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VIROLOGY
Virology 308 (2003) 279–290
www.elsevier.com/locate/yviro

Dual role of tumor suppressor p53 in regulation of DNA replication and oncogene E6-promoter activity of *Epidermodysplasia verruciformis*-associated human papillomavirus type 8

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Received 26 July 2002; returned to author for revision 4 September 2002; accepted 28 November 2002

Abstract

Human papillomavirus 8 (HPV8) is a representative of *Epidermodysplasia verruciformis* (EV)-associated viruses. Transient assays in the human skin keratinocyte cell line RTS3b have shown that its replication depends *in trans* on expression of the viral proteins E1 and E2, similarly to other HPVs. Using deletion mutants and cloned subfragments of the noncoding region (NCR) of HPV8 we identified a 65-bp sequence in the 3′ part of the NCR to be necessary and sufficient to support replication *in cis.* The origin of replication (ori) of HPV8 is composed of the sequence motifs "CCAAC" (nt 57–73) and M29 (nt 84–112), which are highly conserved among the majority of EV HPVs. Analysis of M29 revealed an unconventional binding site of the E2 protein and an overlapping DNA recognition site of the tumor suppressor protein p53. Both these factors competitively bind to M29. In transient replication assays p53 acted as a potent inhibitor of ori activity, most probably in a DNA-binding-dependent fashion. The minimal ori sequences are also functionally critical for the E6 oncogene promoter P_{175}. In contrast to its effect on replication, p53 stimulated promoter activity depending on its interaction with M29. Our observations suggest that p53 is involved in controlling the balance between DNA replication and gene expression of HPV8.
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Keywords: HPV; *Epidermodysplasia verruciformis*; Origin of DNA replication; Tumor suppressor p53; HPV8-E2 protein

Introduction

Human papillomaviruses (HPVs) are ubiquitously occurring small, unenveloped, double-stranded DNA viruses responsible for induction of a broad spectrum of tumors of the skin and mucosa in man. More than 100 HPV types have been characterized and many additional types are suspected to exist (Howley and Lowy, 2001). The most characteristic feature of HPVs is their specific tropism for stratified epithelia. HPV particles gain access to proliferating basal keratinocytes, probably through microwounds, and can thereafter establish a persistent infection of the epithelium. The complete virus life cycle is strictly bound to differentiation of the cells within the stratified epithelium. This phenomenon results in a programmed expression of the early and late viral proteins.

The synthesis of viral DNA can take place in form of either a maintenance replication, characterized by establishment of episomal viral genomes at low copy numbers in the basal stratum of the epithelium, or as extensive, vegetative DNA replication in the differentiating keratinocytes (Chow and Broker, 1994). There is evidence that these separate phases employ two different molecular replication mechanisms: bidirectional replication via θ-DNA structures and the rolling circle modus, respectively (Flores and Lambert, 1997; Yang and Botchan, 1990).

Due to the lack of a generally applicable in vivo DNA replication system for HPVs, most of the data concerning the involved mechanisms originate thus far from transient replication assays with immortalized cells (Ustav and Stenlund, 1991). According to these, replication of the viral DNA is initiated at the origin of DNA replication (ori)

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doi:10.1016/S0042-6822(02)00133-2

element within the noncoding region (NCR) of the virus and depends *in trans* primarily on its physical interactions with the two viral proteins E1 and E2 (Chow and Broker, 1994; Lambert, 1991; Stenlund, 1996). E1 is a sequence-specific DNA-binding, replication-initiating factor with properties of an ATPase, helicase, and DNA unwinding protein (Sedman and Stenlund, 1998). The postulated role of E2 is the recruitment of E1 to the ori locus via its ability to form complexes with E1 and due to high-affinity E2-binding sites located within all known HPV ori elements (Berg and Stenlund, 1997; Bonne-Andrea et al., 1997). Both these factors collaborate in an assembly of a functional preinitiation complex with host proteins, which constitute the actual replication machinery.

Data on the structure of ori elements in papillomaviruses and on factors involved in their activity are to date almost exclusively limited to bovine papillomavirus 1 (BPV 1) and the mucosa-specific types HPV11 (Lu et al., 1993) and HPV18 (Lee et al., 1997; Sverdrup and Khan, 1995). In contrast little attention has been paid in this regard to cutaneous HPVs except for HPV1 (Gopalakrishnan and Khan, 1994). Epidemiological studies of the last years have shown an overwhelming majority of skin-specific HPVs to belong to the group of so-called EV HPVs. The prototypes of these viruses have been isolated from lesions of patients suffering from the rare skin cancer syndrome *Epidermodysplasia verruciformis* (EV). There are strong indications that several of these HPV types are causally involved in the development of skin neoplasia. This is supported by the fact that in contrast to the plurality of EV-associated HPVs in benign tumors, only a few virus types, predominantly HPV5 and HPV8, could be detected in nearly all EV cancers and that their E6 proteins possess cell-transforming potential in vitro (Iftner et al., 1988). Whereas the oncoprotein E6 of HPV16 and HPV18 interacts with and leads to degradation of the tumor suppressor protein p53 (Scheffner et al., 1990), no such property could be observed for the corresponding proteins of the EV viruses (Steger and Pfister, 1992).

Based on DNA sequence data, EV HPVs form a separate group of phylogenetically related papillomaviruses (Myers et al., 1997). EV-HPVs are distinguished by a peculiar organization of their NCRs, which among other regulatory sequences also contain the ori element. The NCRs of all EV-associated HPVs are relatively shorter and the majority display a pattern of characteristic, highly conserved sequence motifs, not recognizable in other papillomaviruses (Ensser and Pfister, 1990; Fuchs and Pfister, 1997; Krubke et al., 1987). All these elements are heavily involved in interactions with cellular proteins, some of which could be proven to play an important role in the activity of the viral promoters (May et al., 1991, 1994; Pajunk et al., 1997).

In this report we describe identification and characterization of the ori sequence of HPV8, a typical representative of the oncogenic EV HPVs, and discuss the importance of interactions with p53 in the regulation of viral replication and gene expression.

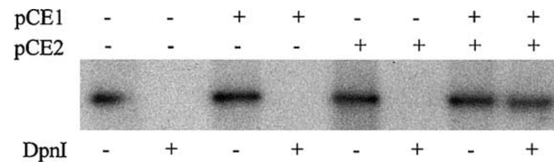

Fig. 1. HPV8-E1 and -E2 are necessary *in trans* for DNA replication. pNCR8-CAT (3.5 μg) was transfected either alone or together with 2 μg of either pCE1 or pCE2 expression vector or with 2 μg of each expression vector. Low-molecular-weight DNA was isolated and linearized with *Bam*HI. Half of each sample was then digested with *Dpn*I (+ or −). The preparations were finally analyzed by Southern blot hybridization as described in the text.

Results

Mapping of the minimal ori element of HPV8

We initially confirmed that similarly to other papillomaviruses studied in this regard, the ability of HPV8 DNA to replicate transiently in vitro depended *in cis* on sequences within the viral NCR and *in trans* on the expression of the viral E1 and E2 proteins. To this end we cotransfected the E1 and E2 expression vectors pCE1 and pCE2 either alone or together with the replication test plasmid pNCR8-CAT, which contains the NCR region (nt 7077–558) of HPV8 (Stubenrauch et al., 1992). As shown in Fig. 1, transfection of this test plasmid alone or with either of the expression vectors did not give rise to detectable replication, whereas transfection of all three DNAs together resulted in replication. Analysis of 5′-deletion mutants of the HPV8 NCR, pNCR8CAT-7642, -7535, -7422, and -112 (Stubenrauch et al., 1992) for their replicational activity revealed that the ori-containing region resides between positions 7642 and 112 within the E6 ORF proximal part of the NCR (data not shown). In contrast, no replication could be observed for plasmid pNCR8-5′-Luc, which contained a part of the L1 ORF and the L1 proximal NCR sequences (nt 7077–9). The ori fragment of the HPV8 genome thus defined displays four sequence motifs, highly conserved among the majority of known EV-associated and genetically related HPVs. These include two binding sites of the viral E2 protein (P3 and P4) as well as the two unique motifs "CCAAC" (nt 57–73) and M29 (nt 84–112) originally defined as conserved elements by sequence alignment of the HPV8, 19, and 25 NCR regions (Krubke et al., 1987). In order to dissect the HPV8 ori region functionally a series of subfragments has been generated by PCR (Fig. 2A), cloned into the vector pUC8 (plasmids p81–p88), and subjected to transient replication tests. As shown in Fig. 2B the 65-bp NCR fragment in the test plasmid p88, encompassing the CCAAC and M29 motifs (nt 53–117), proved to be necessary and sufficient *in cis* to drive DNA replication of the test plasmids used and can therefore be regarded as the minimal ori element of HPV8. Furthermore, comparison of the test constructs p85 through p88 clearly shows that both CCAAC and M29 are equally essential in mediating ori activity.

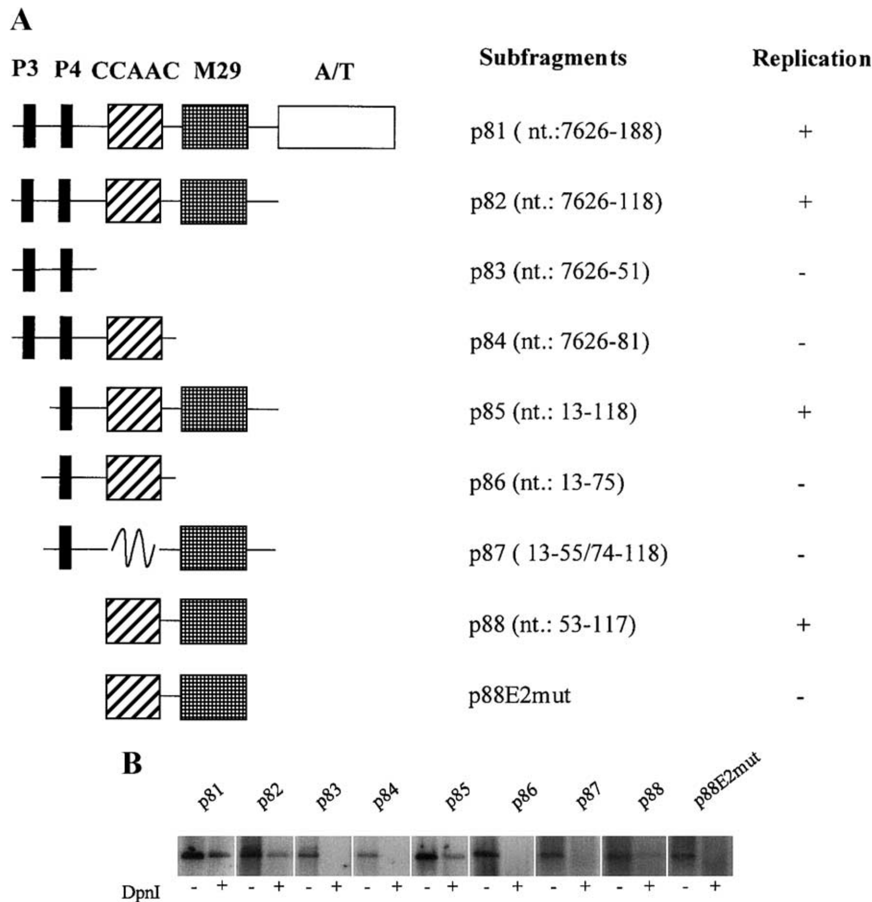

Fig. 2. Mapping of the HPV8 origin of DNA replication (ori). (A) The NCR subfragments of HPV8 which were cloned into pUC8 (p81–p88) and used in transient replication assays to map the minimal ori. P3, P4, E2-binding site; CCAAC motif, M29, A/T, conserved sequence elements of EV HPVs, HPV8 nucleotides 57–73, 84–112, 84–112, respectively. (B) Functional mapping of the HPV8 ori. Transient replication analysis of the various plasmids in RTS3b cells. Low-molecular-weight DNA was isolated and linearized with *Bam*HI. Half of each sample was then digested with *Dpn*I (+ or −). The preparations were finally analyzed by Southern blot hybridization.

The minimal ori element of HPV8 contains a noncanonical E2-binding site

HPV8 NCR and L1 ORF contain five known binding sites of the viral transcriptional regulator/replication protein E2 (P0–P4; Ensser and Pfister, 1990). To test for functional dependence of the HPV8 ori element on interactions of E2 with these sites we analyzed in transient replication assays a number of NCR mutants, disabled in binding of E2 protein to these target sequences. All mutated constructs tested in this regard turned out to replicate at rates directly comparable to those of the wild-type NCR. Surprisingly, this also held true for the plasmid P01234, which contained all five known E2-binding sites (E2BS) in a functionally inactive form (data not shown). Inspection of the minimal ori sequence, however, disclosed a palindromic motif, 5′-ATCGN$_4$CGAT-3′, within the M29 element which overall resembles the general E2 consensus binding site 5′-ACCGN$_4$CGGT-3′ (Androphy et al., 1987; Fig. 3A). In order to determine the capacity of E2 to physically interact with this motif we performed gel retardation tests using the oligonucleotide M29E2, which spans the sequence in question within the M29 element and purified HPV8-E2 protein, synthesized in a baculovirus expression system. As can be seen in Fig. 3, M29E2 was able to generate a complex with the E2 protein (Fig. 3B, lane 2), migrating in parallel to the one formed by the oligonucleotide P2C, which carries a well-characterized, high-affinity E2-binding site (Fig. 3B, lane 8). The DNA–E2 complex did not appear when the binding reaction was run in the presence of an excess of the nonlabeled target oligos (Fig. 3B, lanes 3 and 4). In contrast, however, both heterologous oligonucleotide SP1 and a mutated form of M29E2 (M29E2mut) which contains two nucleotide exchanges within the conserved left flank of the putative E2-binding palindrome failed to compete in an interaction of M29E2 with the E2 protein. Since the latter findings argue for a sequence-specific DNA–protein interaction it may be assumed that the M29 element contains an aberrant, E2-binding site. Consequent to the previous nomenclature we denote it further as P5. To prove that this new E2-binding site is indeed involved in DNA replication we introduced the two nucleotide exchanges as described in Fig. 3A into the ori E2-binding site in the plasmid p88 (p88E2mut). In replication tests we dem-

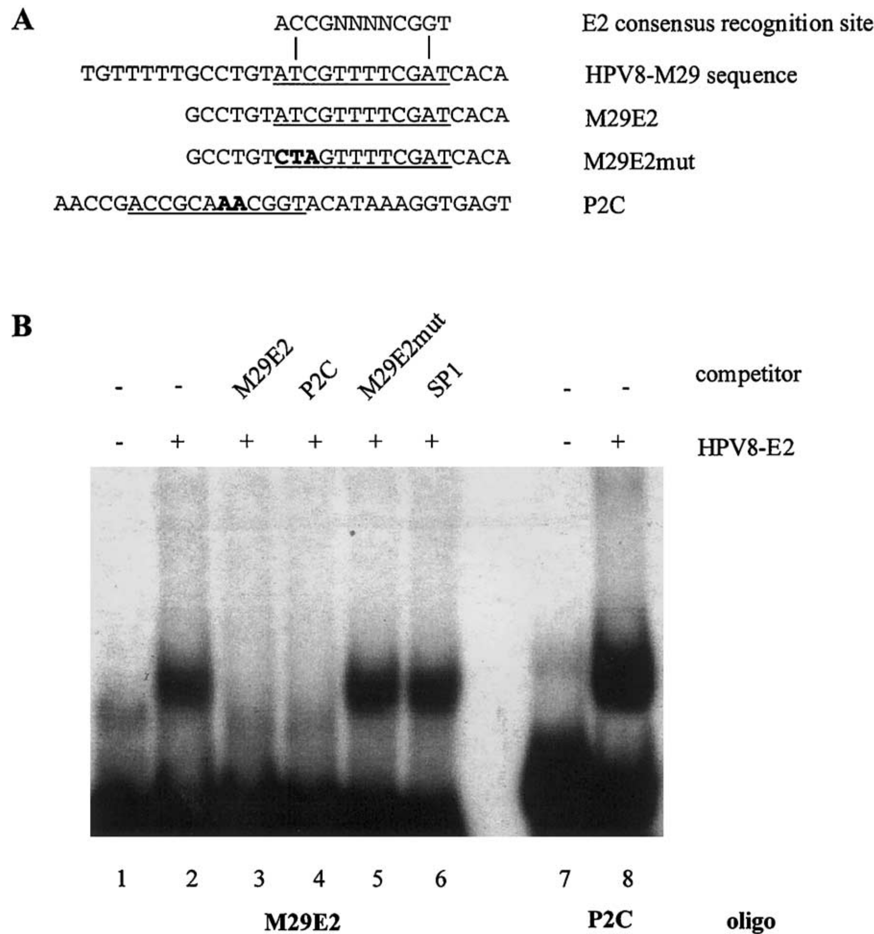

Fig. 3. Analysis of HPV8-E2 binding to the M29 sequence element. (A) Nucleotide sequences of the E2 consensus recognition site, the HPV8-M29 motif, and oligonucleotides M29E2, M29E2mut, and P2C. The mutated nucleotides in M29E2mut and P2C are highlighted in bold letters. The oligonucleotide P2C contains the exchange CC→AA in the HPV8-E2-binding site P2 and has a higher affinity to E2 compared with the wild-type sequence (Stubenrauch et al., 1994). (B) E2 is able to form DNA–protein complexes with the M29 element. Binding was analyzed by adding E2 protein to aliquots of ^{32}P-labeled M29E2 or P2C oligonucleotides. For competition unlabeled oligonucleotides that represent individual E2-binding sites (M29E2, P2C) were added in 100-fold excess. Unlabeled SP1 and M29E2mut oligonucleotides were used as nonspecific competitors. Control reaction mixtures received no protein.

onstrated that the plasmid p88E2mut was no longer able to replicate transiently (Fig. 2). This shows the importance of the cryptic E2 site for ori activity.

Tumor suppressor protein p53 binds to the ori element of HPV8

In the course of computer-assisted analysis to identify cellular factors interacting with regulatory sequences within the ori fragment we became aware that the M29 element and its immediate 5′ vicinity display similarity to a potential p53-binding site, as defined by El-Deiry et al. (1992). In comparison to the p53 consensus binding site which consists of two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by a 0- to 13-bp spacer, the two decamers of the p53-binding site in HPV8 are separated by 14 bp. The p53-binding site in M29 differs from the consensus sequence in the flanking sequences, where there are pyrimidine bases in the conserved purin regions. Interestingly, this site overlaps with the newly defined E2-binding site P5. To verify this observation we tested for direct interaction of p53 protein with the oligonucleotide M29com by means of EMSA (Fig. 4B) with in vitro translated p53. The reactions were performed in the presence of pAb421, an antibody previously shown to interact with the C-terminus of p53 and to activate sequence-specific DNA binding (Hupp et al., 1992). As shown in Fig. 4B a specific complex between p53 and the M29com oligonucleotide was observed only when the antibody pAb421 was present. Competition experiments revealed that the formation of the p53–M29com complex was prevented by the presence of excess unlabeled competitors such as the M29 oligonucleotide or $p53^{+}$ control oligonucleotide, which contains a consensus p53-binding site. In contrast, using the heterologous SP1 or p53-control oligonucleotides the p53–M29 DNA complexes could still be seen. These data clearly show that the ori element of HPV8 is involved in direct physical interaction with the cell cycle regulator protein p53.

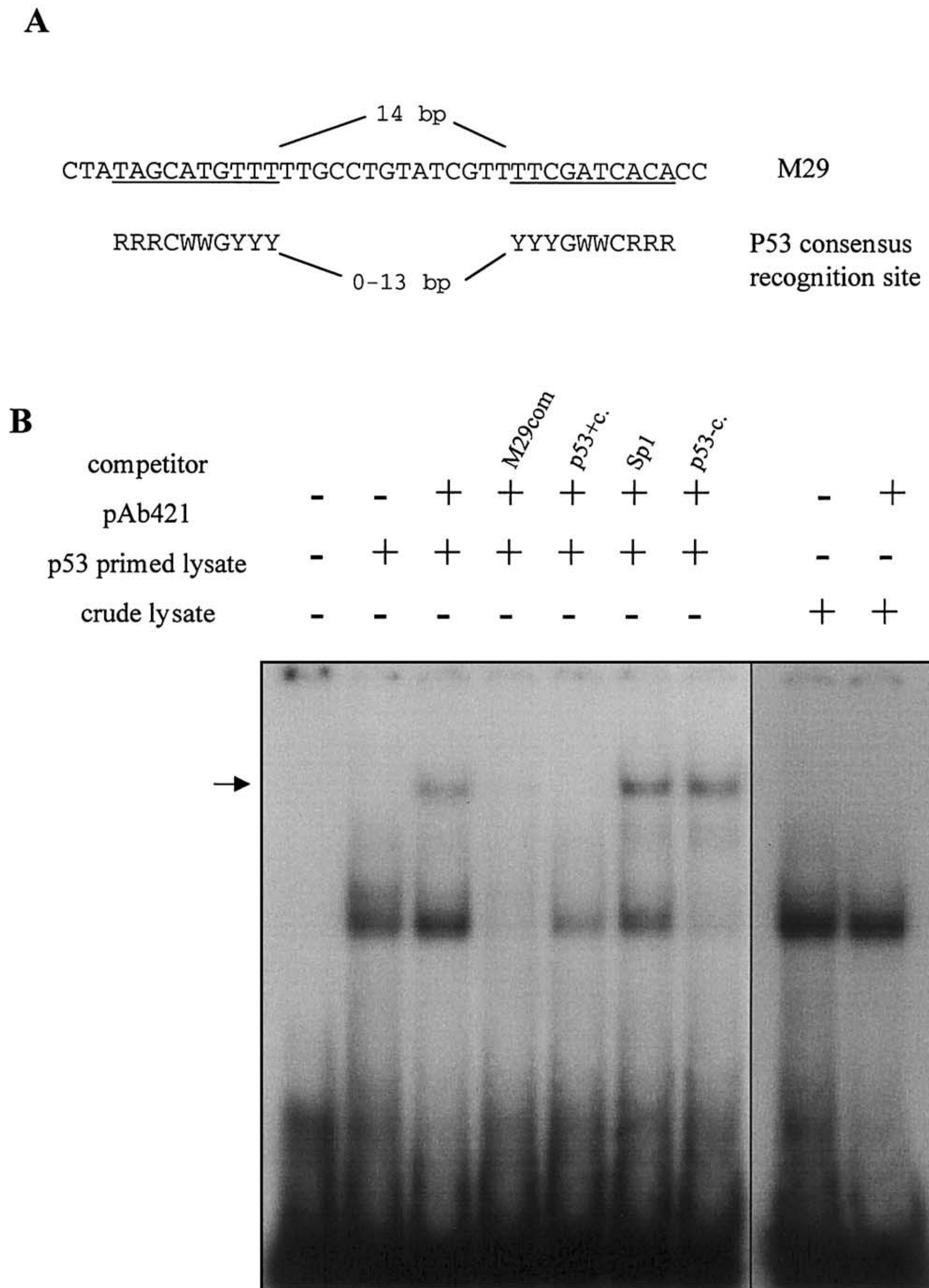

Fig. 4. In vitro translated p53 binds to the HPV8 M29 sequence element. (A) The M29 sequence (top) compared with the p53-binding site consensus sequence. Comparison of the putative p53-binding site in the HPV8 sequence motif M29 with the p53 consensus binding site, consisting of two sequence decamers, which are separated by 0–13 nucleotides. Bases in M29 matching the consensus sequence are underlined. (B) ^{32}P-end-labeled double stranded oligonucleotides corresponding to the M29 sequence were incubated with in vitro translated p53 in the presence of the anti-p53 antibody pAb421. DNA–protein complexes were separated by nondenaturing polyacrylamide gel electrophoresis. Where indicated, experiments were performed in the presence of the unlabeled oligonucleotides M29com and p53+c as specific and p53−c as unspecific competitors. The positions of the p53-specific bands are indicated by the arrow.

p53 inhibits HPV8 DNA replication

Because of the fact that p53 binds to the M29 motif, which is an essential element of the origin, we were interested to see whether p53 influences the DNA replication of HPV8. Cotransfection of human p53 wt expression vector pCp53wt suppressed the replication of the HPV8 replication test plasmid p85 (Figs. 2A and 5). In contrast, cotransfection with the expression vector $pCp53_{175}$, encoding for a p53 mutant protein at amino acid 175, which is not able to bind to DNA, did not inhibit replication (Fig. 5). We convinced ourselves that p53 inhibition of replication is not due to

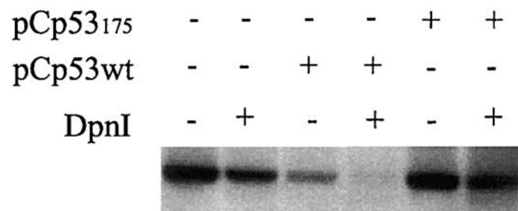

Fig. 5. Wild-type p53 suppresses HPV8 DNA replication. In this assay p53 expression vectors pCp53wt and $pCp53_{175}$ were additionally transfected in transient replication assays. Overexpression of p53wt inhibits HPV8 DNA replication, whereas the p53 mutant 175, which is no longer able to bind to DNA (Nigro et al., 1989) has no effect. Low-molecular-weight DNA was isolated and linearized with *Bam*HI. Half of each sample was then digested with *Dpn*I (+ or −). The preparations were finally analyzed by Southern blot hybridization.

pleiotropic, nonspecific effects following induction of apoptosis. Flow cytometric analysis of annexin-labeled cells gave no evidence of apoptosis up to 72 h post-transfection of pCp53wt (data not shown).

Interaction between HPV8-E2 and p53

It has recently been demonstrated that E2 protein of the high-risk genital HPV type 16 can directly bind p53 (Massimi et al., 1999). In order to test if this might also be the case for HPV8-E2 we expressed HPV8-E1 and -E2 as GST fusion proteins in *Escherichia coli* (Enzenauer et al., 1998) and investigated their ability to bind in vitro translated p53 by a series of GST pulldown assays. The results obtained are shown in Fig. 6. It is clear that E2 retains a significant portion of the input p53 protein, in contrast with no binding to GST-E1 and the GST alone.

HPV8-E2 displaces p53 from its binding site within the ori element

In view of the fact that the binding sites of E2 and p53 proteins completely overlap we asked if these two proteins can bind simultaneously to their overlapping target sequences. To answer this question band-shift tests were performed with constant concentrations of p53 and increasing amounts of E2. As can be seen in Fig. 7, high concentrations of E2 protein were able to displace p53 from its binding site. There was no additional complex detected, indicating that the proteins are not able to bind to the same oligonucleotide and that no protein–protein interaction occurs between DNA-bound E2 and p53. The displacement shows that binding of p53 and E2 to their target sites within the ori element is mutually exclusive.

p53 activates the E6 oncogene promoter of HPV8

Since the M29 motif represents a core element of the HPV8 E6 promoter P_{175} (Pajunk et al., 1997) we analyzed the functional relevance of the p53-M29 interaction for its activity. To construct an E6 promoter-specific test plasmid, the 3′ part of the NCR (nt 10–558) was cloned into the promoterless luciferase vector pALuc. The HPV8 fragment chosen for this purpose encompasses the P_{175} cap site (Stubenrauch et al., 1992) and contains a number of sequence elements conserved among most of the EV-specific HPV types in their E6 proximal NCR parts (P4, CCAAC, M29, A/T). Since P_{175} activity depends on a constitutive enhancer element located in the 5′ region of the NCR, we inserted a dimer of the transcriptional enhancer element M33/AP1 (Horn et al., 1993) in front of the promoter fragment. The resulting reporter gene plasmid pNCR8-3′-Luc was cotransfected with an expression vector for wild-type p53 into RTS3b cells. As shown in Fig. 8 E6 promoter activity increased 2.5- 3-fold upon p53 overexpression. To confirm that this enhancement was mediated by the p53 interaction with DNA, pNCR8-3′-Luc was cotransfected with an expression vector encoding mutated p53 ($PCP53_{175}$), which is not able to bind to DNA (Nigro et al., 1989). It can be seen that the (over)expression of $p53_{175}$ protein had no noticeable effect on the promoter activity of pNCR8-3′-Luc. In order to confirm that the p53-binding site is involved in p53-dependent P_{175} activation we mutagenized plasmid pNCR8-3′-Luc within the 5′ decamer of its p53 recognition site (pNCR8-3′-Luc-p53mut) and tested it in transient promoter assays upon cotransfection of the wild-type p53-expressing vector. Data presented show that the mutated p53-binding site disables its stimulating influence on the P_{175} promoter of HPV8. These results prove that p53 acts as an activator of the E6 promoter by virtue of its binding to the M29 element.

Cotransfection of p53 and E2 expression vectors led to a sixfold activation of pNCR8-3′-Luc compared with a twofold activation by E2 alone (data not shown). This suggests that at relative concentrations of p53 and E2, which led to inhibition of replication, p53 still activates the E6 promoter of HPV8.

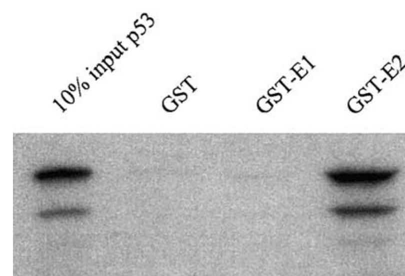

Fig. 6. p53 binds in vitro to HPV8-E2. Protein affinity chromatography of GST, GST-E1, and GST-E2 with in vitro translated p53. GST fusion proteins GST-E1 and GST-E2 or GST protein were immobilized on glutathione Sepharose, incubated with in vitro translated p53, and washed as described in Enzenauer et al. (1998). The bound p53 protein was separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography.

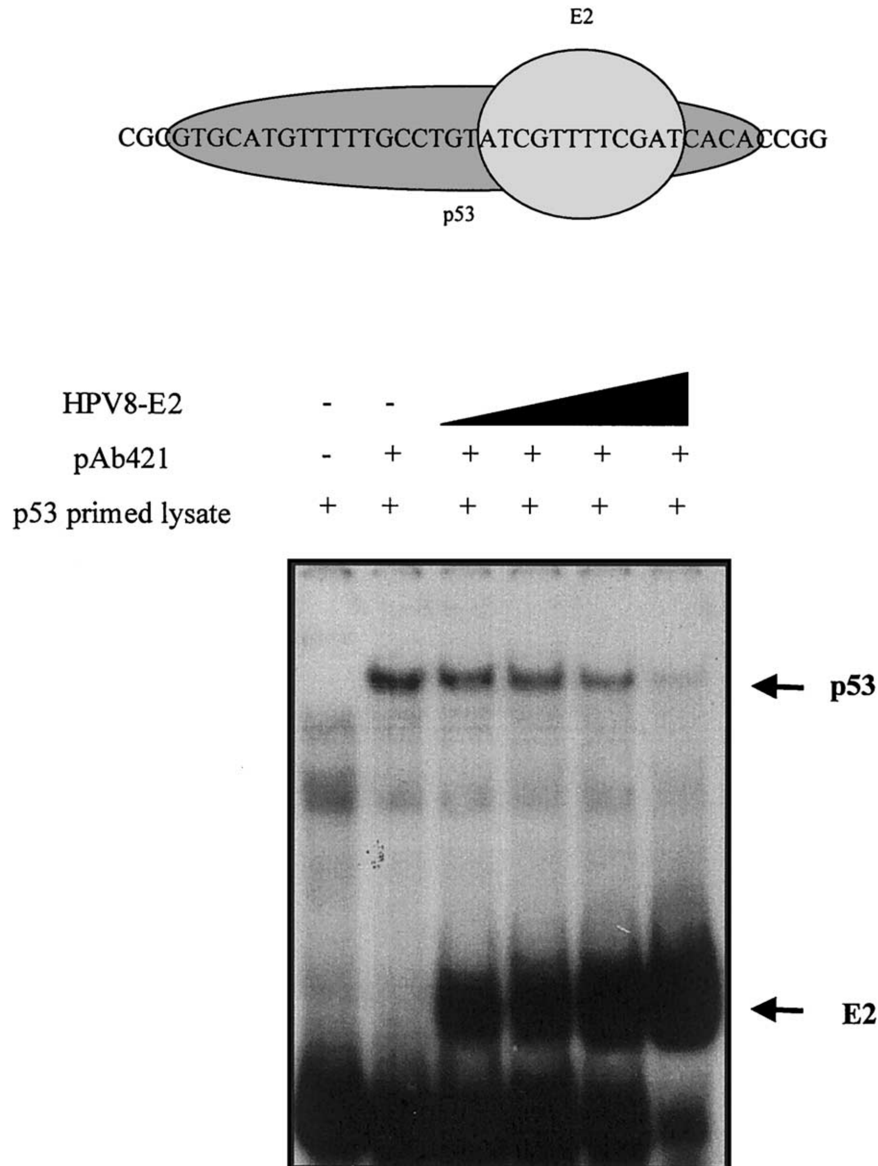

Fig. 7. HPV8-E2 and p53 protein competitively bind to M29. EMSAs were performed with the M29com oligonucleotide, 4 μl of in vitro translated p53 protein, and increasing amounts of purified HPV8-E2 protein.

Discussion

All eucaryotic ori sequences generally display the same structural organization, typically consisting of an origin recognition element, a DNA unwinding element, binding sites for auxiliary transcription factors, and in many cases a local A/T-rich segment (DePamphilis, 1993). The data available so far argue that origins of HPVs also follow this pattern. However, they have been raised primarily for a subgroup of HPVs, infecting almost exlusively the mucosal epithelia. In the course of this study we mapped and characterized an ori element of the EV-associated HPV8, a representative of the most ubiquitous group of skin-specific HPVs.

It could be shown that the ori of HPV8 resides in the E6 proximal part of its NCR. The location of the HPV8 ori is thus similar to that of other papillomaviruses analyzed in this regard, which supports the postulated, highly conserved functional architecture of their genomes. However, in contrast to other HPVs the NCRs in EV viruses are much more compact and show a unique, group-specific sequence organization (Fuchs and Pfister, 1997).

The smallest NCR fragment necessary and sufficient to fulfill the ori function in HPV8 consists of a 65-bp DNA fragment containing two sequence elements, CCAAC and M29, which are highly conserved exclusively among most of the EV HPVs. Interestingly, the very same fragment could also be shown to constitute an essential part of the E6 oncogene promoter (Horn et al., 1993; Pajunk et al., 1997). This is reminiscent of the organization of the regulatory

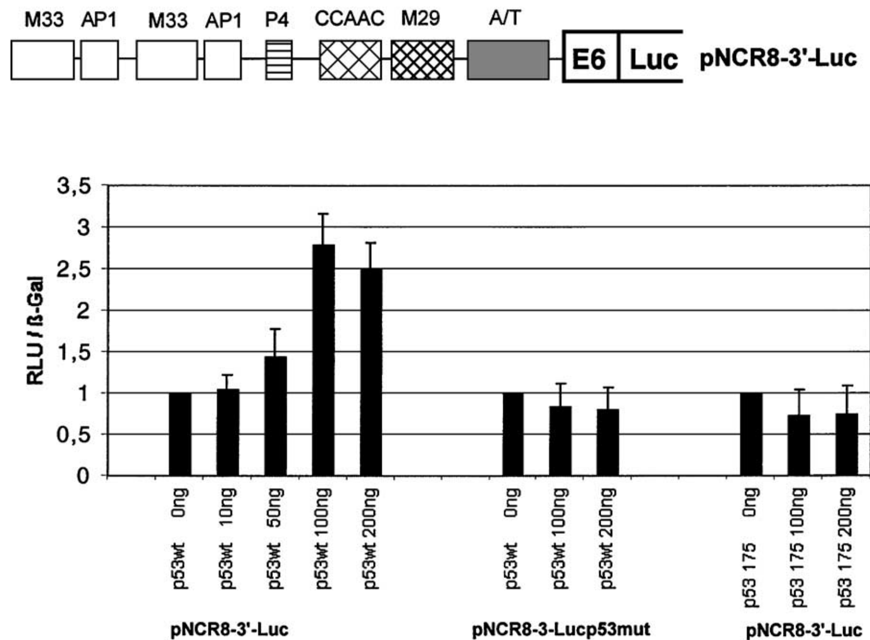

Fig. 8. p53 activates the E6 oncogene promoter of HPV8. The structure of the HPV8 NCR fragment cloned into pALucis is shown on top. Different graphic symbols mark the localization of characteristic sequence motifs (a dimer of M33/AP1, P4, CCAAC, M29, A/T). Luciferase activity in extracts from RTS3b cells transfected with pALucNCR8-3′-Luc and pALucNCR8-3′-Lucp53mut and p53 expression vectors (wild-type and mutant 175). The pCMV-β-Gal plasmid was cotransfected as an internal control for transfection efficiency. Promoter activities are presented as luciferase/β-galactosidase activity ratios.

sequences of polyomaviruses, characterized by intermingled sequence modules, serving both replication and transcription.

The exact roles of the CCAAC and M29 motifs in DNA replication have thus far not been clear. The sequence analysis of the CCAAC element, however, strongly suggests that it may serve as a binding site for the essential HPV replication factor E1. As shown below, an alignment of the CCAAC sequence of HPV8 with the consensus binding site for E1 proteins (O'Conner et al., 1995) displays only two mismatches over a stretch of 20 bp: (E1 consensus site) TWNTWATWNHWWWYWAYAAT and (CCAAC sequence) TTGTTATTGCCAACAACCAT.

It could be demonstrated here that a part of the second ori element of HPV8, M29, adopts in its turn the function of an E2-BS. The newly identified E2 target sequence 5′-ATCGN_4CGAT-3′ (P5) differs from the consensus of an E2-BS (5′-ACCN_6GG/TT-3) originally defined for the bovine papillomavirus 1 (Li et al., 1989) and from a novel E2-BS 5′-ACACN_5GGT-3′, recently reported to be active in supporting HPV transcription and replication (Newhouse and Silverstein, 2001). P5 seems to represent an E2-BS of relatively high affinity, comparable at least to that of P2C (Stubenrauch and Pfister, 1994). Taken together these data argue that the actually active E2-BSs may also display a considerable degree of sequence variation in their symmetric flanks, thus far regarded as conserved parts of the E2-binding palindrome. Interestingly, the M29 E2-BS cannot be recognized by the BPV1-E2 protein (G. Steger, personal communication).

Although the minimal ori element of HPV8 as defined by assays with p88 is characterized by a rather high content of A and T residues (~60%), there is no clearly recognizable continuous stretch of these nucleotides. Interesting in this regard is the fact that the 34-bp A/T sequence located 3′ from the ori of HPV8 and characteristic of most of the EV-associated viruses turned out to be dispensable for its transient in vitro replication ability.

Our analysis of the M29 sequence furthermore revealed the presence of an element very closely resembling a putative binding site of the tumor suppressor protein p53. p53-BS consists of two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0- to 13-bp spacer (El-Deiry et al., 1992). Both decamers within M29 differ from this consensus in containing pyrimidine bases in its conserved purine regions, as well as by the spacer element of 14 bp (Fig. 4A). Nevertheless, we demonstrated that this sequence can indeed act as a functional p53-BS.

Having thus identified an authentic p53-binding site within the ori element, we asked if and how p53 may influence the replication of HPV8. At least in transient in vitro assays, p53 completely inhibited HPV8 DNA replication, while in contrast, the DNA-binding-defective H175 mutant of p53 displayed no effect at all. These findings argue that physical interactions between p53 and the ori sequence may represent one of the mechanisms controlling the course of skin infection by HPV8. Moreover, we detected formation of a complex of p53 and E2 in the absence of DNA. The latter is in line with previous data raised for HPV16-E2 (Massimi et al., 1999). We suggest that the

observed displacement of p53 from its BS by E2 and perhaps also the DNA-independent, direct p53–E2 interactions can be instrumental in replicational repression by p53. The same inhibitory effect of p53 on amplificational replication has previously been reported in vitro for bovine papillomavirus 1 and HPV11 in CHO cells by Lepik et al. (1998). However, in this case the authors concluded that while the DNA-binding domain is essential for the repression activity of p53, there is no need for a p53 response element within the ori. Irrespective of this discrepancy, it is tempting to speculate that p53–E2 interactions may play a general role in regulating papillomavirus replication.

The results presented show that besides its influence on the function of the ori element, p53 may also affect the activity of the overlapping E6 oncogene promoter P_{175} (Pajunk et al., 1997) depending on the binding of p53 to its cognate sequences. Surprisingly, however, the effects of p53 upon HPV8 replication and transcription turned out to be opposite, since overexpression of p53 resulted in activation of the P_{175} activity. Stimulation of P_{175} activity could be observed in the p53-negative RTS3b cell line used throughout this study as well as in normal primary keratinocytes isolated from breast skin (data not shown).

Taken together, these observations strongly suggest that E2 and p53 are involved in the control of the balance between viral DNA replication and oncogene expression in the course of skin infection with EV HPVs. Unfortunately, there is to date only little information concerning the patterns of expression of both these factors in normal and dysplastic human skin epithelium. The p53 protein has been detected in the cytoplasm of the basal cell layer in normal skin and in psoriatic lesions (Helander et al., 1993). In contrast, squamous cell carcinomas of the skin revealed diminuished p53 expression, confined in a subset of cases to the cell nucleus. In regard to cell proliferation it is interesting to note that p53 was reported to accumulate in the cytoplasm during the G_1 phase of the cell cycle and to relocate to the nucleus at the beginning of the S phase (Shaulsky et al., 1990). In the case of EV lesions immunohistochemical p53 positivity could be detected in almost all specimens studied (Pizarro et al., 1995). A study of Padlewska et al. (2001) revealed nuclear staining mostly confined to basal cells. On the other hand, the E2 transcription in HPV5-induced benign EV skin lesions could be shown by in situ hybridization to occur predominantly in the central layers of the stratified epithelium (Haller et al., 1995).

Combining these data with our results one can envisage a scenario in which the gradients of p53 and E2 expression in differentiating skin keratinocytes decide on preference between virus DNA replication and transcription of the HPV8 E6 oncogene. This would fit the limited (maintenance) replication of HPV DNA in basal/suprabasal strata and the productive replication in the middle and upper parts of the epithelium. The demonstrated impact of p53 on HPV replication may also be relevant to the establishment of latent infections, typical for EV viruses.

Materials and methods

Oligonucleotides

Oligonucleotides used to generate subfragments of the HPV8 NCR by PCR consisted of 12–14 nt, with their 5′ ends at positions given in Fig. 2A.

Band-shift experiments were performed with the double stranded oligonucleotides P2C (nt 7486–7516, AACCGACCGCAAACGGTACATAAAGGTGAGT), with a high-affinity E2 recognition site as described in Stubenrauch and Pfister (1994); M29com (nt 79–118, CTATAGCATGTTTTTGCCTGTATCGTTTTCGATCACACC); M29E2 (nt 94–117, GCCTGTATCGTTTTCGATCACACC); M29E2mut (nt 94–117, GCCTGTCTGGTTTTCGATCACACC); SP1, with a bona fide SP1-binding site (GATCTAAACCCCGCCCAGCG); p53+control oligonucleotide (TACAGAACATGTCTAAGCATGCTGGGG); and p53-control oligonucleotide (TACAGAATCGCTCTAAGTCGCCTGGGG), all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Nucleotide numbering of the HPV8 sequence is according to Fuchs et al. (1986).

Plasmids

Expression vectors

The E1 expression vector pCE1 was generated by insertion of the HPV8 *Pvu*II fragment (nt 828–2937) into the *Bgl*II site of the vector pCB6, downstream of the CMV immediate-early promoter. The HPV8-E2 expression vector pCE2 was constructed by inserting the whole E2 open reading frame of HPV8 (nt 2682–4222) into pCB6 (Stubenrauch and Pfister, 1994).

The eucaryotic expression vector pCp53wt contains the wild-type human p53 cDNA (Nigro et al., 1989), whereas its mutant form $pCp53_{175}$ encodes for an amino acid exchange from arginine to histidine at position 175 and is no longer able to bind DNA. p53 expression vectors pSp53wt for in vitro translation and pGEX-2Tp53 for the expression of GST-fused p53 were as described elsewhere (Zimmermann et al., 1999). The GST fusion vectors for HPV8-E1 and E2 are described in Enzenauer et al. (1998).

Replication test plasmids for HPV8

The plasmids p81 (nt 7626–188), p82 (nt 7626–118), p83 (nt 7626–51), p84 (nt 7626–81), p85 (nt 13–118), and p86 (nt 13–75) were generated by PCR amplification of the HPV8 fragments, which were then cloned into the *Sma*I site of pUC8. In p87 the CCAAC element (nt 56–75) was replaced by a linker sequence (CATGGAGCTCTATAG). p88 (nt 34–115) was generated by ligation of a doublestranded synthetic oligonucleotide into the *Sma*I site of pUC18. To construct P01234, the *Eco*47III-*Xho*I restriction fragment of the vector P34 (Stubenrauch and Pfister, 1994)

was inserted into P012 (Stubenrauch and Pfister, 1994), in which the same sequence was previously deleted.

Luciferase gene reporter plasmids

The HPV8 E6 promoter plasmid pNCR8-3′-Luc was generated by cloning of an HPV8 PCR amplicate (nt 10–558) between the *Hin*dIII–*Kpn*I sites and a dimer of a double stranded oligonucleotide corresponding to the HPV8 M33/AP1 enhancer (nt 7422–7474, Horn et al., 1993) into the *Hin*dIII site of pALuc.

Site-directed mutagenesis

In order to introduce nucleotide exchanges into HPV8 sequences, site-directed mutagenesis was performed by using the QuikChange site-directed mutagenesis kit (Stratagene). To mutate the E2- and p53-binding motives within the ori element of HPV8 the sense/antisense oligonucleotide pairs p88E2mutA 5′-GCATGTTTTTGCCTGTCTGGTTTTCGATCACACC-3′/p88E2mutB 5′-GGTGTGATCGAAAACCAGACAGGCAAAAACATGC-3′ and pNCR8-3′-Luc-mutA 5′-CCATCGTCTATAGTCGCTTTTTGCCTGTATCG-3′/pNCR8-3′-Luc-mutB 5′-CGATACAGGCAAAAAGCGACTATAGACGATGG-3′ were used. After obtaining positive clones, the constructs were sequenced to confirm the mutated nucleotides in the context of the HPV8 sequences.

Cell culture

The spontaneously immortalized human skin keratinocyte cell line RTS3b (Purdie et al., 1993), which contains no papillomavirus DNA, was maintained in F12–DMEM (1:4) supplemented with hydrocortisone (0.4 mg/ml), 10^{-10} M cholera toxin, transferin (5 mg/ml), 2×10^{-11} M triiodothyronin, 1.8×10^{-4} M adenin, insulin (5 mg/ml), epidermal growth factor (10 ng/ml), 1% penicillin–streptomycin, and 10% fetal calf serum.

Transient replication assay

RTS3b cells were transfected with plasmid DNA with Fugene (Roche, Mannheim) according to the manufacturer's protocol. Transient replication assays were done with 6×10^6 cells in 100-mm plates and 2 μg of the expression vectors pCE1, pCE2, pCp53wt, and pCp53$_{175}$ and 3 μg of ori plasmids; 72 h after transfection, low-molecular-weight DNA was isolated according to the protocol established by Ustav and Stenlund (1991). The plasmid DNA was linearized with *Bam*HI as a single cutter. To distinguish between replicated and unreplicated DNA, half of each sample was additionally treated with *Dpn*I to remove the unreplicated, methylated input DNA. The DNA samples were analyzed by electrophoresis in 0.8% agarose gels followed by Southern blot hybridization. The DNA was transferred to Hybond N^+ membrane (Amersham) and hybridized to a ^{32}P-labeled pNCR8-CAT probe generated by using the random primer labeling kit (Gibco). Blots were subjected to autoradiography at −70°C with intensifying screens.

Transient gene expression assay

Twenty-four hours prior to transfection 2×10^5 RTS3b cells were plated into 6-well culture dishes. Each plate received equal amounts of DNA composed of 500 ng of Luciferase gene reporter plasmid, 200 ng of the expression vector pCMV-β-Gal, and up to 200 ng of the p53 expression vectors pCp53wt or pCp53175. The total amount of plasmid DNA was kept constant in cotransfection by the addition of plasmid pCMV1. Cells were harvested 24 h after transfection in PBS, resuspended in 100 mM potassium phosphate, pH. 7.8, containing 1 mM dithiothreitol, and lysed by 4 freeze–thaw cycles. Luciferase activity was quantified as described by DeWet et al. (1987). The promoter activity was expressed as ratio of luciferase to β-Gal activities.

Expression and purification of HPV8 E2 protein from insect cells

High Five insect cells (*Trichoplusia ni* 5B1-4, Invitrogen) were cultivated in Express Five serum-free medium (Gibco) at 27°C. The infection of cells was performed as described by Stubenrauch and Pfister (1994); 72 h postinfection cells were resuspended in a total volume of 10 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche, Mannheim). The suspension was frozen in liquid Nitrogene and then thawed at 42°C three times to lyse the cells. The crude lysate was centrifugated at $15{,}000 \times g$ and the supernatant was applied to a 5-ml HiTrap heparin–Sepharose column (Pharmacia). HPV8 E2 protein was eluted with 0.6 M KCl.

GST fusion protein purification and in vitro binding assays

GST fusion protein expression and purification was done as described previously (Enzenauer et al., 1998). In vitro translations were performed using the TNT-coupled reticulocyte lysate system (Promega) as instructed by the manufacturer. For p53 EMSAs the p53 protein was in vitro synthesized with the modification that no radiolabeled amino acids were used.

Electromobility shift assay (EMSA)

^{32}P-end-labeled oligonucleotides (250 pg) were incubated with HPV8-E2 protein in a total volume of 20 ml of binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM dithiothreitol, 4 mM spermidine, 5 mg/ml of bovine serum albumin, 5% glycerol (v/v) and 1 mg poly(dI-dC)·poly(dI-dC)). For competition experiments, the reactions were supplemented with different amounts of ho-

mologous and heterologous unlabeled oligonucleotides. After 20 min at room temperature, the samples were electrophoresed in 5% native polyacrylamide gels.

For each p53 EMSA test, 4 μl of p53-primed reticulocyte lysate was diluted in 20 μl of DNA-binding buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% NP-40 (v/v), 10% glycerol (v/v), 5 mM dithiothreitol) and 50 ng of salmon sperm DNA as a non specific competitor. Where required, reactions were performed in the presence of unlabeled competitor oligonucleotides. Reactions were incubated for 10 min at ambient temperature with the labeled oligonucleotides, followed by the addition of 1 ml of anti-p53 monoclonal antibody pAb421 (Hupp et al., 1992). The samples were incubated for a further 30 min and finally subjected to 4% nondenaturing polyacrylamide gel electrophoresis. The gels were vacuum dried and exposed to X-ray film overnight at −80°C.

Acknowledgments

The authors thank Dr. M. Scheffner (University of Cologne, Germany) and Dr. A. Storey (Cancer Research UK, London, UK) for helpful discussions and A.S. for the kind gift of the p53 antibody pAb421. We are indebted to Dr. H. Zimmermann for providing us with pGEX2T-p53 and pSp64-p53 and S. Hoberg for the pNCR8-3′-Luc construct. This work was supported by Grant (ZMMK) from the Cologne Center for Molecular Medicine to H.J.P. and P.G.F.

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