently mutated gene in human cancers, indicating that the p53 protein plays a key role in the regulation of cellular proliferation. Experimental evidence suggests that this function is at least partly dependent upon the ability of p53 to bind DNA in a sequence-specific manner and activate gene transcription. The list of cellular genes known to be targets of p53-mediated transactivation is increasing steadily and includes a number of genes involved in the growth arrest and apoptosis pathways. In this study, we have identified a consensus p53 binding sequence in the regulatory region of HPV77. Although p53 binding sites have previously been identified within the genomes of the SV40 (Bargonetti *et al*., 1991), HIV (Gualberto and Baldwin, 1995) and recently hepatitis B (Ori *et al*., 1998) viruses, in all of these cases the binding of wild-type p53 to its recognition sequence led to the repression of viral transcription. Our finding that this HPV77 motif mediates a p53-dependent increase in HPV77 promoter activity following exposure to physiological UVB radiation is, therefore, the first example of a positive p53-dependent response element within a viral genome. We also show that this upregulation of E7 expression, which has a transforming potential similar to HPV16 E7, occurs within the context of the HPV77 genome. These data provide a fascinating example of the way in which this virus has adapted to exploit its host cellular environment, and in view of the association of this HPV type with skin cancer in the RTR population (Delius *et al*., 1998), suggest a possible mechanism by which HPV77 and UVR may act as cofactors in skin carcinogenesis.

The p53 binding site in the HPV77 NCR possesses several unique features that distinguish it from all of the consensus sequences associated with known cellular targets of p53. These include the absence of the conserved cytosine and guanine at positions 4 and 7, respectively, in the second decamer, as well as the fact that the first decamer is a perfect palindrome. An examination of the HPV77 NCR identified two further decamers located 12 bp upstream and 4 bp downstream of the two contiguous decamers of the El-Deiry consensus sequence. One of these additional decamers contained four errors and the other three errors and a base insertion between the C and G. Together with the two contiguous decamers in the core sequence, these HPV77 sequences fulfil the criteria for a functional p53-responsive element (Kern *et al*., 1992; Bourdon *et al*., 1997). In contrast, homologous sequences in the corresponding genomic regions of the closely related types 3, 10, 28 and 29 failed to meet these criteria and, when tested, equivalent sequences from HPV10 and 29 failed to bind p53.

Gel-retardation analysis revealed that *in vitro* translated p53 forms two distinct complexes with the *WAF1*, *bax* and HPV77 motifs. Moreover, the major species in the HPV77 DNA complex appeared to correspond to the faster migrating of the two *WAF1–bax* DNA complexes, whereas for both *WAF1–bax* consensus sequences, the slower migrating complex constituted the major species. Further analyses using hybrid oligonucleotides showed the identity of the base at position 4 of the second decamer to be crucial in determining the amount of DNA–protein complex formed. Whether these differently migrating complexes contain differentially modified forms of p53 remains an open question. Martinez *et al*. (1997) reported that the *WAF1* and *mdm2* p53 binding sites interact with distinct sets of wild-type p53-containing complexes in electrophoretic mobility shift assays. Here, we have developed a methodology for isolating the species of cellular p53 associated with the various consensus binding sequences, which should prove useful in resolving this question.

As outlined above, the composition of the HPV77 p53 consensus sequence differs from all of the other p53 binding sites identified to date. We were therefore interested in comparing the ability of these motifs to confer p53 responsiveness on a heterologous promoter. Our results show that p53 inducibility is 2- to 3-fold lower when the p53 consensus sequence is derived from the HPV77 NCR rather than the *bax* or *WAF1* gene promoters. These data could not be explained by differences in the amount of p53 bound by the various consensus sequences, since gel-retardation analysis revealed that the level of p53 binding was similar for the HPV77 and *bax* sequences. Furthermore, it was evident from a comparison of the *WAF1* and *bax* consensus sequences that the amount of *in vitro* translated p53 bound by a motif correlated poorly with its ability to confer p53 inducibility on a foreign promoter. In the context of the NCR, however, we found that the ability of the HPV77 motif to confer p53 responsiveness on its homologous promoter was comparable to that of the *WAF1* sequence. The discrepancy between the two sets of results implies that other elements in the HPV77 NCR might contribute to the stimulation of HPV77 promoter activity, although the essential role of the p53 consensus sequence was highlighted by the finding that mutation of this motif completely abrogated the response. It is also possible that the discrepancy might be due to the different composition of the p53 binding sites in the heterologous and homologous promoter constructs; in the former, these comprise two repeats of the 20 bp El-Deiry consensus sequence, whereas the HPV77 NCR and *WAF1* gene promoter both contain a single copy of the El-Deiry motif flanked by two additional decamers. In addition, it cannot be excluded that the HPV77 p53 consensus sequence may display some degree of promoter specificity that causes it to function suboptimally in the context of a foreign promoter. Such promoter specificity has been reported previously for several well characterized enhancer sequences including the octamer binding site (Scholer *et al*., 1989). The contrasting data may also be a result of major differences in the experimental protocols; the former set of experiments was performed in a p53-null osteosarcoma line transfected with a p53 expression vector, whereas the latter used primary skin keratinocytes that had been irradiated to increase the levels of endogenous p53. It seems likely, therefore, that the intracellular populations of p53 would have differed in the two experiments. In any event, our results clearly show that in the appropriate cell type, under physiological conditions, wild-type p53 activates the HPV77 and *WAF1* promoters with similar efficiencies.

DNA sequencing studies suggest that >50% of cutaneous SCCs contain point mutations in one or both p53 alleles (Brash *et al*., 1991). The HPV status of these lesions has

not been determined however. The question of whether p53 mutation constitutes an early or late event in malignant transformation is clearly crucial in determining the significance of the results obtained in the current investigation. Recently, Ren *et al.* (1997) observed that p53 mutations present in morphologically normal skin appeared to possess little or no pre-cancerous potential. Other investigators (Pelisson *et al*., 1994; Stark *et al*., 1994; Khorshid *et al*., 1996) have reported that few viral warts or dysplastic lesions from RTRs display a pattern of p53 immunoreactivity suggestive of gene mutation. These combined data imply that the acquisition of p53 mutations is a late event in RTR skin carcinogenesis and that consequently the p53 status of premalignant HPV77-positive lesions is likely to be wild type.

The discovery that p53 can activate transcription from the HPV77 promoter was somewhat unexpected in view of the well documented ability of the HPV16 and 18 E6 oncoproteins to target p53 for ubiquitin-mediated degradation (Scheffner *et al*., 1990). Indeed, the correlation between the oncogenic potential of anogenital HPVs and the ability of their E6 proteins to inactivate p53 suggests that this constitutes a major function of E6 in transformation. Preliminary results obtained in this laboratory using rodent cell assays indicate that the transforming activity of the HPV77 E6 protein is comparable to that of HPV18 E6 (J.Pennington and A.Storey, personal observations). These combined data would seem to question the relevance of p53-mediated transactivation of the HPV77 promoter. In the current study, however, we have shown that HPV77 E6, like the E6 proteins of high-risk EV-specific HPV types 5 and 8, cannot target p53 for degradation in either *in vitro* or *in vivo* systems. Moreover, our results clearly demonstrate that, irrespective of the recognition sequence, expression of the HPV77 E6 protein fails to abrogate p53-mediated transactivation. This finding raises further questions about the relevance of the p53 consensus sequence in the HPV77 NCR, since it would seem to imply that any p53-dependent increase in HPV77 promoter activity would be accompanied by the transactivation of cellular targets of p53 such as *p21*/*WAF1* and *bax*, whose products are known to promote cell cycle arrest and apoptosis, respectively. In any event, it is not inconceivable that HPV77 proteins might act downstream of p53 to block apoptosis or cell cycle arrest. For example, we have recently demonstrated that the HPV77 E7 protein has transforming potential and it is possible that it may also share the ability of HPV16 E7 to bind p21 and inhibit its activity (Funk *et al*., 1997). Certainly, the fact that the EV-specific HPVs 5 and 8 are strongly associated with skin cancer, despite the inability of their E6 proteins to target p53 for degradation, points to the existence of another as yet unidentified mechanism by which HPVs may bypass the tumour suppressor functions of p53.

In conclusion, we have identified HPV77 as a novel transcriptional target of p53, a finding that offers a likely explanation for the localization of HPV77-associated lesions at sun-exposed sites. The detection of HPV77 DNAs in lesions of varying degrees of dysplasia, combined with our results demonstrating increased expression of the HPV77 E7 oncogene by activated p53, suggests a possible mechanism whereby HPV77 and UVR might act as cofactors in skin carcinogenesis, which may involve both a direct transforming property of virally encoded proteins combined with the mutagenic effects of UV.

Materials and methods

Oligonucleotides

The sequences of the oligonucleotides used to generate the heterologous reporter gene constructs were as follows: HPV77(2) sense, 5′-AGCTTAAACATGTTTGCAAATCCCCA AACATGTTTGCAAATCCCCCCC-3′; antisense, 5′-GGGGGGGATTTGCAAACATGTTTGGGGATTTGCAAACATGTTTA-3′; WAF(2) sense, 5′-AGCTTGAACATGTCCCAACAT GTTGGAACATGTCCCAACATGTTGCC-3′; antisense, 5′-GGGCAACATGTTGGGACAT GTTCCAACATGTTGGGACATGTTCA-3′; bax(2) sense, 5′-AGCTTAGACAAGCCTGG GCGTGGGCAGACAAGCCTGGGCGTGGGCCCC-3′; antisense, 5′GGGGCCCACGCC CAGGCTTGTCTGCCCACGCCCAGGCTTGTCTA-3′. The equivalent HPV10 and 29 NCR sequences to those of the HPV77 p53 motif were taken from Figure 1A. The sequences of the oligonucleotides used for gel mobility shift and streptavidin-coupled Sepharose binding assays are as follows: HPV77 wild type, 5′-AAACATGTTTGCAAATCCCC-3′; HPV77 mutants, 5′-AAACATTAATGCAAATTAAC-3′, 5′-AAACATGTTTGCACATCCCC-3′ and 5′-AAACATGTTTGCAAATGCCC-3′; *WAF1* wild type, 5′-GAACATGTCCCAACATGTTG-3′; *WAF1* mutants, 5′-GAACATGTCCCAAAATGTTG-3′ and 5′-GAACATGTCCCAACATCTTG-3′; HPV77/*WAF1* hybrid, 5′-AAACATGTTTCAACATGTTG-3′; *WAF1*/HPV77 hybrid, 5′-GAACATGTCCGCAAATCCCC-3′; *bax*, 5′-AGACAAGCCTGGGCGTGGGC-3′. Where required, oligonucleotides were biotinylated at the 5′ end. Mutagenesis was performed on the pHPV77NCRE6E7 plasmid using the p53 binding site mutant oligos described above using Pfu polymerase (Stratagene).

Plasmids

The pBKCMV–HPV77/4 construct containing the HPV77 NCR and the E6, E7 and L1 ORFs, and used as a source of HPV77 DNAs for DNA sequencing and cloning, is described elsewhere (Delius *et al*., 1998). The HPV77(2)CAT heterologous promoter construct contains two copies of the p53 consensus sequence from the HPV77 NCR, and was based on the pG13CAT plasmid (Kern *et al*., 1992) that contains copies of the RGC p53 consensus decamer cloned upstream of the polyoma virus early promoter and CAT gene. First, pG13CAT was digested with *Hin*dIII and *Sma*I to remove the RGC p53 motifs. The HPV77(2) sense and antisense oligonucleotides were then annealed in equimolar quantities to generate a double-stranded oligonucleotide with *Hin*dIII–*Sma*I sticky ends, which was cloned into the vector. The WAF(2) and bax(2) sense and antisense oligonucleotides were used to generate the WAF(2)CAT and bax(2)CAT constructs in a similar fashion. To obtain the HPV77NCR–CAT construct used for reporter gene analysis, a 0.8 kb fragment encompassing the entire HPV77 NCR and ~100 bp of the L1 ORF was first obtained by PCR amplification of pBKCMV–HPV77/4 plasmid DNA using the following HPV77 primers: forward, 5′-CGGGATCCTTTATTACAGATTGGTG-3′; reverse, 5′-CGCTCGAGCCATCACTTGTAGA CAT-3′. This fragment was cloned into the *Bam*HI and *Xho*I sites of pBLCAT3Δ, a promoterless CAT plasmid generated from the previously described pBLCAT3 construct (Luckow and Schutz, 1987), by deleting a 424 bp *Hin*dIII–*Aat*II fragment adjacent to the multiple cloning site. This modification was necessary because the deleted vector sequences had been found to confer UV inducibility (K.Purdie, personal observations). The HPV77 p53 recognition sequence 5′-AAACATGTTTGCAAATCCCC-3′ in HPV77NCR–CAT was mutated to 5′-AAACATTAATGCAA ATTAAC-3′ to generate the HPV77mtNCR–CAT construct.

The pWWP-CAT construct contains the 2.4 kb *WAF1* promoter region cloned upstream of the CAT reporter gene as described previously (El-Deiry *et al*., 1993, 1995). The construct used for *in vitro* translation of p53, SP64 p53, has been described previously (Thomas *et al*., 1995). For *in vitro* translation of HPV77 and 16 E6 proteins, the HPV77 and 16 E6 ORFs were cloned into plasmid SP64. The pcDNA3–p53 and pRSVp53 plasmids used for the eukaryotic expression of p53 have been described previously (Pim *et al*., 1997). For eukaryotic expression of HPV E6 proteins, the E6 ORFs of HPVs 77, 16 and 18 were cloned into plasmid pcDNA3 (Invitrogen). To generate the GST–E6 fusion constructs, E6 ORFs were amplified by PCR and cloned into the *Bam*HI and *Eco*RI sites of the pGex 2T vector (Pharmacia).

The pHPV77NCRE6E7 plasmid was generated by amplifying a fragment of the pBKCMV–HPV77/4 construct using the forward NCR primer and a reverse primer located at the 3′ end of the E7 gene, CACGAATTCTTACGCACAGCGAGGACACAC. This fragment was cloned between the *Bam*HI and *Eco*RI sites of a modifed pcDNA3 plasmid in which the CMV promoter had been deleted by digesting the plasmid with *Nru*I and *Bam*HI and end-filling, followed by religation.

Cell culture

Primary keratinocyte cultures were established as outlined previously (Rheinweld and Green, 1975) from normal adult skin discarded after abdominal reduction surgery. Cultures of primary and RTS3b keratinocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25% Ham's F12 medium, 10% fetal calf serum (FCS) and various mitogens as previously described (Navsaria *et al*., 1995). Established osteosarcoma cell lines Saos-2 and HT1080 were maintained in DMEM supplemented with 10% FCS. Where UV irradiation was required, cultures were first washed twice in phosphate-buffered saline (PBS) and aspirated. Dishes were then irradiated with a dose of 15 mJ/cm^2 in an ultraviolet cross-linker (UVP products) containing lamps emitting predominantly UVB light (280–320 nm), according to the manufacturer's instructions, and culture medium then replaced.

RT–PCR

Total RNA was isolated using RNazol (Biogenesis) and 1 µg was used in the RT–PCR reaction with the GeneAmp kit (Perkin Elmer) following the manufacturer's guidelines. E7-specific message was amplified using the same reverse primer used for cloning; the forward primer was CACGGATCCATGCATGGGCCAAAGCCGACA.

Gel mobility shift assay

The p53 protein was translated *in vitro* using the TNT coupled reticulocyte lysate system (Promega) in accordance with the manufacturer's instructions, with the modification that no radiolabel was used. For each reaction, 4 µl of p53- or water-primed reticulocyte lysate were diluted in 20 µl of DNA binding buffer (final concentrations: 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% NP-40, 10% glycerol) containing 1 ng ^{32}P-end-labelled double-stranded oligonucleotide, 1 µg salmon sperm DNA as a non-specific competitor and 5 mM dithiothreitol (DTT). Reactions were incubated for 10 min at ambient temperature, at which point 1 µl of anti-p53 monoclonal antibody pAb1801, DO-1 or pAb421 (Wade-Evans and Jenkins, 1985; Banks *et al*., 1986; Vojtesek *et al*., 1992) was added and reactions incubated for a further 30 min. Where required, reactions were performed in the presence of a 30- or 50-fold excess of unlabelled competitor oligonucleotide. Reaction products were separated by 4% non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Streptavidin-coupled Sepharose binding assay and Western blot analysis

For each reaction, 1 µg of biotinylated double-stranded oligonucleotide was incubated with 25 µl of a 10% suspension of streptavidin-conjugated Sepharose beads (Zymed, CA) in TE–NaCl (TE pH 7.5, 1 M NaCl) for 30 min at ambient temperature. Next, the beads were washed twice in 200 µl TE–NaCl and twice in 200 µl DNA binding buffer (25 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM $ZnCl_2$, 2 mM DTT, 0.1% NP-40 and 10% glycerol). The beads were then incubated at ambient temperature with 100 µg of protein extract prepared as described previously (Thomas *et al*., 1996) from cultured cells 16 h after UV treatment and diluted in DNA binding buffer to a total volume of 200 µl. After 30 min, beads were washed three times with 200 µl DNA binding buffer and resuspended in SDS–PAGE sample buffer. Protein was eluted from the beads by boiling, separated by SDS–PAGE and results visualized by Western blot analysis. Western blot analysis of p53 expression was performed as described previously (Thomas *et al*., 1996), with the modification that blots were probed with DO-1.

Transfections and CAT assays

Transfections of established cell lines were performed using standard calcium phosphate precipitation methodology. Primary keratinocyte cultures were transfected using lipofectin (Gibco-BRL) according to the manufacturer's instructions. Typically, each 60 mm tissue culture dish was transfected with 2 µg of CAT construct. Where applicable, cultures were co-transfected with 100 ng of pcDNA3 p53 and/or 300 ng of pcDNA3 18E6 or pcDNA3 77E6. The total amount of plasmid DNA was kept constant in co-transfections by the addition of an appropriate quantity of pcDNA3 control plasmid. Cells were harvested 48–72 h after transfection and CAT assays performed as described previously (Thomas *et al*., 1995). Results were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics).

p53 binding and degradation assays

GST fusion proteins were expressed and binding assays performed with *in vitro* translated radiolabelled p53 as described previously (Thomas *et al*., 1995), except that proteins were bound to glutathione-linked Sepharose beads (Pharmacia Biotech). For *in vitro* degradation assays, p53 and E6 proteins were translated *in vitro* using the TNT system (Promega) in the presence of [^{35}S]cysteine. Protein levels were then quantified by SDS–PAGE, stained in Coomassie Brilliant Blue and equal amounts of each E6 protein incubated with p53 at 30°C. Aliquots were removed at the 0 and 2 h time points, and the extent of degradation determined by SDS–PAGE and autoradiography.

In the *in vivo* degradation assay, p53-null Saos2 cells were seeded onto coverslips and co-transfected with 100 ng of pRSVp53 and 300 ng of pcDNA3E6 plasmid or pcDNA3 vector control. The cells were fixed after 36 h in methanol:acetone (1:1) and stained for p53 using DO-1 antibody. Briefly, coverslips were incubated in DO-1 hybridoma supernatant for 1 h at room temperature, washed three times in PBS, and incubated for 35 min in biotin-conjugated rabbit anti-mouse immunoglobulin (Dako), diluted 1:1000 in DMEM, 10% FCS. After three washes in PBS as before, the coverslips were incubated for a further 35 min in streptavidin-conjugated horseradish peroxidase (Dako). Permanent staining was carried out using 3,3′-diaminobenzidine in 0.3% nickel chloride as the chromogen (Bartek *et al*., 1991). For each E6 protein investigated, p53-positive stained cells were counted and expressed as a proportion of total cell numbers in five random fields chosen from three coverslips.

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