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Influence of Papillomavirus Early Proteins on the Expression of Tumor-Progression Promoting Genes

"Look to this day, for yesterday is already a dream and tomorrow is only a vision.
But today well lived makes every yesterday a dream of happiness, and every tomorrow a vision of hope."

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And the last line is, as always through these years, reserved for my dearest friend Katie. There is not much left to say this time. Same as usual, live your dreams!

INDEX

I SUMMARY

Human papillomaviruses (HPV) are the known cause for cancers of the cervix and are associated with a variety of other human malignancies including head and neck, and skin. The cottontail-rabbit papillomavirus (CRPV) serves as a suitable animal model to study the development and progression of these cancers *in-vivo*. Our group has previously demonstrated that CRPV-induced skin lesions express elevated levels of metalloproteinase-9, a protease contributing to cancer progression by extracellular matrix remodelling. Based on our previous findings that the CRPV early protein 2 (E2) can activate a truncated human MMP-9 promoter fragment, we hypothesized that enhanced MMP-9 expression in the rabbit lesions is a consequence of activation of the rabbit MMP-9 promoter by CRPV E2. In order to elucidate the mechanism involved in MMP-9 promoter activation a library of genomic DNA isolated from rabbit skin was constructed, and the sequence of the MMP-9 promoter was identified. Promoter deletion mutants were cloned and the minimum required fragment for E2-mediated MMP-9 promoter activation was determined to be -717 bp in length. Selective mutation of transcription factor binding sites within the promoter sequence revealed a high importance of both of the two identified AP-1 binding sites in the rabbit MMP-9 promoter. Using the transactivation-deficient c-Jun mutant TAM67, a strong inhibitory effect on promoter activation after challenge with E2 could be observed, suggesting an important role of c-Jun in the activating AP-1 transcription-factor complex. The same mechanism could be shown in the human system during this study. Furthermore, it could be determined that in both, the rabbit and the human system, the activation of the MMP-9 promoter by E2 requires the phosphorylation of the MAP-kinase ERK, as inhibition of the cascade by the chemical inhibitor PD098059 resulted in a significant decrease of promoter activation. Co-transfection of E2 and siRNA directed against ERK or a dominant-negative mutant of the latter led to similar results.

It has been described previously that the high-risk HPV E2 is located within both nucleus and cytoplasm. Mutations in the domains responsible for protein localization allowed for investigation of the role of E2 localization in the activation of the MMP-9 promoter. It was observed that MMP-9 activation was strikingly decreased when the protein was mainly cytoplasmic. Additionally, HPV6bE2 which has been described to be solely nuclear did also induce MMP-9 promoter activation.

It can hence be concluded that CRPV E2 and HPV16 E2 both activate the respective MMP-9 promoters via an AP-1 and ERK dependent mechanism. As direct binding of the E2 proteins to the promoter can be roled out, this mechanism has to be further investigated. It can, however, be hypothezised that the interaction of E2 with its potential interation partner is taking place within the nucleus.

II ZUSAMMENFASSUNG

Infektionen mit humanen Papillomviren sind ursächlich für die Entstehung des Zervixkarzinoms und werden mit weiteren malignen Tumorerkrankungen des Menschen in Verbindung gebracht. Das einzige verfügbare Tiermodell zum Studium der Papillomavirus-assoziierten Tumorentstehung ist das Cottontail rabbit Papillomvirus (CRPV) Modell. Nach subkutaner Applikation der viralen DNA in Kaninchen kommt es zur Ausbildung von Papillomen, welche sich ohne Kofaktoren zu infiltrierenden Tumoren entwickeln. In vorausgehenden Studien unserer Gruppe konnte gezeigt werden, dass die CRPV-induzierten Hautläsionen des Kaninchens eine erhöhte Expression der Matrixmetalloproteinase-9 (MMP-9) aufwiesen. Dieser Protease wird eine wichtige Rolle in der Tumorigenese zugeschrieben, da sie an der Umstrukturierung und dem Abbau der extrazellulären Matrix beteiligt ist. Aufgrund der früheren Studien bei denen gezeigt wurde, dass das E2 Protein von CRPV in der Lage ist, eine verkürzte Form des humanen MMP-9 Promotors zu aktivieren, entstand die Vermutung, dass die gesteigerte Expression von MMP-9 in den CRPV-induzierten Hautläsionen eine Folge der Aktivierung des Kaninchen-MMP-9 Promotors ist. Um den zugrundeliegenden Mechanismus zu untersuchen, wurde zunächst eine DNA-Bibliothek mit genomischer DNA aus Kaninchenhaut erstellt und die Sequenz des Kaninchen-MMP-9 Promoters ermittelt. Promoter-Deletionsmutanten wurden kloniert und wir konnten nachweisen, dass 717 bp des Promoters ausreichend für die E2 induzierte Aktivierung sind. Selektive Mutationen von Transkriptionsfaktor-Bindestellen ergaben, dass beide im Kanichenpromoter identifizierten AP-1 Bindestellen von grosser Bedeutung für die Aktivierung sind. Auch Kotransfektion der transaktivierungsdefizienten c-Jun Mutante TAM67 zeigte eine deutliche Hemmung der E2 gesteuerten Promotoraktivierung, was auf eine bedeutende Rolle dieses fakultativen Bestandteils des AP-1 Komplexes hindeutet. Dieser Mechanismus konnte in dieser Arbeit auch im humanen System unter Zuhilfename des humanen Papillomavirus 16 (HPV16) aufgezeigt werden. Des Weiteren konnte die Abhängigkeit der Induktion des MMP-9 Promotors von einer Aktivierung der MAP-Kinase ERK sowohl im Kaninchen als auch im humanen System demonstriert werden. Die Inhibition der ERK-Kaskade durch den niedermolekularen Inhibitor PD098059, wie auch durch Kotransfektion von ERK siRNA oder dominant-negativen ERK Mutanten, mündete in einem Rückgang der E2-vermittelten Promotoraktivität.

Es wurde zuvor beschrieben, dass das E2 Protein von Hochrisiko HPV-Typen im Nukleus sowie im Zytoplasma der infizierten Zelle zu finden ist. Mutationen in den Domänen des E2, die für die Proteinlokalisation verantwortlich sind, boten die Möglichkeit, die Rolle der Lokalisation von E2 in der Aktivierung des MMP-9 Promotors zu klären. Mutiertes E2, welches überwiegend im Zytoplasma der Zellen nachweisbar war, zeigte ein wesentlich geringeres Potential zur MMP-9 Promoteraktivierung. Zudem konnte gezeigt werden,

dass HPV6b E2, welches ausschliesslich nukleär vorliegt, ebenfalls in der Lage ist den MMP-9 Promotor zu aktivieren.

Die hier vorliegenden Ergebnisse lassen die Schlussfolgerung zu, dass sowohl CRPV E2 als auch HPV16 E2 die respektiven MMP-9 Promotoren über einen AP-1 und ERK-abhängigen Mechanismus aktivieren. Da ausgeschlossen werden kann, dass die Proteine direkt den jeweiligen Promotor binden, bedarf der genaue Mechanismus weiterer Aufklärung. Es kann jedoch postuliert werden, dass eine Wechselwirkung von E2 mit einem potentiellen Interaktionspartner im Nukleus stattfindet.

III ABBREVIATIONS

AP-1	**a**ctivator **p**rotein-**1**
APS	**a**mmonium **p**er**s**ulfate
ATF	**a**ctivating **t**ranscription **f**actor
BCA	**bi**c**i**nchinonic **a**cid
BMK	**b**ig **M**AP-**k**inase
BPV	**b**ovine **p**apilloma**v**irus
BS	**b**inding **s**ite
BSA	**b**ovine **s**erum **a**lbumine
Cdk	**c**yclin-**d**ependent **k**inase
CR	**c**onserved **r**egion
CRPV	**c**ottontail **r**abbit **p**apilloma**v**irus
DAG	**d**i**ag**lycerin
DBD	**D**NA-**b**inding **d**omain
DMSO	**d**i**m**ethyl **s**ulf**o**xide
DNA	**d**eoxyribo**n**ucleic **a**cid
dNTPs	**d**esoxyribo**n**ucleosid **t**ri**p**hosphate
ds	**d**ouble**s**tranded
EGF	**e**pidermal **g**rowth **f**actor
E	**e**arly
EBV	**E**pstein-**B**arr **v**irus
ECM	**e**xtra**c**ellular **m**atrix
ERK	**e**xtracellular signal-**r**egulated **k**inase
FAK	**f**ocal **a**dhesion **k**inase
H_2O_{bidest}	doubledestilled water
HDAC	**h**istone **d**e**ac**etylase
HEPES	N-(2-**h**ydrox**e**thyl)**p**iperazin-N'-(2-**e**than**s**ulfonic acid)
HNSCC	**h**ead and **n**eck **s**quamous **c**ell **c**arcinoma
HPV	**h**uman **p**apilloma**v**irus
hTERT	human telomerase
IGF	**i**nsulin-like **g**rowth **f**actor
kb	**k**ilo **b**asepairs
kDa	**k**ilo**da**lton
JNK	c-**J**un **N**-terminal **k**inase
L	**l**ate
LCR	**l**ong **c**ontrol **r**egion
LMP	**l**ate-**m**embrane **p**rotein
MAPK	**m**itogen-**a**ctivated **p**rotein **k**inase

MAPKK	**m**itogen-**a**ctivated **p**rotein **k**inase **k**inase
MAPKKK	**m**itogen-**a**ctivated **p**rotein **k**inase **k**inase **k**inase
mRNA	**m**essenger **RNA**
MMP	**m**atrix-**m**etallo**p**roteinase
NES	**n**uclear **e**xport **s**equence
NLS	**n**uclear **l**ocalization **s**equence
OD	**o**ptical **d**ensity
ORF	**o**pen **r**eading **f**rame
PAA	**p**oly**a**cryl**a**mide
PAGE	**p**olyacrylamide **g**el **e**lectrophoresis
PAI	**p**lasminogen **a**ctivator **i**nhibitor
PBS	**p**hosphate **b**uffered **s**aline
PKC	**p**rotein **k**inase **C**
PMA	see TPA
RNA	**r**ibo**n**ucleic **a**cid
RTK	**r**eceptor **t**yrosine **k**inase
SAPK	**s**tress-**a**ctivated **p**rotein **k**inase
SDS	**s**odium **d**odecyl**s**ulfate
STAT	**s**ignal **t**ransducers and **a**ctivators of **t**ranscription
TBP	**T**ATA-box **b**inding **p**rotein
TBS	**t**ris **b**uffered **s**aline
TEMED	**te**tra**me**thyl**d**iamine
TGF	**t**ransforming **g**rowth **f**actor
TIMP	**t**issue-**i**nhibitor of **m**etallom**p**roteinases
tPA	**t**issue-type **p**lasminogen **a**ctivator
TPA	12-otetradecanoylphorbol-13-acetate
TRE	**T**PA-**r**esponse **e**lement
Tris	**tris**-(hydroxymethyl)methylglycine
uPA	**u**rokinase-**p**lasminogen **a**ctivator
uPAR	**uPA r**eceptor
URR	**u**pstream **r**egulatory **r**egion
wt	**w**ild**t**ype

1 INTRODUCTION

1.1 HUMAN PAPILLOMAVIRUSES

Human papillomaviruses (HPVs) are small non-enveloped dsDNA viruses with a diameter of approximately 55 nm. The ikosaedric capsid that surrounds the viral DNA is composed of 72 capsomers (Klug and Finch, 1965) made up of the major capsid protein L1 and the minor capsid protein L2. The viral DNA is between 7.2 kb and 8 kb in length and maintained in a supercoiled closed circular episome associated with histone like particles (Favre *et al.,* 1977; Pfister and zur Hausen, 1978). Depending on HPV type, up to 10 open reading frames (ORFs) are occupying the transcriptionally active strand. The genome (Figure 1) can be devided into two coding regions which encode a variety of proteins which have been termed Early (E) or Late (L) proteins depending on the time of their expression in the infected cell (Ozbun and Meyers *et al.,* 1997). While the early proteins have a mainly regulatory function, the late genes encode the capsid proteins. Gene expression is controlled by a long-control region (LCR), also termed upstream regulatory region (URR), which covers about 12% of the viral genome and contains several transcription-factor binding sites.

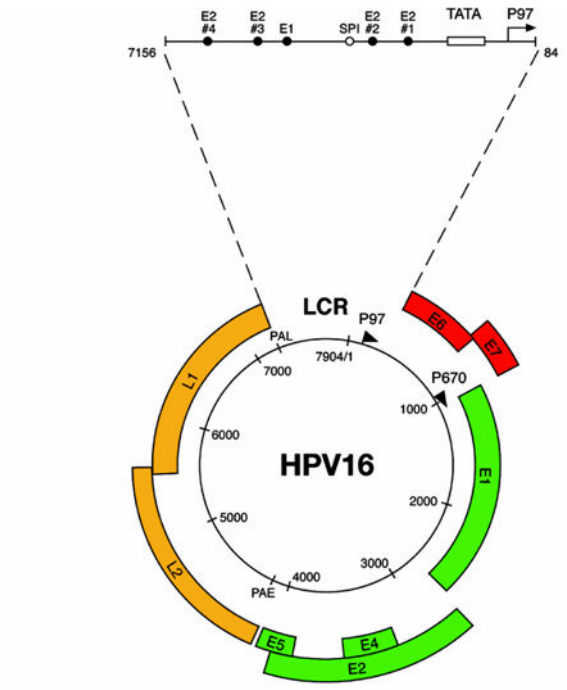


Figure 1: Organization of the HPV16 genome. The genome is shown as a black circle with the early (p97) and late (p670) promoters marked by arrows. The six early ORFs E1, E2, E4 and E5 (green), E6 and E7 (red) are expressed from either p97 or p670 at different stages during epithelial cell differentiation. The late ORFs L1 and L2 (yellow). All the viral genes are encoded on one strand of the double-stranded circular DNA genome. The long control region (LCR) is enlarged to allow visualization of the E2-binding sites and the TATA element of the p97 promoter. The location of the E1- and SP1-binding sites is also shown. Modified from Doorbar, 2006.

1.1.1 The regulatory early proteins 1 and 2

The E1 and E2 proteins are the major regulatory proteins and required for papillomavirus replication and transcription of the viral genes. They are highly conserved among different HPV types.

The E1 protein is the largest of the HPV proteins with a size of about 70 kDa and is expressed throughout the HPV life cycle (Klumpp and Laimins, 1999). E1 is a DNA-binding protein which posseses ATP-ase activtiy (Hughes *et al.,* 1993). It also functions as an ATP-dependent DNA helicase (Hughes *et al.,* 1993; Yang *et al.,* 1993), unwinding double-stranded DNA molecules by utilizing energy obtained by the hydrolysis of ATP (Patel and Picha, 2000). The binding of E1 to viral DNA it rather week in the absence of E2, however, interaction with E2 enables efficient binding of E1 to the DNA (Mohr, 1990; Frattini and Laimins, 1994; Kuo *et al.,* 1994; Sedman *et al.,* 1997) where its monomers subsequently form a ring-shaped hexameric assembly which initiates the helicase activity (Sedman and Stenlund, 1998). E1 has further been shown to bind DNA polymerase alpha subunits and recruit those to the viral origin of replication (Masterson *et al.,* 1998; Conger *et al.,* 1999).

The E2 protein contains approximately 360 amino acids and is 48 kDa in size. Is is composed of two domains which are seperated by a flexible hinge region (Giri and Yaniv, 1988). The N-terminal part of the protein contains the transactivation domain while the C-terminus is occupied by the DNA-binding domain (DBD) (Sakai *et al.,* 1996) and mediates the interaction of E2 with E1 (Sedman *et al.,* 1997). Dimerized, the DBD recognizes E2-binding sites (E2BSs) consisting of the palindromic sequence 5'-AAC$(N)_6$GTT-3' (McBride *et al.,* 1991). The LCR of high-risk HPVs contains four highly conserved E2-binding sites suggesting that this specific arrangement is important for viral function (Stubenrauch *et al.,* 1998). Two adjacent binding sites are located between binding sites for the cellular transcription factors Sp-1 and TBP (TATA-box binding protein), resulting in transcriptional repression (Dong *et al.,* 1994; Romanczuk *et al.,* 1990; Thierry & Howley 1991) by steric inhibition of Sp-1 and TBP binding (Demeret *et al.,* 1997; Dong *et al.,* 1994; Stubenrauch *et al.,* 1996; Tan *et al.,* 1994). The remaining two E2BSs are positioned further upstream of the promoter and the binding of E2 to any of these sites mediates transcriptional activation (Romanczuk *et al.,* 1990). E2 is suggested to be the major regulator of E6 and E7 expression. Loss of E2 expression can be detected in late stages of cervical cancer due to integration of the viral genome into the the host DNA and resulting disruption of the E2 ORF (Dürst *et al.,* 1992; Klaes *et al.,* 1999). This may subsequently lead to increased cellular proliferation and further progression of carcinogenesis induced by E6 and E7 (Corden *et al.,* 1999; Pett *et al.,*

2004). Additionally, overexpression of HPV16 and -18 E2 proteins have been been shown to induce cell cycle arrest and apoptosis in cervical carcinoma cell lines (Desaintes *et al.,* 1997). As mutations eliminating the DNA-binding activity do not prevent E2-mediated apoptosis in HeLa cells (Webster *et al.,* 2000) it has been suggested that the effect of E2 on cell death is a result of its interaction with cellular proteins. Studies have led to the discovery that HPV16 E2 interacts with p53 (Massimi *et al.,* 1999) and may induce apoptosis in a p53-dependent manner (Webster *et al.,* 2000). As high-risk HPV E2 proteins can be detected within both, the nucleus and the cytoplasm due to exportin-1 receptor (CRM1)-dependent nucleo-cytoplasmic shuttling, while low-risk HPV E2 is strictly nuclear, these differences in localization may explain why high- but not low risk HPV E2 has been shown to induce apoptosis *in-vitro* (Blachon *et al.,* 2005). Furthermore, it has been recently shown that apoptosis can be induced by direct interaction of HPV18 E2 with caspase-8 (Thierry and Demeret, 2008).

1.1.2 The early proteins E4 and E5

The function of the E4 protein is largely unknown. It accumulates within the cell at the time of viral amplification, its mRNA being the major transcript found in HPV-induced lesions (Chow *et al*, 1987 a, b). The protein is not involved in transformation or the episomal maintenance of the viral DNA (Neary *et al.,* 1987), but it is thought to play a role in late events of viral infection as these have been demonstrated to be disrupted upon loss of E4 in several experimental systems (Peh *et al.,* 2004; Nakahara, *et al.,* 2005; Wilson *et al.,* 2005). An association has been shown between E4 and the cells keratin cytoskeleton; an interaction that induces the collapse of the cytokeratin filament network, contributing to virus release from the cell (Doorbar *et al.,* 1991; Roberts *et al.,* 1993). The protein also sequesters the Cdk1/cyclin B1 complex to the cytoskeleton, preventing it from accumulating within the nucleus and thus inducing G2 arrest (Nakahara *et al.,* 2002; Raj *et al.,* 2004) and a prolonged S-phase, promoting HPV genome amplification (Knight *et al.,* 2004; Davy *et al.,* 2005; Wilson *et al.,* 2005).

The E5 protein has appoximately 80 amino acids, and is a commonly dimeric hydrophobic membrane protein found predominantly in the membranes of the golgi, the endoplasmic reticulum, and the plasma membrane (Bubb *et al.,* 1988; Halbert and Galloway, 1988; Burkhard *et al.,* 1989; Conrad *et al.,* 1993). While E5 was found to be the major transforming protein in bovine papillomaviruses (BPV) (Schiller *et al.,* 1986; DiMaio *et al.,* 1986; Rabson *et al.,* 1986) where it forms a stable complex with the platelet-derived growth factor β receptor, inducing receptor dimerization and activation (DiMaio *et al.,* 2001), it is only weakly transforming in HPV (Leptak *et al.,* 1991; Leechanachai *et al.,* 1992; Pim *et al.,* 1992). In HPV16 transfected cells, E5 forms complexes with the

epidermal growth factor (EGF) receptors (Hwang *et al.,* 1995) which impacts downstream signalling (Straight *et al.,* 1993; Crusius *et al.,* 1997, 1998; Zhang *et al.,* 2002) and enhances mitogen-activated protein kinase (MAPK) activity (Gu *et al.,* 1995). HPV16 E5 also interacts with the vacuolar ATPase which results in the inhibition of acidification of the endosome (Conrad *et al.,* 1993) and thus in inhibited degradation of internalized growth factor receptors ultimatly leading to increased recycling of the receptors to the cell surface (Straight *et al.,* 1993, 1995). It also interferes with cell-mediated immune functions as it downregulates MHC class I molecules (Cartin and Alonso, 2003; Ashrafi *et al.,* 2005) and perturbs MHC class II antigen maturation (Zhang *et al.,* 2003) to repress host immune responses.

1.2.3 The oncogenes E6 and E7

The HPV16 E6 protein is relatively small with 151 amino acids and 18kDa. It contains four C-x-x-C motifs which probably lead to the formation of two zinc-fingers (Barbosa *et al.,* 1989; Grossman *et al.,* 1989; Kanda *et al.,* 1991). However, the protein itself has no intrinsic enzymatic activity. E6 is located within both, cytoplasm and nucleus of the infected cell (Barbosa *et al.,* 1989). It has been shown to exert transforming functions in various human cell lines, which seems to be caused by the interaction with cellular proteins (Band *et al.,* 1990; Wazer *et al.,* 1995; Reznikoff *et al.,* 1996; Liu *et al.,* 1999). The best studied function of the E6 protein is binding of the tumor supressor gene p53 and the subsequent ubiquitin-mediated degradation of the latter. P53 has been shown to regulate the expression of genes involved in cell cycle control in response to a number of stimuli, including ionizing radiation, cell stress, or viral infection, upon which it mediates either cell cycle arrest or apoptosis (Werness, *et al.,* 1990; Scheffner *et al.,* 1990; Ko *et al*, 1996). Expression of high-risk E6 results in the formation of a trimeric complex with the cellular ubiquitin-ligase E6-AP, a complex which then leads to the ubiquitination of p53, resulting in reduced steady-state levels of p53 (Figure 2) (Huibregdse *et al.,* 1991, 1993; Kao *et al.,* 2000). E6 can further downregulate p53-mediated transcription through the binding of CPB /p300, which are known co-activators of p53 (Lechner *et al.,* 1994; Patel *et al.,* 1999; Zimmermann *et al.,* 1999). Another important function of high-risk E6 is the activation of the catalytic subunit of human telomerase (hTERT) leading to an increase in telomeric length (Klingelhutz *et al.,* 1996; Stoppler *et al.,* 1997; Veldman *et al.,* 2001; Gewin and Galloway, 2001; Oh *et al.,* 2001). Additionally, E6 can activate hTERT transcription by binding directly to the transcription factor myc and recruiting it to the hTERT promoter (Veldman *et al.,* 2001). It has been suggested that the induction of chromosomal instability by E6, resulting from the loss of G_1/S checkpoint control due to the degradation of p53, is an important aspect for the long-term progression of latently high-risk HPV infected cells (Kessis *et al*. 1996).

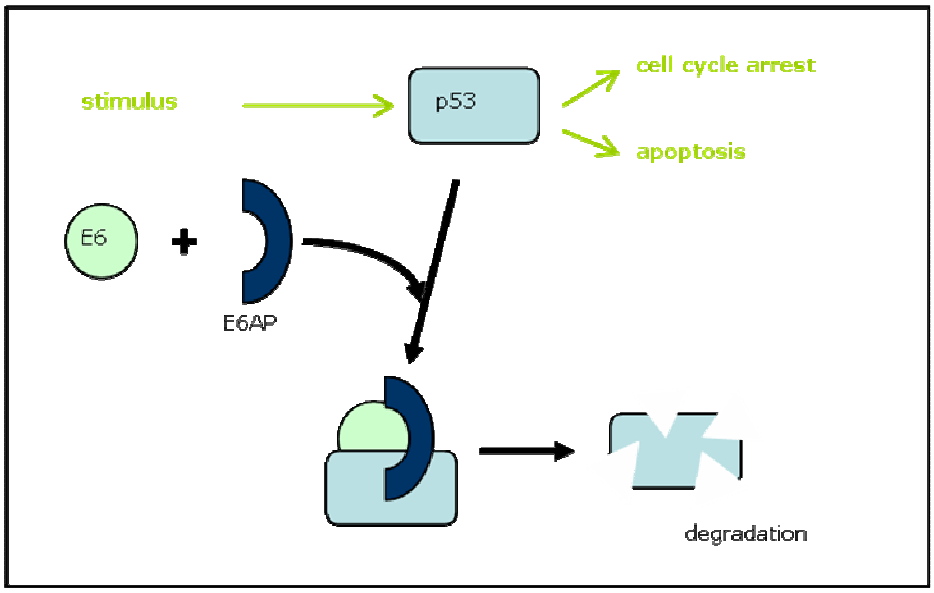


Figure 2: Effect of the E6 protein on p53. Upon DNA damage activated p53 induces either cell cycle arrest or apoptosis. E6 (green) binds to p53 (light blue) when complexed with E6AP (dark blue) and thereby targets p53 for apoptosis.

HPV16 E7 is composed of 98 amino acids and has a molecular weight of approximately 21 kDa. The protein appears to be situated predominantly within the nucleus and was shown to be phosphorylated *in-vivo* (Smotkin and Wettstein, 1987; Firzlaff *et al.,* 1989; Barbosa *et al.,* 1990; Smith-McCune *et al.,* 1999). The N-terminal region of the protein contains three highly conserved regions (CR), CR-1, CR-2, and CR-3 (Barbosa *et al.,* 1990; Dyson *et al.,* 1992). The CR-1 domain comprises the amino terminus and a domain corresponding partially to the conserved region 1 of the adenovirus E1A protein, a protein driving cells into S-phase. The CR-2 domain corresponds to the conserved region 2 of the adenovirus E1A protein completely, as well as to an analogous region in the cell-transforming SV40 large T antigen (Phelps *et al.,* 1989). It further contains an LXCXE motif essential for binding the retinoblastoma protein (Rb) (Chellappan *et al.,* 1992; Dyson *et al.,* 1992). Both, the CR-1 and the CR-2 domains impact the immortalizing potential of E7. The CR-3 domain consists of two zinc-finger motifs important for dimerization as E7 is predominantly present in a dimeric state (McIntyre *et al.,* 1993; Braspenning *et al.,* 1998; Phelps *et al.,* 1992). The C-terminal portion of the protein contains two C-x-x-C motifs which form an unconventional zinc-finger region showing similarity to those found within the E6 protein and suggesting for an evolutionary relationship between the two (Phelps *et al.,* 1989). The most intensely studied function of the E7 protein is its effect on the cell cycle mediated by its interaction with the so-called pocket proteins Rb, p107, and p130 (Berezutkaya *et al.,* 1997; Dyson *et al.,* 1989; Classon and Dyson, 2001). These pocket proteins bind the E2F family of transcription factors. Even though binding of E7 and E2F occurs at separate sites within the pocket of the proteins, the binding of E7 brings about the release of E2F (Lee *et al.,*

1998), resulting in the constitutive activation of E2F-dependent genes which hence lead to unchecked cell proliferation (Berezutkaya *et al.,* 1997; Huang *et al.,* 1993; Patrick *et al.,* 1994; Nguyen *et al.,* 2002). Furthermore, binding of E7 to Rb targets the protein for ubiquitin-mediated degradation, leading to a reduction in Rb protein levels (Figure 3) (Phelps *et al.,* 1992; Boyer *et al.,* 1996; McCaffrey *et al.,* 1999; Gonzales *et al.,* 2001). While many other interactions of E7 with cellular proteins have been described and are of importance, such as binding to cyclin A/cdk-2 complexes and p107, histone deacetylases (HDACs), and TBP (Tommassino *et al.,* 1993; McIntyre *et al.,* 1996; Massimi *et al.,* 1997; Phillips *et al.,* 1997; Brehm *et al.,* 1999; Longworth and Laimins, 2004), they will not be described in detail.

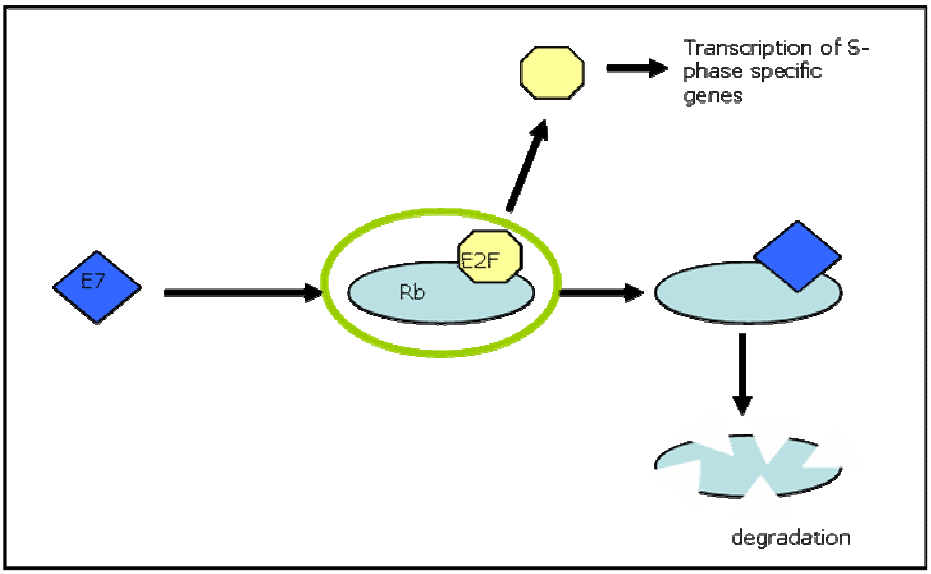


Figure 3: Effect of HPV E7 on Rb. The transcription factor E2F (yellow) is bound to Rb and released at the transition into S-phase. Binding of E7 (blue) to Rb (light blue) releases E2F, resulting in continuous activation of E2F-regulated genes and deregulated cellular proliferation. Furthermore, the binding of E7 to Rb targets the latter for degradation.

However, it should be mentioned that E7 was found to bind to the transcription factor activating protein 1 (AP-1) (described in detail under 1.6) and its subunits c-Jun, JunB, JunD, and c-Fos, an interaction that may be important in tumorigenesis (Antinore *et al.,* 1996; Nead *et al.,* 1998).

1.1.3 The structural proteins L1 and L2

The capsid proteins L1 (57 kDa) and L2 (75 kDa) are expressed in the late stages of the viral life cycle (Stoler *et al.,* 2000). Both proteins are synthesized in the cytoplasm of highly differentiated suprabasal cells (Ozbun and Meyers, 1997) and then are relocalized to the nucleus in order to package the HPV genomes. However, L1 capsomers are

translocated into the nucleus through the nuclear core complex (Nelson *et al.,* 2002), in contrast to L2, which uses the means of two nuclear localization sequences (NLSs) (Day *et al*. 2004).

For formation of the HPV virion, the L1 proteins form pentameric stuctures which are stabilized by disulfite bonds. Upon packaging of the HPV genome, 72 pentameres are formed which contain the major (L1) and minor (L2) capsid proteins at a ratio of about 30:1. While the L1 capsomers have been shown to expose neutralizing epitopes towards the viral surface which show vary extensively between different genotypes (Figure 4) (Dillner *et al.,* 1999), it was proposed that the incorporation of L2 protein into the capsomer contributes to efficient DNA-packaging (Stauffer *et al.,* 1998), and enhances the infectivity of the virus (Roden *et al.,* 2001).

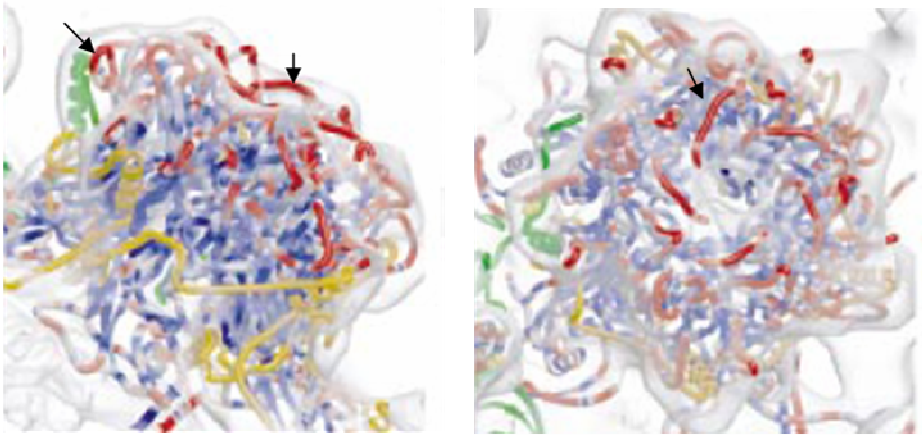


Figure 4: L1 capsomer structure. Two L1 pentamers are shown, colored by sequence conservation among a set of 49 different HPV types (HPV Compendium 1997: http://hpv-web.lanl.gov). Highly variable positions are red, fully conserved positions are blue and positions of average variation are white. All the hypervariable regions lie on the outward-facing surface of the pentamer. The C-terminal arm has several exposed, hypervariable residues (red and indicated by arrows). Modified from Modis *et al*., 2002.

1.1.4 HPV Infection and Cancer

Papillomaviruses are highly species- and tissue specific. Human papillomaviruses infect the squamous epithelial cells of the skin and mucous membranes and bring about the formation of hyperproliferative lesions (warts). The life cycle of the virus is tightly linked to the differentiation of human keratinocytes and initial infection requires the availability of a cell that is still proliferating (zur Hausen, 1996). Within the first stage of the viral life cycle, the virus infects basal epithelial cells and establishes its genome episomally, maintaining a low copy number of 50-100 genome copies per cell. As the infected cell differentiates, the productive stage in the viral life cycle is initiated, where the late genes are expressed and the copy number is enhanced (Dürst *et al.,* 1985; Chow and Broker,

1994; McMurray, *et al.,* 2001). At the end of this stage, the virus DNA is encapsidated in the viral capsomers and the viruses are released by the differentiated cell (Figure 5).

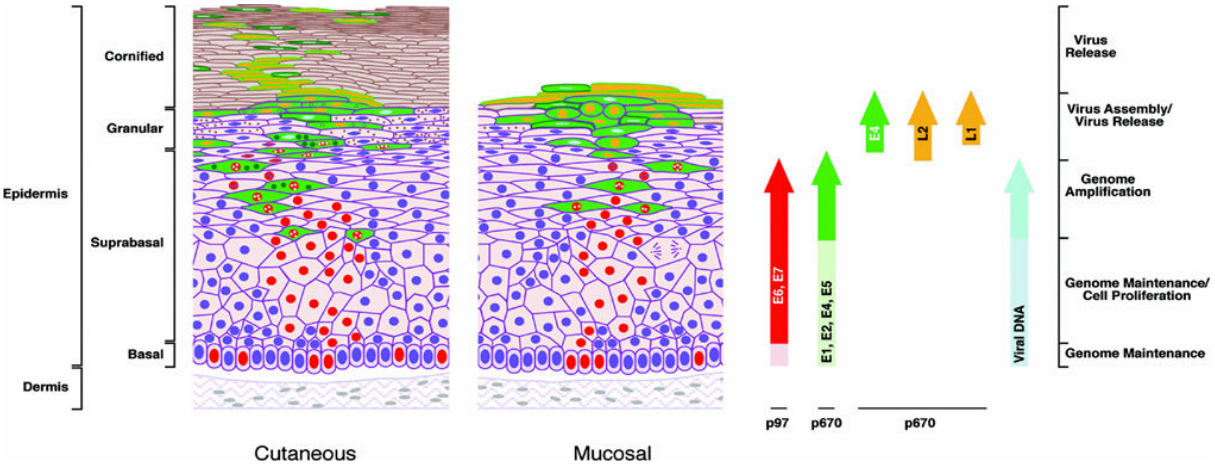


Figure 5: The viral life cycle in differentiating epithelium. The key events that occur following infection are shown diagrammatically on the left. The different cell layers present in the epithelium are indicated on the left. The timing and extent of expression of the viral proteins are summarized using arrows at the right of the Figure and indicated by coloring of nuclei or whole cells. Modified from Doorbar, 2006.

Transmission of HPV often occurs through microlesions in the skin, where cells in the basal layer are exposed to the surface (Oriel *et al.,* 1971). Anogenital HPV types are most commonly transmitted through sexual contacts and infection with those types can rarely be detected in young women prior to first intercourse (Fairley *et al.,* 1992; Andersson-Ellström *et al.,* 1994; Rylander *et al.,* 1994). Additionally, close correlations between the number of sexual partners and HPV infection have been described (Critchlow *et al.,* 1995). Furthermore, oral-genital and oral-anal contacts may lead to an infection of oral sites with anogenital HPV types (Kashima *et al.,* 1992, Gillisson, 2004).

Most HPV types cause only benign cutaneous warts that quickly regress and are considered to be low risk. However, some types of HPV have been associated with human cancers and have been designated high risk. The high-risk HPV types most frequently found in human cancers are HPV16 and HPV18. Cervical cancer is the best-documented human cancer caused by infection. Here, development of malignant tumors is strongly correlated with high-risk HPV infection as their DNA can be detected in up to 99.8% of all cervical cancers. With about 500,000 cases reported worldwide, and about 275,000 cancer deaths, cervical cancer is the second most common cause of cancer deaths in women (Parkin *et al.,* 2005). The most commonly found HPV type in cervical cancer is HPV16 (up to 63%), followed by HPV18 (10-14%), HPV45 (~8%), and HPV31 (~5%) (Bosch *et al.,* 2002; Clifford *et al.,* 2003; Munoz *et al.,* 2003).

In addition to cervical cancer, HPV infection has also been described to play a role in several other malignancies such as of the penis, vulva, vagina, anus, head and neck, and skin (zur Hausen, 1996).

HPV infections of the head and neck region can be accounted for by oral-genital and oral-anal transmission as described above (Kreimer *et al.,* 2004). HPV DNA can be detected in up to 25% of all squamous cell carcinomas of the head and neck region (HNSCCs) (Gillison, 2004) with the highest incidence in tumors of the oropharynx (~35%), followed by the oral cavity (~25%), and the larynx (~25%) with the most common HPV type being HPV16 (Chen *et al*., 2005). There are molecular, pathological, and prognostic differences between HPV-positive and –negative oropharyngeal cancers that include the presence of wt-p53 (Balz *et al.,* 2003), higher sensitivity to radiation therapy (Mellin *et al.,* 2000, Lindel *et al.,* 2001; Strome *et al.,* 2002), and significantly better prognosis for HPV-positive HNSCCs (Gillison 2000).

1.2 THE COTTONTAIL RABBIT PAPILLOMAVIRUS (CRPV)

The cottontail rabbit papillomavirus (CRPV), also known as Shope Papillomavirus was the first animal papillomavirus to be discovered (Shope and Hurst, 1933) and also the first mammalian DNA tumor virus. It was first described by Shope in 1933 to induce cutaneous papillomas (warts) in cottontail rabbits under natural conditions and in domestic rabbits under experimental conditions (Shope, 1935).

In its natural host, CRPV causes a premissive infection where the systemic regression of warts can be detected in a variable proportion of rabbits as a consequence of a specific cell-mediated immune response (Evans and Ito, 1966; Kreider and Bartlett, 1981) resulting in clearance of the infection. Progression of persistent warts into invasive carcinomas as a result of an abortive infection can be observed in approximately 25% of cottontail rabbits. It was found that, when introduced into the skin of domestic rabbits, CRPV induces the formation of papillomas within approximately 8-12 weeks with about 80% of these papillomas progressing into infiltrating and metastazising tumors within the course of a year without the need of co-factors (Shope, 1935). This makes it the "smallest" available animal model available to study the progression of benign papillomas to infiltrating carcinomas that mimics the development and progression of epithelial neoplasia associated with high-risk HPV. Furthermore, it allows for analysing the function of separate papillomavirus proteins and their mutants *in-vivo*. Hence it was found in a study by Jeckel *et al*. (2002) that mutations in a single amino acid in the transactivation domain of CRPV E2 within the context of the CRPV genome led to a drastic decrease of infection, causing only few papillomas none of which progressed into infiltrating carcinomas. One of the proteins shown to be increased in papillomas and carcinomas as compared to uninfected skin is the protease MMP-9 (Behren *et al.,* 2005) which is known to play a role in tumor development and -progression.

1.3 TUMOR PROGRESSION

The invasion of tumor cells into blood and lymph vessels and the establishment of metastases is often a critical stage in tumorigenesis for the patient. While the primary tumor is locally restricted and therefore accessible for treatment, the infiltration into surrounding tissues and organs drastically reduces survival rates.

Tumor invasion itself is a multistage process which can be divided into three major parts, attachment, intravasation, and extravasation (Figure 6). These three steps, however, involve the detachment of malignant cells from the primary tumor, attachment of the cells to the structural barriers such as the basement membranes and surrounding stromal collagenous extracellular matrix (ECM), proteolysis of the barrier, intravasation of the tumor cells into blood and lymph vessels, circulation, attachment to the ECM at a distant site, and extravasation from the vessels to form a new tumor at the new site (Liotta *et al.,* 1993; Stetler-Stevenson, *et al.,* 1993). Upon reaching a certain size, the metastasis, as the primary tumor need to induce the formation of blood vessels, a process termed neovascularization, in order to carry nutrients to all parts of the tumor. Degradation of stromal ECM is also considered essential in tumor-induced angiogenesis (Stetler-Stevenson *et al.,* 2001).

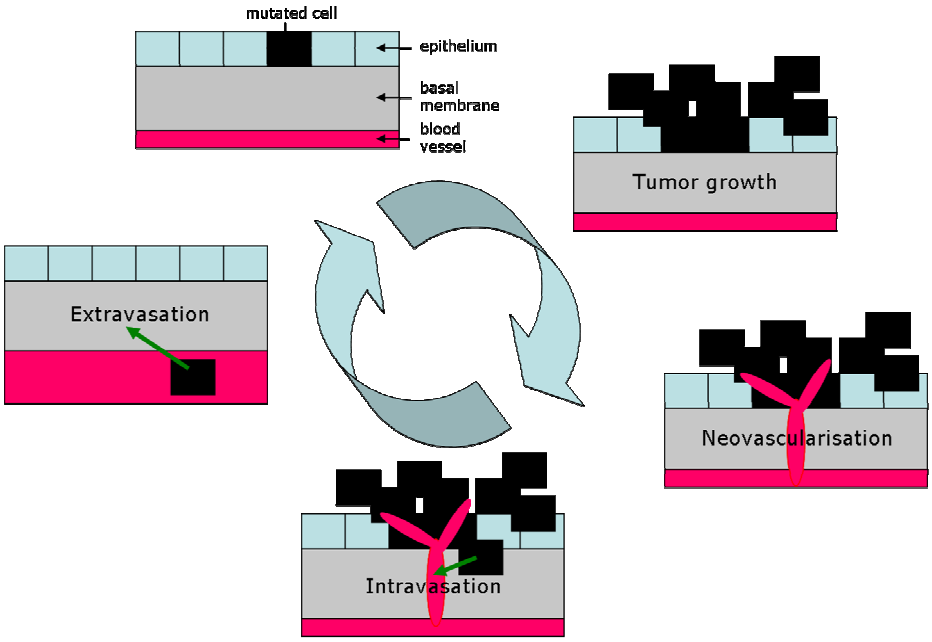


Figure 6: Stages of tumor formation and progression. Initiation occurs when a single cell aquires a mutation which offers a growth adventage over the surrounding cells. At a certain size of the developing tumor requires neovascularization to supply the cell mass with nutrients. Some cells may detach from the cell mass, invade the surrounding tissue, and enter the blood or lymph vessels. At distant sites, the cells exit the circulation, and may be able to establish and form metastases.

The exact mechanisms required for tumor invasion are still to be uncovered, but it has been shown to involve the maintainance of a complex equilibrium of proteases, protease inhibitors and –activators, growth factors, pro-and anti-angiogenetic factors, and other biological effectors.

1.4 PROTEASES

The activity of proteases is required for the enzymatic degradation of matrices such as the extracellular matrix (ECM). Proteases known to play a role in this action include the members of the family of matrix metalloproteinases (MMPs) and the urokinase-plasminogen activator system. The MMPs are able to degrade all components of the ECM, an activity required for physiological processes such as wound healing, angiogenesis, and invasion of immune cells into tissues, but also for pathological events as tumor development and –progression (McCawley and Matrisian, 2000). Binding of the serine-protease urokinase-plasminogen activator (uPA) to its receptor (uPAR) leads to the conversion of plasminogen to plasmin which is able to cleave peptides such as laminin and fibronectin (Sidenius and Blasi, 2003).

The family of matrix-metalloproteinases consists of more than 20 members which can be divided into several subgroups due to differences in structure and substrate specificity. These subgroups include collagenases, stromelysins, gelatinases, membrane-type MMPs, and others. The common structure of all MMPs includes a signal peptide, a propeptide, a catalytic domain with a highly conserved zinc-binding site, a hinge region, and a hemopexin-like domain (Figure 7). Most of the MMPs are secreted as inactive precursors (zymogens) that are proteolytically activated outside the cell by cleavage of the propeptide which exposes the catalytic site of the proteins (Figure 7) (Westermarck and Kähäri, 1999; Lee *et al.,* 2004). MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs) which bind to the zinc-binding site at an equimolar ratio (Gomez *et al.,* 1997).

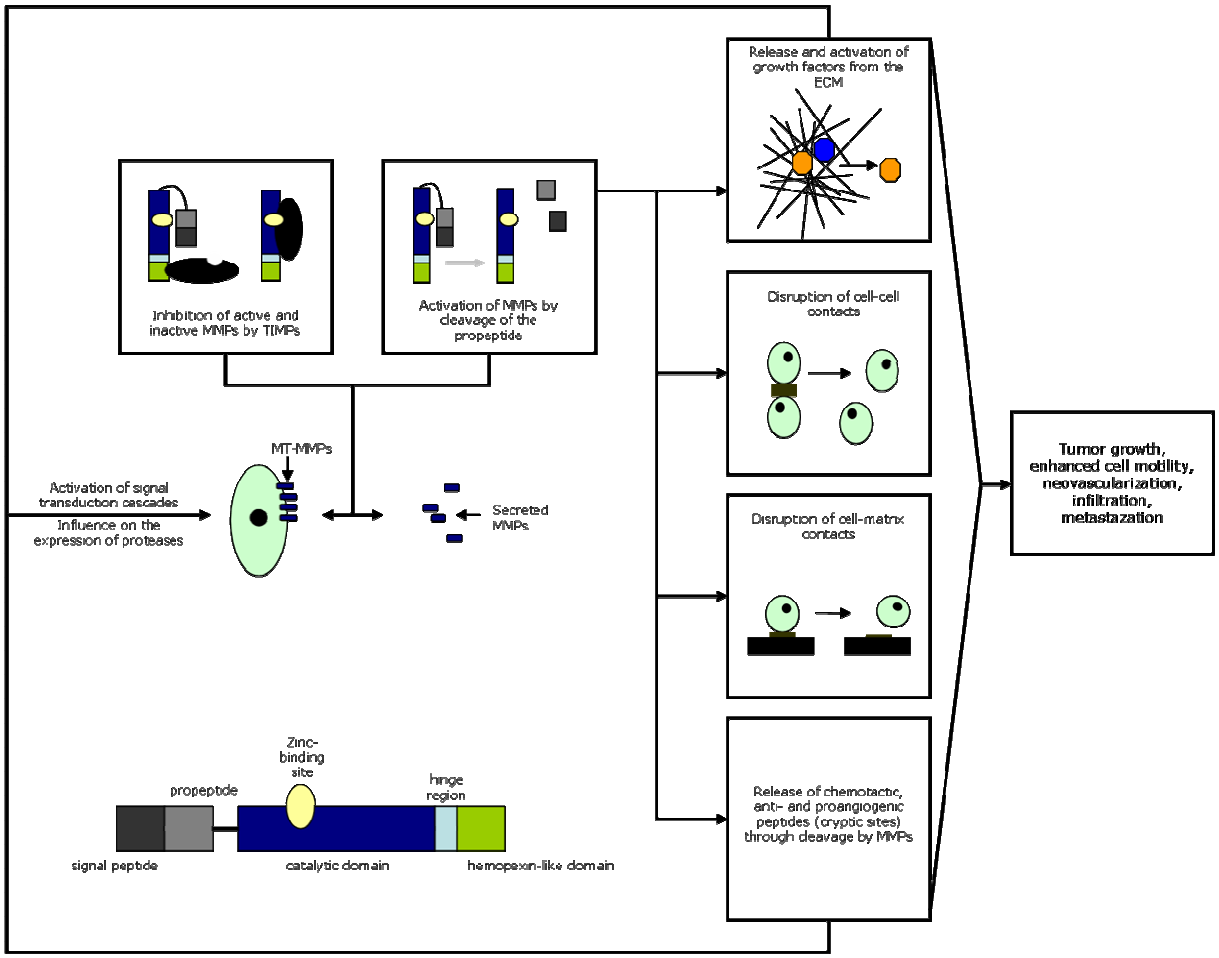


Figure 7: Matrix-metalloproteinases in tumor development and –progression. Illustration of the structure and key roles of MMPs in tumorigenesis.

One of the most important subgroups of MMPs is the group of the gelatinases. This group includes two members, MMP-2 (72kDa), which is expressed in a variety of normal and transformed cells, and MMP-9 (92kDa), which can be detected in keratinocytes, monocytes, and many types of malignant cells (Westermarck and Kähäri, 1999). Substrates of both enzymes include collagen V, a major component of the ECM, collagen I, vitronectin, fibronectin, and gelatin (Price *et al.,* 1997). High MMP-2 expression was detected in gliomas, adenocarcinomas, and melanomas amongst others (Chintala *et al.,* 1999; Hofmann *et al.,* 2000). Overexpression of MMP-9 was detected in many different epithelial cancers such as of the cervix (Davidson *et al*., 1999), head-and-neck, and skin (Juarez *et al.,* 1993; Ikebe *et al.,* 1999; Magary *et al.,* 2000; Davidson *et al.,* 1999; Jones *et al.,* 1999). The overexpression of both gelatinases correlates with the lymph node status of patients with squamous-cell carcinomas of the head-and-neck. For decades, these protases have been investigated under the aspect of degradation of the ECM and the creation of a matrix defect. Only within the last couple of years the focus of researchers has shifted to events that play major roles in tumorigenesis besides infiltration.

It was found that MMP-9 and uPA play an important role in the migration of cells, the release and activation of growth factors, the supply of chemotactic substances, and have been shown to interact with a great number of biological effectors. MMP-9 can induce the motility of tumor cells through the degradation of cell-cell and cell-matrix molecules such as E-cadherin and CD44 and thereby promote the detachment of small cell clusters from the primary (Seiki, 2003; Noe *et al.,* 2001). After proteolytical processing of matrix proteins such as laminin and fibronectin, cryptic sites that can induce chemotaxis and also participate in the regulation of MMP expression may be exposed (Brassart *et al.,* 2001; Björklund and Koivunen, 2005). Furthermore, growth factors that are localized within the ECM such as the insulin-like growth factor II (IGFII) or the transforming growth factor beta (TGF-beta) are released by the action of MMP-9 and may subsequently enhance tumor growth (Yu and Stamenkovic, 2000; Whitelock *et al.,* 1996). Despite these factors promoting tumor growth, the degradation of the ECM also leads to the formation of pro-angiogenic factors. Endostatin and restin, for example, are generated by the cleavage of Collagen XVIII or XV (Patterson and Sang, 1997; Marneros and Olsen, 2001; Ferreras *et al.,* 2000).

The urokinase plasminogen activator (uPA) system consists of the serine proteases uPA and tPA (tissue-type plasminogen activator), the endogenous plasminogen activator inhibitors (PAI-1 and -2), and the uPA receptor (uPAR) (Sidenius and Blasi, 2003). Many studies describe a correlation between uPA/uPAR expression and the diagnosis of cancer (Curino *et al.,* 2004; Nielsen *et al.,* 2005; Ohba *et al.,* 2005). It has also been suggested that the uPA system is involved in multiple steps of tumor progression such as cell migration, adhesion, and signal transduction (Duffy, 2004). The latter can be the result of direct activation of growth factors such as the pro-hepatocyte growth factor (pro-HGF) (Naldini *et al.,* 1995) or an indirect effect as observed after the conversion of plasminogen to plasmin by uPA and the subsequent activation of released cytokines (Sato *et al.,* 1990; George *et al.,* 2005). In some cells, the binding of uPA to uPAR results in the activation of signal-transduction cascades such as the extracellular-regulated kinase (ERK) or the signal transducers and activators of transcription (STAT) pathway (Jo *et al.,* 2002), influencing the expression of other tumor-progression promoting genes.

MMP-9 and uPA are often found to be co-expressed and the expression of both is mainly regulated by the mitogen-activated protein kinase (MAPK) pathway.

1.5 THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAYS

The MAPKs are signal-transduction cascades which traffic signals from the outside of a cell to its nucleus via a number of phosphorylation events. Within the nucleus, the MAPKs

induce changes in the gene expression pattern as a response to the stimulus by direct or indirect modulation of several transcription factors (Robinson and Cobb, 1997). To date, five MAP-kinases have been identified, the extracellular-regulated kinases 1/2 (ERK1/2 or p42/p44), the c-jun N-terminal kinases 1, -2, and -3 (JNK), the p38 kinase with its isoforms p38-α, -β, -γ, and –δ, ERK3/4, and ERK5 (also known as Big Map-Kinase-1, BMK-1) (Chen *et al.,* 2001). However, the most important members are the ERK-cascade as "classical" MAPK, and p38 and JNK, the stress-activated protein kinases (SAPK) (Figure 8).

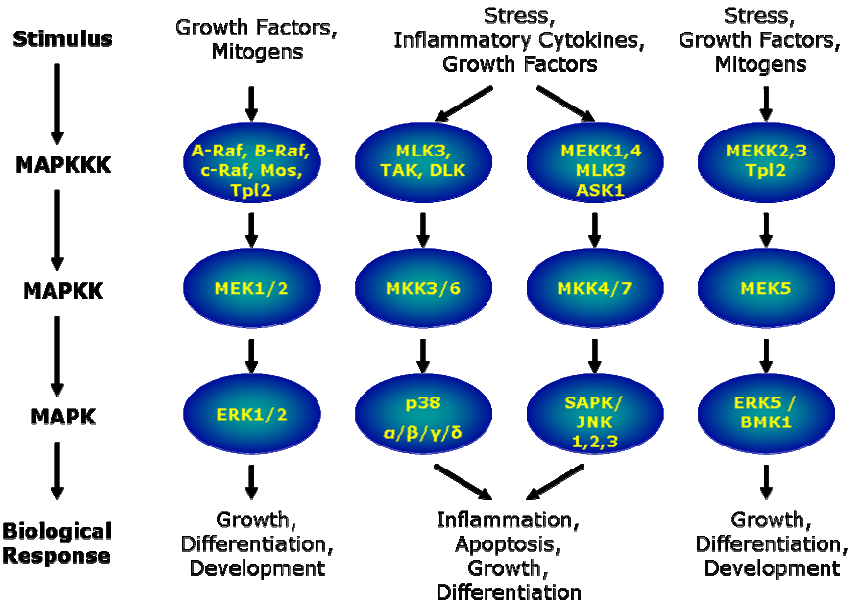


Figure 8: The mitogen-activated protein kinases. Summarizing illustration of the MAPK pathways, their stimuli, and responses. Modified from Cell Signaling Technologies.

A broad spectrum of stimuli is involved in the activation of MAP-kinases, while it can be generalized that the ERK-cascade is stimulated by growth factors, protein kinase C (PKC), and tumor-inducing substances such as phorbol ester (PMA), which mimics Diaglycerin (DAG) derived from the IP3 pathway. JNK and p38 on the other hand are activated by stress factors such as UV-light, osmotical and mechanical stresses, and cytokines (Pearson *et al.,* 2001). All three signal-transduction cascades are constructed similarly and consist of three kinases involved in passing the signal. The first phosphorylation step is mediated by an interaction between MAPK-kinase-kinases (MAPKKKs or MEKKs) with GTP-binding proteins of the Ras/Rho family. In this step, the MAPKKKs are phosphorylated at serin/threonine side-chains and subsequently phosphorylate MAPK-Kinases (MAPKKs or MEKs) which then dually phosphorylate the MAP-Kinases at tyrosin and threonine side-chains (Arbabi and Maier, 2002). Upon activation, the MAP-Kinases then relocate to the nucleus by yet to be determined

mechanisms. All three MAPK-pathways can interact *in-vitro* and *in-vivo* but one of the common features is their ability to influence the transcription factor complex AP-1 through the activation of a diverse set of intranuclear targets (Pearson *et al*., 2001)).

The ERK-cascade is the best characterized of the three MAP-Kinase pathways. It has been described to play a keyrole in cell proliferation and is activated mainly through the action of growth factors (Lewis *et al.,* 1998). This activation usually occurs through autophosphorylation of ligand-bound receptor tyrosine kinases (RTKs), recruitment of the GTP/GDP conversion factor son of sevenless (SOS), activation of members of the Ras family of proteins and their interaction with effectors of the Raf proteins (Daum *et al.,* 1994; Kolch, 2000). Activated Raf can subsequently phosphorylate MEK1 and -2 which leads to the activation of ERK1 and -2.
Ras and Raf isoforms are of special interest in the regulation of growth processes. Mutated Ras has been detected in about 30% of all human malignancies and mutations in Raf proteins have been described to be present in high percentages in certain kinds of tumors (Barbacid, 1987; Bos, 1989). Inhibition of the ERK-cascade has been shown to reduce invasion in transformed cell lines and primary tumors. It was further described that ERK1/-2 as well as JNKK participate in the regulation of *in-vitro* invasion of NIH3T3 cells that have been stably transfected with oncogenic Ras (Janulis *et al.,* 1999).
However, besides its influence on cell proliferation and invasion the ERK-cascade has more recently also been connected to an increase in cell motility. By activating Calpain-2, a protease involved in the detachment and migration of cells and the eventual degradation of focal adhesion kinase (FAK) and other adhesion proteins, ERK governs the migration of cells (Bhatt *et al.,* 2002; Carragher *et al.,* 2003; Huttenlocher *et al*,. 1997).
Apart from the above mentioned activators, several viral proteins such as the late membrane protein-1 (LMP-1) of the Epstein-Barr virus (EBV) and the E5 protein of HPV have also been shown to induce the ERK-cascade and hence influence cell proliferation and the expression of several genes which promote tumor progression (Roberts and Cooper, 1998; Crusius *et al.,* 2000).

To date, four p38 isoforms have been described; p38-α, -β, -μ, and –δ (Enslen *et al.,* 1998). Of these, p38-α and –β are expressed ubiquitously, whereas p38-μ, and –δ-expression is tissue specific (Eckert *et al.,* 2003). All four isoforms share a common amino acid motif (Thr-Gly-Tyr) at which they can become dually phosphorylated (Raingeaud *et al.,* 1995). Inflammatory processes are closely connected to the p38-pathway, while their role in tumorigenesis remains widely unknown. Studies point to an enhanced activation of p38 in infiltration events and propose a role for p38 in the regulation of different tumor-progression promoting proteases such as uPA, MMP-1, MMP-9, and MMP-13 (Bernstein and Colburn, 1989; Reunanen *et al.,* 2002; Han *et al.,*

2002). Active forms of p38 could also be detected in tumors of the lung, breast, and stomach (Greenberg *et al.,* 2002; Salh *et al.,* 2002). On the contrary, a close connection between an enhanced p38 activity with senescence and apoptosis is being discussed in other studies (Kummer *et al.,* 1997; Holmes *et al.,* 2003; Haq *et al.,* 2002). The role of the p38-pathway therefore seems to be individually determined by the cell line, the stimulus, and potential interactions with other signalling proteins.

The JNK-cascades belong to the SAPKs. They are activated by the same stimuli as the p38 cascade and are encoded by three different genes, JNK1, JNK2, and JNK3. While JNK1 and JNK2 are expressed ubiquitously, the expression of JNK3 is limited to certain tissues (Davis, 2000). Within the nucleus, the JNK proteins phosphorylate a set of transcription factors such as ATF-2 and c-jun, and thereby play part in the regulation of the transcription-factor complex AP-1. They also phosphorylate c-jun at residues Ser63 and Ser73 within its N-terminal transactivation domain (Hibi *et al.,* 1993). This stabilizes and activates c-Jun, which results in the enhanced transcriptional activation of c-Jun dependent genes (Musti *et al.,* 1997). Furthermore, the activity of JNK seems to be connected to apoptosis as well as to the transformation of cells, as has been described for the p38-cascade (Pedram *et al.,* 1998; Tournier *et al.,* 2000).

1.6 THE TRANSCRIPTION FACTOR AP-1

The activator protein 1 (AP-1) transcription-factor complex is formed by different dimer compositions of members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) protein families, and some members of the activating transcription factor (ATF) (Bakiri *et al.,* 2002), JDP, and musculoaponeurotic fibrosarcoma (MAF) subfamilies (Matsushima-Hibiya *et al.,* 1998; Li *et al.,* 1999). The Jun and Fos family members belong to the immediate early genes (IE) (Herschman *et al.,* 1991). These IE genes are induced within short periods after the treatment of cells with a variety of physiological, pathological and oncogenic stimuli, like the epidermal growth factor EGF (Kajanne *et al.,* 2007), tumor necrosis factor TNF-a (Brenner *et al.,* 1989), and 12-otetradecanoylphorbol-13-acetate (TPA or PMA) (Espino *et al.,* 2006). Hence, AP-1 is involved in the induction and regulation of cellular responses reaching from proliferation to apoptosis (Angel and Karin, 1991; van Dam and Castellazzi, 2001). Whereas members of the Jun protein family are able to form homo- (Grondin *et al.,* 2007) as well as heterodimers (Turner *et al.,* 1989), the Fos protein family members always heterodimerize (Smeal *et al.,* 1989). Depending on stimulus, tissue type, and duration of induction, the proteins which form the AP-1 transcription factor vary (Hess *et al.,* 2004). Furthermore, the composition of the dimer defines which genes are further induced (Chalmers *et al.,* 2007). Fos and Jun proteins have first been described as the viral

oncoproteins v-Fos and v-Jun in the Finkel-Biskis-Jinkins osteosarcoma virus (Curran *et al.,* 1982) and avian sarcoma virus 17 (Maki *et al.,* 1987), respectively. All AP-1 proteins are characterized by an evolutionary conserved leucin zipper domain (bZIP) which is needed for the dimerisation and a basic domain which allows for the interaction with the DNA backbone (Turner *et al.,* 1989; Smeal *et al.,* 1989) after the dimer is formed. Several different DNA sequences are recognized by AP-1, the most affine of which is the TPA response element (TRE; consensus sequence 5'-TGAG/CTCA-3'), but different dimers also bind to the cAMPrespose element (CRE), the MAF recognition elements (MAREs) and the antioxidant response elements (AREs) (Sng *et al.,* 2004; Hess *et al.,* 2004).
A couple of mechanism exist by which AP-1 activity is regulated including changes in transcription of subunit-encoding genes, control of the stability of mRNAs, and post-translational processing (Hess *et al*., 2004). The most extensively studied mechanism of post-translational control is stress- or mitogen-induced hyperphosphorylation of c-Jun by MAPKs.

AP-1 is required for the transcription of HPV (Offord and Beard, 1990; Kyo *et al*., 1997) in addition to several other cellular transcription factors that regulate HPV transcription from the HPV enhancer region (Chong *et al.,* 1991). However, only few transcription-factor binding sites have been found in all HPV types studied, as was the case for AP-1 (del Mar Pena and Laimins, 2001). There may be more than one binding site for AP-1 in the LCR, such as 3 in HPV16 and 2 in HPV18. Mutagenesis of one binding site in HPV18 leads to suppression of the HPV transcription (Butz and Hoppe-Seyler, 1993). In addition, similar to the regulation of cellular events, the composition of the AP-1 transcription factor complex is essential for HPV transcription (Soto *et al.,* 1999). Previous studies have shown that the induction of changes in the AP-1 composition influences transcription of the HPV genome (Rösl *et al.,* 1997) and therefore that AP-1 composition is pivotal for HPV transcription (Soto *et al.,* 1999). Fusing HPV18 positive HeLa cells with normal fibroblasts yields two different kinds of hybrid cell lines, one of which is non-tumorigenic in immunodeficient nude mice, whereas the other hybrid cell line and HeLa cells induce tumors under the same experimental settings (Soto *et al.,* 1999). Treatment with TNF-α induces down-regulation of HPV expression within the non-malignant hybrid cells (Rösl *et al.,* 1994) due to enhanced expression of Fra-1 and resulting changes in the AP-1 composition from predominantly Jun/Jun or Jun/Fos to Jun/Fra-1 heterodimers. This suppression of viral transcription is not detectable in HeLa cells or in tumorigenic hybrid cells after incubation with TNF-α. Furthermore, ectopical expression of c-fos in non-tumorigenic cells resulted in a change in AP-1 composition towards Jun/Fos herterodimers over Jun/Fra-1 dimers and subsequent conversion into tumorigenic cells (Soto *et al.,* 1999).

1.7 AIM OF THIS STUDY

The action of Matrix-metalloproteinse 9 has been associated with tumor-initiation and –progression. MMP-9 overexpression was shown in a variety of human cancers. In CRPV induced papillomas and carcinomas as compared to uninfected rabbit skin, an expression of MMP-9 has been observed. Further, it has been shown that CRP-viruses encoding transactivation-deficient E2 proteins are impaired in the induction of papillomas.

The aim of this study was to clone and sequence the rabbit MMP-9 promoter and to investigate the mechanism by which the Papillomavirus early proteins, with emphasis on E2, induce activation of the MMP-9 promoter. This was meant to be schieved by meant of promoter deletion mutants and transcription-factor binding site mutants. Additionally, MAP-kinases involved in the activation of the promoter were to be investigated using chemical inhibitors of the respective MAPKs.
All experiments carried out in the rabbit system were parallel to be carried out in the human system using HPV16 early proteins and the human MMP-9 promoter to enable the comparison of the two systems.

Localization studies using HPV16 E2 were to be conducted in later stages of the study to obtain an insight whether the localiation of the proteins impacts the induction of MMP-9 expression.

2 EXPERIMENTAL PROCEDURES

2.1 MATERIALS

2.1.1 Chemicals

All chemicals used in this study were of analytical grade. They were purchased from Carl Roth (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), Sigma (Munich, Germany), and Jena Biosciences (Jena, Germany) unless stated otherwise.

2.1.2 Oligonucleotides

Table 1: List of oligonucleotides.

Name	Sequence
AP1	5'-GTAATACGACTCACTATAGGGC-3'
AP2	5'-ACTATAGGGCACGCGTGGT-3'
MMP9 Promoter for	5'- GCCTCAGAGACCCACTCCTTCCG-3'
MMP9forwBegin	5'-AACCCAGTCGCCTATACCTG-3'
MMP9forwSeq	5'-GCCGGCCGTGAGCCTGTCCTAC-3'
MMP9forwSeq2	5'-GGGTGAGAAGTAGAGCTGGGGCTG-3'
MMP9neu4	5'-CAGCCGGCCAGCCTTCTCTGACTCCAAG-3'
MMP9PromreversSeq	5'-CCCGCAGGCTACGTCCTCCCTCCTGG-3'
MMP9PromrevSeq2	5'-CGGAAGGAGTGGGCTCTCTAGG-3'
MMP9PromSabfor	5'-GGGAGAGGAAGCTGAGTC-3'
MMP9Sabrev	5'-AGGCAAGTGCTGACTCAG-3'
NZWMMP9revnest2	5'-ATGGTGAGGGGAGCAGCGTCTGGC-3'
MMP9ForKpn2	5'-AACCCATTCGCC*GGTACC*TGGTCCTCTC-3'
MMP9RevKpn2	5'-GAGCGCCA*GGTACC*AGGGGCTG-3'
MMP9PhumKpnF	5'-GCA*GGTACC*TACCCACTTCTATACCTGGG-3' (*Kpn*I)
MMP9PhumKpnR	5'-*GGTACC*AGAGGCTCATGGTGAGGG-3' (*Kpn*I)
rMP-1550	5'-GGAACT*GGTACC*AGAGCTGGGACG-3' (*Kpn*I)
rMP-1225	5'-GAACGGGGA*GGTACC*AGCTGAGG-3' (*Kpn*I)
rMP-953	5'-GGGGAGAGAATGCCA*GGTACC*TTGTAC-3' (*Kpn*I)
rMP-717	5'-GTCACAGCAT*GGTACC*AACACCTGCC-3' (*Kpn*I)
rMP-493	5'-CCAGCT*GGTACC*GGGAGGAGG-3' (*Kpn*I)
rMP-144	5'-GTTCTTT*GGTACC*TTCTCATGCTGGGGC-3' (*Kpn*I)
rMPrev	5'-GAGCGCCA*GGTACC*AGGGG-3' (*Kpn*I)
MMP9Ende	5'-GCTCCCCTCACCATGAGCC-3'
hMP-1785	5'-TAA*GGTACC*TGGCACATAGTAGGCC-3' (*Kpn*I)

hMP-1261	5'-AAT*GGTACC*ATGGAGCAGGGC-3' (*Kpn*I)
hMP-829	5'-ATT*GGTACC*TCACATCAATTTAGGGACAAAG-3' (*Kpn*I)
hMP-670	5'-TC*GGTACC*CCTGAAGATTCAGC-3' (*Kpn*I)
hMPrev	5'-CTGCCAGA*GGTACC*CTCATG-3' (*Kpn*I)
rMP-AP1-distal-for	5'-CGCCCAGCTGGAGCCGGGAGGAGGAAGC**G**GAGTCAGGAG GAGGGC-3'
rMP-AP1-distal-rev	5'-GCCCTCCTCCTGACTC**C**GCTTCCTCCTCCCGGCTCCAGC TGGGCG-3'
rMP-AP1-proximal-for	5'-CCCCCCTGCACCGGCCC**G**GAGTCAGGCACTTGCCTGC-3'
rMP-AP1-proximal-rev	5'-GCAGGCAAGTGCCTGACTC**C**GGGCCGGTGCAGGGGGG-3'
rMP-MyoDmt-for	5'-CACTCTGCAAATACTTGCAACAGCC**GC**GTGCCGGGCCAA GAGC-3'
rMP-MyoDmt-rev	5'-GCTCTTGGCCCGGCAC**GC**GGCTGTTGCAAGTATTTGCAG AGTG-3'
rMP-E47mt-for	5'-GCGTCTCCCACG**A**AGGTGGCAGGGACTCAAGTACTTGAG CCG-3'
rMP-E47mt-rev	5'-CGGCTCAAGTACTTGAGTCCCTGCCACCT**T**CGTGGGAGA CGC-3'
rMP-ETS1-for	5'-CCCAGGGTGACACCAGCA**T**GAAGCTGGGTGAGAAGTAGA GC-3'
rMP-ETS1-rev	5'-GCTCTACTTCTCACCCAGCTTC**A**TGCTGGTGTCACCCTG GG-3'
rMP-ETS2-for	5'-GGCTGGAACCCAGGCCCTCCAGTGCA**T**GATGCAAGCATC C-3'
rMP-ETS2-rev	5'-GGATGCTTGC**A**TCATGCACTGGAGGGCCTGGGCTCCAGC C-3'
hMP-E2mut-for	5'-GCAGCTTAGAGCCCAATAAC**A**TGGTTT**T**GTGATTCCAAG-3'
hMP-E2mut-rev	5'-CTTGGAATCAC**A**AAACCA**T**GTTATTGGGCTCTAAGCTGC-3'
hMP-AP1-distal-for	5'-GAAGCAGGGAGAGGAAGCT**TT**GTCAAAGAAGGCTGTCAG G-3'
hMP-AP1-distal-rev	5'-CCTGACAGCCTTCTTTGAC**AA**AGCTTCCTCTCCCTGCTT C-3'
hMP-AP1-proximal-for	5'-CACACCCTGACCCCT**TT**GTCAGCACTTGCCTG-3'
hMP-AP1-proximal-rev	5'-CAGGCAAGTGCTGAC**AA**AGGGGTCAGGGTGTG-3'
EEcoRIF	5'-TAT*GAATTC*ATCCTCCAAAATCGGATC-3' (*Eco*RI)
EXhoR	5'-ATA*CTCGAG*ATCGTCAGTCAGTCA-3' (*Xho*I)
eGFPtagF	5'-TA*GAATTCTA*ATTGGTGAGCAA-3' (*Eco*RI)

16E2I73A	5'-GGCTGTATCAAAGAATAAAGCATTACAAGCA**GC**TGAACTGC AACTAACGT-3'
16E2I73A-r	5'-ACGTTAGTTGCAGTTCA**GC**TGCTTGTAATGCTTTATTCTTTG TACAGCC-3'
NLS1 (107/108)	5'-GTGTATTTAACTGCACCAA**A**A**C**GATGTATAAAAAAACAT GGATATACAGTGGAAGTGC-3'
NLS1 (107/108)rev	5'-GCACTTCCACTGTATATCCATGTTTTTTTATACATC**G**T**T**TTG GTGCAGTTAAATACAC-3'
NLS2 (241/244)	5'-CACAGACGACTATCCAGCGA**A**AAAGATCA**CG**GCCAGACAC CGGAAACCCC-3'
NLS2 (241/244)rev	5'-GGGGTTTCCGGTGTCTGGC**CG**TGATCTTT**T**TCGCTGGATA GTCGTCTGTG-3'
NESmt L-P	5'- GCATTACAAGCAATTGAACTGCAAC**C**AACGTTAGAAACAA TATATAACTCAC-3'
NESmt L-P rev	5'-GTGAGTTATATATTGTTTCTAACGTT**G**GTTGCAGTTCAATT GCTTGTAATGC-3'
NESdel (del217-237)	5'-GCTGTATCAAAGAATAAAGCATTACAAGCA_GAAACAATA TATAACTCACAATATAGTAAT-3'
NESdel (del217-237)rev	5'-ATTACTATATTGTGAGTTATATATTGTTTC_TGCTTGTAAT GCTTTATTCTTTGATACAGC-3'
NLSDBDmt	5'-GATGCTAATACTTTAAAATGTTTA**G**GATAT**G**GATTTA**C**AAAG CATTGTACATTGTATACT-3'
NLSDBDmt rev	5'-AGTATACAATGTACAATGCTTT**G**TAAATC**C**ATATC**C**TAAACA TTTTAAAGTATTAGCATC-3'
16E2 306/307	5'-ACTTTAAAATGTTTAAGATATAGATTT**GC**A**GC**GCATTGTACA TTGTATACTGCAGTGTC-3'
16E2 306/307 rev	5'-GACACTGCAGTATACAATGTACAATGC**GC**T**GC**AAATCTATA TCTTAAACATTTTAAAGT-3'
QPRCPlauHRev	5'-CCAGCTCACAATTCCAGTCA-3'
QPRCPlauHFor	5'-GTCACCACCAAAATGCTGTG-3'
QPCRJUNhFor	5'-TAACAGTGGGTGCCAACTCA-3'
QPCRJUNhRev	5'-TTTTCTCTCCGTCGCAACTT-3'
QPCRMMP9hFor	5'-CGACGTCTTCCAGTACCGA-3'
QPCRMMP9hRev	5'-CTCAGGGCACTGCAGGAT-3'
QPCRACTBhFor	5'-GCACAGAGCCTCGCCTT-3'
QPCRACTBhRev	5'-CCTTGCACATGCCGGAG-3'
QPCRGAPDHhFor	5'-CTCTGCTCCTCCTGTTCGAC-3'

QPCRGAPDHhRev	5'-TTAAAAGCAGCCCTGGTGAC-3'
qPCRHPV16E2	5'-ATGGAGGACTCTTTGCCACG-3'
qPCRHPV16E2rev	5'-CCTGTTGGTGCAGTTAAATACAC-3'
QPCRHPV16E6for	5'-ACTGCAATGTTTCAGGACCC-3'
QPCRHPV16E6rev	5'-TCAGGACACAGTGGCTTTTG-3'
QPCRHPV16E7for	5'-CCCAGCTGTAATCATGCATG-3'
QPCRHPV16E7rev	5'-TGCCCATTAACAGGTCTTCC-3'
rMMP9qPCRfor	5'-CTGGGCAAGGGCGTCGTGGTC-3'
rMMP9qPCRrev	5'-CGTGGTGCAGGCGGTGTAGGAG-3'
qPCRCRPVE2	5'-ATGGAGGCTCTCAGCCAGCGCTTAG-3'
qPRCCREPE2rev	5'-CCTTTCTCTACTGGTATCCTGCAATGTCCATGG-3'
QuantiTect MAPK1 Primer	Hs_MAPK1_1_SG QuantiTect Primer Assay (QT00065933)
QuantiTect MAPK3 Primer	HS_MAPK3_1_SG QuantiTect Primer Assay (QT00000532)
pCMV-HA/myc SeqF	5'- GAT CCG GTA CTA GAG GAA CTG AAA AAC-3'
pCMV-HA/myc SeqR	5'- TTA CAA ATA AAG CAA TAG CAT CAC-3'

Restriction sites are indicated in italics, restriction endonucleases are given in parentheses, nucleotide exchanges are marked in bold.

2.1.3 siRNAs

Table 2: List of siRNAs.

Name	Sequence (5' → 3')
siRNA MAPK3 sense	r(CGU CUA AUA UAU AAA UAU A)dTdT
siRNA MAPK3 antisense	r(UAU AUU UAU AUA UUA GAC G)dGdG
siRNA MAPK1 sense	r(CAU GGU AGU CAC UAA CAU A)dTdT
siRNA MAPK1 antisense	r(UAU GUU AGU GAC UAC CAU G)dAdT
siRNA Raf1 sense	r(GAC GUU CCU GAA GCU UGC C)dTdT
siRNA Raf1 antisense	r(GGC AAG CUU CAG GAA CGU C)dTdT

2.1.4 Plasmids

Table 3: List of plasmids.

Name	Characteristic trait	Reference
pCAT3 Basic	chloramphenicol acetyltransferase gene (*CAT)* without promoter	Promega, Mannheim, Germany
pCATrMPFL	full length rabbit MMP-9 promoter; *CAT*	this study
pCATrMP-1225	1225bp of the rabbit MMP-9 promoter; *CAT*	this study

pCATrMP-953	953bp of the rabbit MMP-9 promoter; *CAT*	this study
pCATrMP-717	717bp of the rabbit MMP-9 promoter; *CAT*	this study
pCATrMP-493	493bp of the rabbit MMP-9 promoter; *CAT*	this study
pCATrMP-144	144bp of the rabbit MMP-9 promoter; *CAT*	this study
pCATrMP AP-1 proximal mt	1550bp of the rabbit MMP-9 promoter with a mutated proximal AP-1 site; *CAT*	this study
pCATrMP AP-1 distal mt	1550bp of the rabbit MMP-9 promoter with a mutated distal AP-1 site; *CAT*	this study
pCATrMP ETS 1 mt	1550bp of the rabbit MMP-9 promoter with a mutated ets 1 site; *CAT*	this study
pCATrMP ETS 2 mt	1550bp of the rabbit MMP-9 promoter with a mutated ets 2 site; *CAT*	this study
pCATrMP-1225 E47mt	1225bp of the rabbit MMP-9 promoter with a mutated E47 site; *CAT*	this study
pCATrMP-1225 MyoD mt	1225bp of the rabbit MMP-9 promoter with a mutated MyoD site; *CAT*	this study
pCATrMP-953 E47 mt	953bp of the rabbit MMP-9 promoter with a mutated E47 site; *CAT*	this study
pCATrMP-953 MyoD mt	953bp of the rabbit MMP-9 promoter with a mutated MyoD site; *CAT*	this study
pSG5	SV40 and T7 promoter controlled; ampicillin resistence	Stratagene, La Jolla, CA, USA
pSG5 CRPV E2	*CRPV E2*; ampicillin resistence	Jeckel et al., 2002
pSG5 CRPV E6	*CRPV E6*; ampicillin resistence	Prof. Iftner, Tübingen
pSG5 CRPV E7	*CRPV E7*; ampicillin resistence	Prof. Iftner, Tübingen
pCAThMPFL	full length human MMP-9 promoter; *CAT*	this study
pCAThMP-1785	1785bp of the human MMP-9 promoter; *CAT*	this study
pCAThMP-1261	1261bp of the human MMP-9 promoter; *CAT*	this study
pCAThMP-829	829bp of the human MMP-9 promoter; *CAT*	this study

pCAThMP-670	670bp of the human MMP-9 promoter; *CAT*	this study
pCAThMP AP-1 proximal mt	full length human MMP-9 promoter with a mutated proximal AP-1 site; *CAT*	this study
pCAThMP AP-1 distal mt	full length human MMP-9 promoter with a mutated distal AP-1 site; *CAT*	this study
pCAThMP E2mt	full length human MMP-9 promoter with a mutated E2 binding site; *CAT*	this study
pMethionine neo	neomycin resistence	Grant et al., 1997
pMeth TAM67	*TAM67* (truncated c-jun); neomycin resistence	Grant et al., 1997
pcDNA3	CMV, Sp6, and T7 promoter controlled; neomycin and ampicillin resistence	Invitrogen, Karlsruhe, Germany
pcDNA3 ERK1 KR	*ERK1*:ERK1 K71R	Dr. A. Behren, Heidelberg
pcDNA3 ERK2 KR	*ERK2*:ERK2 K52R	Dr. A. Behren, Heidelberg
pcDNA3 MEKK1 Km	*MEKK1*:ERK1 K432A	Dr. A. Behren, Heidelberg
Uro2345	2345bp of the urokinase-plaminogen activator promoter	Blasi et al.
pCMV-HA	CMV promoter controlled; HA-tag upstream of MCS	TakaraBio Europe/Clontech, St.- Germain-en-Laye, France
pCMV-HA HPV6bE2	*HPV6bE2*	this study
pCMV-HA HPV6bE6	*HPV6bE6*	this study
pCMV-HA HPV6bE7	*HPV6bE7*	this study
pCMV-HA HPV16E2	*HPV16E2*	this study
pCMV-HA HPV16E6	*HPV16E6*	this study
pCMV-HA HPV16E7	*HPV16E7*	this study
pCMV-HAeGFP	*eGFP*	this study
pCMV-HAeGFP HPV6bE2	*eGFP; HPV6bE2*	this study
pCMV-HAeGFP HPV6bE6	*eGFP; HPV6bE6*	this study
pCMV-HAeGFP HPV6bE7	*eGFP; HPV6bE7*	this study
pCMV-HAeGFP HPV16E2	*eGFP; HPV16E2*	this study
pCMV-HAeGFP HPV16E6	*eGFP; HPV16E6*	this study
pCMV-HAeGFP HPV16E7	*eGFP; HPV16E7*	this study
pCMV-HA HPV16E2 NLS1	*HPV16E2*:E2 NLS1 mt	this study
pCMV-HA HPV16E2 NLS2	*HPV16E2*:E2 NLS2 mt	this study

pCMV-HA HPV16E2 NESmt	*HPV16E2*:E2 NESmt	this study
pCMV-HA HPV16E2 NESdel	*HPV16E2*:E2 NESdel	this study
pCMV-HA HPV16E2 NLSdel	*HPV16E2*:E2 NLSdel	this study
pCMV-HA HPV16E2 I73A	*HPV16E2*:E2 I73A	this study
pCMV-HA HPV16E2 307/307	*HPV16E2*:E2 306/307	this study
pCMV-HAeGFP HPV16E2 NLS1	*eGFP; HPV16E2*:E2 NLS1 mt	this study
pCMV-HAeGFP HPV16E2 NLS2	*eGFP; HPV16E2*:E2 NLS2 mt	this study
pCMV-HAeGFP HPV16E2 NESmt	*eGFP; HPV16E2*:E2 NESmt	this study
pCMV-HAeGFP HPV16E2 NESdel	*eGFP; HPV16E2*:E2 NESdel	this study
pCMV-HAeGFP HPV16E2 NLSdel	*eGFP; HPV16E2*:E2 NLSdel	this study
pCMV-HAeGFP HPV16E2 I73A	*eGFP; HPV16E2*:E2 I73A	this study
pCMV-HAeGFP HPV16E2 306/307	*eGFP; HPV16E2*:E2 306/307	this study
pCMV dsRED-Express	CMV promoter controlled *dsRed*; kanamycin/neomycin resistence	TakaraBio Europe/Clontech, St.- Germain-en-Laye, France

2.1.4 Cell Lines and Bacterial Strains

Table 4: List of cell lines and bacterial strains.

Name		Supplier
E. coli XL1-Blue	chemically competent bacteria	Stratagene, La Jolla, CA, USA
E. coli NEB5alpha	chemically competent bacteria	New England Biolabs, Frankfurt/Main, Germany
Rab-9	spontaneously immortalized rabbit epithelial cell line	ATCC
C33A	HPV-negative human cervical cancer cell line	ATCC
HaCat	spontaneously immortalized human keratinocytes	Prof. Dr. A. Alonso, DKFZ

HPV16 E6	HPV16 E6 immortalized primary human keratinocytes	Prof. Dr. F. Roesl, DKFZ
HPV16 E7	HPV16 E7 immortalized primary human keratinocytes	Prof. Dr. F. Roesl, DKFZ
HPV16E6/E7	HPV16 E6 and E7 immortalized primary human keratinocytes	Prof. Dr. F. Roesl, DKFZ
NIH 3T3	spontaneously immortalized mouse fibroblasts	DSMZ, Braunschweig, Germany

2.1.5 Antibodies

Table 5: List of antibodies.

Antibody	Supplier	Species	Dilution
Actin	Santa Cruz (sc-1616)	rabbit polyclonal	1:100 (IF) 1:3000 (WB)
c-jun	Cell Signaling Technology (#9165)	rabbit polyclonal	1:1000
Phospho-c-Jun (Ser63) II	Cell Signaling Technology (#9261)	rabbit polyclonal	1:1000
c-fos	Cell Signaling Technology (#2250)	rabbit polyclonal	1:1000
c-Raf	Cell Signaling Technology (#9422)	rabbit polyclonal	1:1000
GFP	Roche (11 814 460 001)	mouse monoclonal	1:1000
Monoclonal Anti-HA Clone HA-7	Sigma (H 9658)	mouse monoclonal	1:100 (IF) 1:3000 (WB)
JNK	Santa Cruz (sc-474)	rabbit polyclonal	1:2000
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling Technology (#9251)	rabbit polyclonal	1:1000
SV40 KT3 tag (KT3)	Santa Cruz (sc-58664)	mouse monoclonal	1:100 (IF) 1:1000 (WB)
MMP-9 (C-20)	Santa Cruz (sc-6840)	goat polyclonal	1:1000
p38 MAPK	Santa Cruz (sc-535-G)	goat polyclonal	1:3000

Phospho-p38 MAP Kinase (Thr180/Tyr182)	Cell Signaling Technology (#9211)	rabbit polyclonal	1:1000
p44/42 MAP Kinase	Cell Signaling Technology (#9102)	rabbit polyclonal	1:3000
Phospho-p44/42 MAP Kinase (Thr202/Tyr204) (E10)	Cell Signaling Technology (#9106)	mouse monoclonal	1:1000
goat anti-mouse IgG-HRP	Santa Cruz (sc- 2005)	goat	1:3000-1:5000
donkey anti-rabbit IgG-HRP	Santa Cruz (sc- 2313)	donkey	1:3000-1:5000
donkey anti-goat IgG, HRP	Santa Cruz (sc- 2020)	donkey	1:3000-1:5000
IRDye 680 Conjugated Goat Anti-Mouse IgG	926-32220 Licor	goat polyclonal	1:10000
PF-780 Goat Anti-Rabbit IgG	PK-PF780-AK-R1 PromoKine	goat polyclonal	1:2000
IRDye 800 Conjugated Donkey Anti-Goat IgG	926-32214 Licor	donkey polyclonal	1:10000
AlexaFluor488 Goat Anti-Mouse IgG (H+L)	A-11001 Molecular Probes	mouse	1:500
Cy3-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)	111-165-003 Dianova (Jackson ImmunoResearch)	rabbit	1:200
Cy3-conjugated AffiniPure Rabbit Anti-Goat IgG, Fc Fragment Specific	305-165-008 Dianova (Jackson ImmunoResearch)	goat	1:200

2.2 MOLECULAR BIOLOGY

2.2.1 Amplification of DNA

DNA was amplified using a BioRad Mini Thermocycler (BioRad, Munich, Germany) using either Taq (New England Biolabs, Frankfurt/Main, Germany), Pfu (Fermentas, St. Leon-Rot, Germany), or Hot-Start FidelITaq Polymerase (USB, Staufen, Germany) with supplied buffer according to the manufacturers' recommendations and dNTPs purchased from Fermentas. Amplification was carried out according to the protocol described below.

94°C	2 min	
94°C	30 sec	
*	45 sec	25-35x
72°C	30 sec – 90 sec[#]	
72°C	5 min	
4°C	∞	

* Annealing temperature was usually about 2°C below melting temperature of the respective primers used or determined by gradient PCR.

[#] Elongation time was dependent on the length of the desired fragment.

2.2.2 Agarose Gel Electrophoresis

Gel electrophoresis was carried out in horizontal electrophoresis chambers (BioRad, Munich, Germany). 1.0 – 2.0 % agarose gels were prepared by dissolving UltraPure Agarose (Invitrogen, Karlsruhe, Germany) in 1x Tris-acetate-EDTA (TAE) buffer and adding of 10µl of ethidium bromide (1mg/ml) to each 150ml of agarose solution. 10x loading buffer was added to the DNA samples for subsequent electrophoresis at 100V for 30 to 75 minutes depending on DNA size and gel concentration. After electrophoresis, DNA fragments were visualized under UV light (302 nm) and photographed.

2.2.3 Construction of a DNA-library

A DNA library was constructed using the GenomeWalker Kit (Clonetech, Saint-Germain-en-Laye, France). DNA from a New Zealand White rabbit's skin was digested using the supplied endonucleases following the manufacturer's manual. Amplification of the DNA region of interest was carried out using the supplied adaptor-specific primers (AP1 and

AP2) and gene-specific primers (MMP9neu4 und MMP9revnested2). The PCR protocols used were the two-step protocols suggested in the manual:

PCR1:
primer 1: AP1
primer 2: MMP9neu4

94°C	2 sec	5x
72°C	3 min	

94°C	2 sec	20x
67°C	3 min	

67°C	4 min
4°C	∞

PCR2:
primer 1: AP2
primer 2: MMP9revnested2

94°C	2 sec	7x
72°C	4 min	

94°C	2 sec	32x
67°C	4 min	

67°C	4 min
4°C	∞

2.2.4 DNA-Sequencing

Sequencing of the major part of the MMP-9 promoter was conducted at GATC Biotech (Constance, Germany). The remainder of the sequence was analyzed by V. Benes (EMBL Heidelberg, Germany) after cloning of the fragment into the pCAT3 Basic reporter gene vector (Promega).

All other constructs and mutants were sequenced for confirmation at GATC Biotech (Constance, Germany).

2.2.5 Cloning

2.2.5.1 Restriction Reactions

Restriction of vector and insert DNAs was carried out using restriction endonucleases from Fermentas or New England Biolabs and was performed in the supplied buffers and at appropriate temperatures. Vectors were digested for 1 to 2 hours whereas fragments were usually incubated overnight at room temperature.

2.2.5.2 Alkaline-Phosphatase Treatment

After digestion, vectors were treated with 1 µg Calf-intestinal Alkaline-Phosphatase (CIAP; Fermentas, St. Leon-Rot, Germany) for dephosphorylation of ends.

2.2.5.3 Ligation Reactions

Ligations were performed using the Ligate-IT Rapid Ligation Kit (USB, Staufen, Germany) with a 3:1 insert:vector ratio as determined by gel electrophoresis for all samples otherwise following the supplied protocol.

2.2.6 Transformation of chemically competent bacteria

2.2.6.1 Preparation of chemically competent *E.coli*

For preparation of chemically competent cells, the following solutions were used:

Medium YT**

8 g Bacto-Trypton
8 g Bacto-Hefeextrakt
5 g NaCl

Add H_2O_{bidest} to yield 1 liter
Adjust pH to 7.5

Following autoclavation the medium was supplemented with:

20 ml 1 M $MgSO_4$
10 ml 1 M KCl

TFB I

	Stock solution	in 200 ml
30 mM potassium acetate	0.5 M	12 ml
50 mM mangane chloride	1 M	10 ml
100 mM rubidium chloride	0.5 M	40 ml
10 mM calcium chloride	0.5 M	4 ml
15 % glycerol		30 ml
H_2O_{bidest}		104 ml

Adjust pH to 5.8

Store at 4°C

TFB II

	Stock solution	in 50 ml
10 mM NaMOPS	0.1 M	5 ml
75 mM calcium chloride	0.5 M	7.5 ml
10 mM rubidium chloride	0.1 M	1 ml
15 % glycerol		7.5 ml
H_2O_{bidest}		29 ml

Adjust pH to 7.0

Store at 4°C

2-2.5 ml YT** medium were inoculated with a single *E.coli* NEB5α-colony and incubated overnight on a rotary shaker (200 rpm). Two 5 ml tubes with YT** were each inoculated with 50 µl of the overnight culture at 37°C and 200 rpm. At an OD_{600} of 0.8 each culture was added to a new flask containing 100 ml pre-warmed YT**-medium. This main culture was incubated on a rotary shaker until it reached an OD_{600} of 0.4 -0.55 and then was transferred to sterile and chilled 50 ml centrifuge tubes and kept on ice for 5 minutes. After centrifuging at 4,000 g at 4°C for 5 minutes, the supernatant was decanted and the tubes placed back on ice. 10 ml of TFB I were added to each of the tubes and cells were resuspended before centrifuging under the previously mentioned conditions. The supernatant was again decanted, the cells were carefully resuspended in 2 ml TFB II, and aliquoted into 50 µl fractions which were shock-frozen in liquid nitrogen and stored at -80°C until further use.

2.2.6.2 Transformation of cells

For transformation, aliquoted competent cells were thawed on ice. 10-100 ng of DNA were added to the cells and the mixture was incubated on ice for 30 minutes. After incubation, the mixture was heat-shocked in a thermo-block at 42°C for 2 minutes and

returned to ice for another 5 minutes. 200µl of LB medium were added and the cells were subsequently incubated for 1 hour at 37°C and 250rpm. 100-200µl of the cell suspension were then plated into LB agar containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.7 Mutagenesis

Site-directed mutagenesis was carried out using the Site-directed mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) or the Site-directed mutagenesis XL Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's recommendations.

2.2.8 Plasmid Purification

Plasmid purification was carried out using either the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

2.2.9 Isolation of genomic DNA from cells and tissue samples

Genomic DNA from cultured cells and tissue samples was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.2.10 RNA Isolation

Isolation of RNA from cultured cells was conducted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the supplied protocol including an On-Column DNase digestion using the RNase-Free DNase Set (Qiagen, Hilden, Germany).

2.2.11 Quantification of DNA and RNA Samples

The amount of DNA or RNA present in samples was determined using a NanoDrop Biophotometer (PeqLab Biotechnologie, Erlangen, Germany).

2.2.12 Reverse Transcription (RT)- PCR

For reverse transcription, the amount of RNA of samples to compare was equalized with RNase-free water. RT-PCR was carried out using the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol using the supplied reagents without addition of RNase inhibitor.

2.2.13 Quantitative End-point PCR

Quantitative End-point PCR was carried out as described in 2.2.1 using the below depicted protocol and cycle numbers as appropriate for the respective gene.

94°C	5 min	
94°C	30 sec	
60 (67)°C	30 sec	30-34 x
72°C	25 sec	
72°C	5 min	
4°C	∞	

67°C in the annealing step were used for rabbit DNA.

2.3 CELL BIOLOGY

2.3.1 General Cell Culture

All cells were maintained in the appropriate culture medium at 37°C and 5% CO_2.

2.3.2 Cell Culture Media and Supplements

DMEM High Glucose (4.5 g/l) with L-Glutamine (PAA, Cölbe, Germany)	for cultivation of C33A, HaCat, and NIH3T3 cells; supplemented with 10% FBS and 1% AbAm
DMEM/Ham's F-12 with L-Glutamine (PAA, Cölbe, Germany)	for cultivation of Rab-9 cells; supplemented with 10% FBS and 1% AbAm
Keratinocyte SFM (Invitrogen, Karlsruhe, Germany)	supplemented with EGF and BPE as supplied with the Kit (Invitrogen, Karlsruhe, Germany)
AntibioticAntimycotic (AbAm) 100x conc (Invitrogen, Karlsruhe, Germany)	for supplementation of media
Foetal Bovine Serum Standard Quality EU approved (PAA, Cölbe, Germany)	for supplementation of media

2.3.3 Thawing and Freezing of Cells

Frozen cells were thawed quickly in a 37°C water bath and transferred to petridishes containing the appropriate culture medium. After 12-18 hours medium was exchanged to remove the remainder of DMSO from the freezing medium.
For freezing, cells were grown in cell culture flasks to approximately 90% confluency, washed in PBS and trypsinized. The resulting cell suspension was centrifuged at 1,600 g for 4 minutes and the pellet resuspended in freezing medium (regular cell culture medium containing 20% FBS and 20% DMSO or PromoCell CryoSFM). The cell suspension was then transferred to cryo-vials. These were then placed inside a cell freezer (Nunc) to allow for a constant cooling rate of 1°C per minute and stored at -80°C.

2.3.4 Transfection

Transfection-grade plasmid DNA was obtained by plasmid preparation as previously described (2.2.8.). All transfection reactions were prepared in OptiMEM (Invitrogen, Karlsruhe, Germany). The amount of medium supplied varied with the well size used. Generally, 12-well plates (Corning) were used and medium was exchanged from regular

growth medium to serum-free antibiotic-free DMEM with L-Glutamine (PAA, Cölbe, Germany) just prior to addition of the transfection reaction. Medium was changed back to regular growth medium 5 hours after transfection.

2.3.4.1 Transfection of DNA

Transfection reactions were prepared by providing two microcentrifuge tubes per reaction containing 100µl OptiMEM per well to be treated (12-well format). DNA was added to one of the tubes in an amount determined by previous optimization. Lipofectamine2000 (Invitrogen, Karlsruhe, Germany) was added to the second tube at either 1µl per well (Rab-9), 2µl per well (C33A), or 4µl per well (NIH3T3).

2.3.4.2 Transfection of siRNA

Transfection of siRNAs was conducted with Oligofectamine (Invitrogen, Karlsruhe, Germany) following the manufacturer's recommedations for the amounts of medium to be used. Oligofectamine was used at 3µl with 3µl of 20nM siRNA per reaction.

2.3.5 Nuclear staining

For nuclear staining prior to microscopic investigation or fixation for immunofluorescence, cells were incubated with Hoechst-33342 Dye (Sigma-Aldrich, Munich, Germany) for 30 minutes to 1 hour at 37°C, 5% CO_2.

2.3.6 Preparation of whole cell lysates

Whole cell lysates were prepared by rinsing the cells with ice-cold PBS and adding CST Cell lysis buffer (Cell Signaling Technologies, Frankfurt/Main, Germany) with PMSF added just before addition to the cells. Plates were then incubated at 4°C for 5 minutes and subsequently cell lysates were transferred to a microcentrifuge tube and centrifuged at 13,000rpm for 10 minutes to remove the cell debris. Where necessary, lysates were additionally homogenized using a QiaShredder (Qiagen, Hilden, Germany).

2.4 BIOCHEMISTRY

2.4.1 Concentration of proteins

For concentration of proteins, protein solutions were applied to Amicon columns (Amicon Ultra YM-30; MILLIPORE Corporation, USA) and centrifuged at 5,000 rpm until the desired concentration was reached.

2.4.2 Protein determination

Protein concentrations were determined using the Bicinchinonic Acid (BCA) Assay (Pierce, Darmstadt, Germany). For this, 20µl of Solution B were added to each ml of Solution A. 10µl of the protein solution to be determined were added to 990µl of the mixture of Solutions A and B and incubated at 37°C and 250rpm for 30 minutes prior to measuring absorbance at 562nm.

2.4.3 Immunoprecipitation

For immunoprecipitation, ProteinA/G Plus Agarose (Santa Cruz Biotechnologies, Heidelberg, Germany) was used. Cell lysates were equalized in protein content and 15µl of the 50% agarose slurry were added to a maximum of 500µl lysate and the solution was precleared in a rotator at 4°C for 1 hour. The solution was centrifuged in a tabletop centrifuge at 2,500 rpm for 5 minutes and subsequently the supernatant was transferred to a new microcentrifuge tube. 1 µg of required antibody was added to the lysate and incubated for 1 hour at 4°C. After incubation with the antibody 20µl of agarose slurry were added and the solution subsequently incubated in a rotator at 4°C overnight. Immunoprecipitates were then collected at 2,500 rpm for 5 minutes and the supernatant was carefully discarded. Pellets were washed 4x with 1.0 ml PBS each time repeating the centrifugation step above. After the final wash, the supernatants were again discarded and the pellets resuspended in 40 µl 2x sample buffer, boiled for 2 minutes and agarose beads pelleted by centrifugation. Samples were analysed using SDS-PAGE (2.4.5).

2.4.4 ELISA

2.4.4.1 Preparation of cell lysates for CAT-ELISA

Cells for CAT-ELISA (Roche, Mannheim, Germany) were lysed according to the manufacturer’s manual with the lysis buffer supplied with the kit.

2.4.4.2 CAT-ELISA

Cell debris was pelleted at 13,000 rpm for 5-10 minutes prior to protein determination by BCA. The amount of lysate used was equalized according to protein concentration and ELISA carried out according to the supplied protocol (Roche, Mannheim, Germany). The absorption was determined at 492 nm using an Anthos 2010 MTP-Reader (Anthos Mikrosysteme GmbH, Krefeld, Germany).

2.4.5 SDS-PAGE

SDS-PAGE was performed according to Laemmli [1970]. Solutions needed were stored at 4°C until further use.

Acrylamide stock solution (PAA Protogel TM):

Acrylamide-/Bisacrylamide solution 30%: 0.8% (Roth, Karlsruhe, Germany)

"Upper Tris":

Tris/HCl pH 6.9	0.5	M
SDS	0.4	%

"Lower Tris":

Tris/HCl pH 8.9	1.5	M
SDS	0.4	%

Running buffer (1x):

Glycin	0.2	M
Tris	0.025	M
SDS	1	%
add H_2O_{bidest} to yield 1 liter		

Sample buffer (4x):

Tris/HCl pH 6.9	0.25	M
SDS	8	%
Glycerol	40	%
Mercaptoethanol	20	%
Bromphenol blue	0.002	%

For native gels, solutions without SDS and/or mercaptoethanol were used.

Composition of SDS polyacrylamide gels (0.75 mm thickness):

for 2 gels	collection gel	separation gel
H_2O_{bidest}	6.2 ml	5 ml
"Upper Tris"	2.5 ml	-
"Lower Tris"	-	3.75 ml
PAA	1.3 ml	6.25 ml
APS (10%)	75 µl	125 µl
TEMED	10 µl	15 µl

Ammoniumpersulphate (APS) was prepared as a stock solution of 10% APS in H_2O_{bidest} and stored at -20°C.

For non-native SDS-PAGE, proteins were diluted in 4x SDS sample buffer and boiled for 10 minutes. Electrophoretic separation of the proteins was carried out at 100V.

Protein standard:

PageRuler Prestained Protein Ladder (#SM0671, Fermentas, St. Leon-Rot, Germany): 10 bands; 10-170 kDa

For use, the marker was heated to 70-95°C for 2 minutes and transferred to ice immediately after heating.
Application: 3-5 µl to SDS gel.

2.4.6 Gelatin Zymography

For gelatin zymography, an SDS-gel containing gelatin was prepared according to the protocol given below. Probes were loaded onto the gel with 10x Zymo buffer (2.5 M Tris-HCl pH 6.8, 50% SDS, 0.5% bromphenol blue, 2 ml glycerol, H_2O_{bidest} to 10 ml) and the gel was run at 90V for approximately one hour when the running buffer front was at the bottom of the gelatin gel. This was then washed in 2.5% Triton-X 100 for 2 hours to remove all SDS and hence allow the proteins to refold. The buffer was then exchanged for Incubation Buffer (50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ x $2H_2O$, 50 mM NaCl, 0.05% Brij-35) which allows the proteins (gelatinases) to degrade the gelatin. Incubation was carried out at 37°C overnight with gentle shaking. The gel was then rinsed with cold water, stained with Coomassie R250 (Sigma, Munich, Germany), and destained with 40% Acetic Acid, 10% Methanol until transparent bands became visible.

for 1 gel (1.5 mm)	collection gel	gelatin gel
H_2O_{bidest}	6.2 ml	3.85 ml
"Upper Tris"	2.5 ml	-
"Lower Tris"	-	2.6 ml
PAA	1.3 ml	2.5 ml
Gelatin (5%)	-	1 ml
APS (10%)	75 µl	100 µl
TEMED	10 µl	6 µl

2.4.7 Protein Transfer (Western Blot)

After SDS-PAGE, proteins were transferred from the gel to an Immobilon-P PVDF membrane (Millipore, Schwalbach, Germany) using a BioRad MiniProteanII Wet Blot Chamber (BioRad, Munich, Germany). Prior to transfer, filter paper and membranes were cut equalling approximately the size of the gel. The membrane was activated in methanol for 10 seconds before being equilibrated in transfer buffer (0.2M Glycin, 0.025M Tris, 20% Methanol). For blotting, sponges and filter paper were soaked in transfer buffer and everything was set up according to the manufacturer's recommendations. Transfer time was between 60 to 90 minutes at 80 – 100V.

2.4.8 Immunodetection of proteins

Following the transfer, membranes were blocked for 1 hour at room temperature in either 5% skim milk in TBST or 5% BSA in TBST as suggested for the respective antibody. If no protocol was supplied by the manufacturer, 5% skim milk in TBST was used.
After blocking, the membrane was washed three times for 5 minutes in TBST. The primary antibodies were prepared with the dilutions given under 2.1.4 in either of the blocking buffers and membranes incubated with the antibody solutions overnight at 4°C with gentle agitation. Unbound antibody was then washed off the membrane with TBST (3x 5 minutes) followed by the addition of the secondary antibody in the respective buffer and incubation for 1 hour at room temperature on an orbital shaker (35-40 rpm). Again, unbound antibody was removed from the membrane by washing 3x for 5 minutes. The membrane was then transferred to a clean tray; ECL Plus reagent (GE Healthcare, Munich, Germany) was mixed according to the manufacturer's protocol and added by pipetting it on top of the membrane several times. Without letting it dry, the membrane was then transferred to seran wrap and into an x-ray chamber. HyperFilm ECL Films (GE Healthcare, Munich, Germany) were exposed to the membrane under red light in a

darkroom for appropriate time and subsequently developed in a HyperProcessor (GE Healthcare, Munich, Germany).

2.4.9 Immunofluorescence

2.4.9.1 Coating of Coverslips

For coating, coverslips were rinsed with 100% ethanol and air-dried for a few seconds. They were then placed into 12-well or 24-well plates depending on their size and washed with PBS. After washing coverslips were coated with an appropriate volume of coating medium (described below) for one hour at room temperature. This was followed by washing with PBS and drying for 1 hour at room temperature.

Coating medium

PBS without Ca^{2+} and Mg^{2+}	50	ml
Acetic acid	0.02	%
Rat-tail Collagen I	50	µg/ml

As soon as the coverslips were dried, cells were plated at the desired density and treated as needed.

2.4.9.2 Fixation of Cells

24 hours after seeding or transfection, cells plated on coverslips were fixed with either ice-cold methanol for 20 minutes on ice or with 4% Paraformaldehyde in PBS for 15 minutes at room temperature followed by permeabilization with 0.25% Triton-X 100 in PBS for 20 minutes.

2.4.9.3 Antibody Staining

Following fixation, the coverslips were washed in PBS and blocked in 1% BSA in PBST for 30 minutes. The primary antibody was then diluted in 1% BSA in PBST and applied to the coverslips which have been transferred to a humidity chamber. The coverslips were incubated with the primary antibody for one hour and then washed 2 to 3 times in PBS before applying the secondary antibody solution (in blocking buffer) for another hour. After incubation with the secondary antibody, coverslips were washed 2 times with PBS and rinsed in water before transferring them upside-down onto slides using VectaShield mounting medium (Linaris Biologische Produkte, Wertheim, Germany).

2.5 MICROSCOPY

2.5.1 Bright-field microscopy

Regular analysis of cells using bright-field microscopy was performed using a Hund Wilovert AFL inverse fluorescense microscope (Hund, Wetzlar, Germany) at a magnification of 10x.

2.5.2 Fluorescence microscopy

Microscopic investigation of fluorescently stained and/or eGFP expressing cells was carried out using a Zeiss AxioImager PlanAPOCHROMAT microscope (63x,1.4 oil), an AxioCam MRm R3, and the software AxioVision 4.7 (all Carl Zeiss, Jena, Germany) at the Zeiss Demonstation Center at the DKFZ in Heidelberg or using a Nikon 90i upright automated microscope at 40x magnification, a D1QM camera and the EZ-2.3-Viewer software (all Nikon, Düsseldorf, Germany) at the Nikon Center (Uni Heidelberg).

3 RESULTS

3.1 THE RABBIT MATRIX-METALLOPROTEINASE 9 PROMOTER

The cottontail rabbit papillomavirus (CRPV) induced papillomas and carcinomas in the New Zealand White rabbit (NZW) serve as a model system for the development and progression of epithelial lesions induced by papillomaviruses. Mutations in the transactivation domain of CRPV E2 in the context of the virus genome have been described to abolish the formation of papillomas and carcinomas in the rabbit model. Hence, it could be concluded that the E2 protein is of importance in the initial steps of CPRV-induced tumorigenesis. In a study by Behren et al. (2005) it was discovered that in CRPV E2-induced lesions and carcinomas the expression of matrix-metalloproteinase 9 (MMP-9) is upregulated (Figure 9) when compared to uninfected rabbit skin.

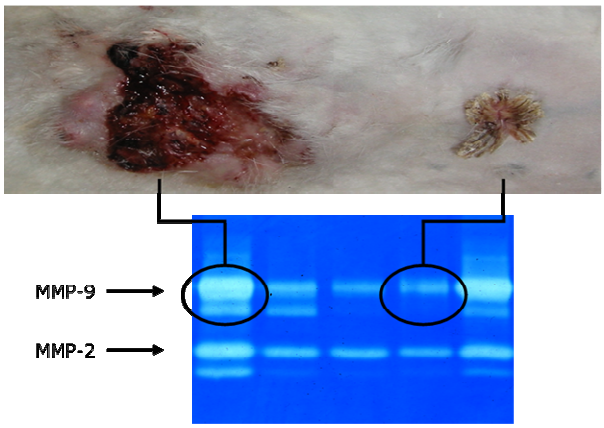


Figure 9: MMP-9 expression in rabbit skin lesions. Gelatin zymography of lysates prepared from papillomavirus-induced lesions of NZW rabbit skin showed expression of MMP-9 (Courtesy of A. Behren).

Furthermore, *in-vitro* experiments have led to the observation that HPV31 E2 as well as CRPV E2 are both able to activate a minimal human MMP-9 promoter fragment in reporter-gene assays (Behren et al., 2005). In order to determine in detail the means by which the CRPV E2 protein achieves the induction of MMP-9 and to investigate whether this observation is comparable to a function of human high-risk papillomavirus E2, the first aim of this study was to sequence and clone the rabbit MMP-9 promoter.

3.1.1 Cloning the rabbit MMP-9 promoter

To determine the sequence of the rabbit MMP-9 promoter a library of genomic DNA isolated from New Zealand White rabbit skin was constructed according to the work-flow described in Figure 10. The genomic DNA was extracted from the skin and subsequently

digested with restriction enzymes. Adapter-sequences were then annealed to the ends of the resulting fragments and a PCR was performed using a primer recognizing part of the adapter sequence and a gene-specific primer designed to bind within the rabbit MMP-9 gene of which the sequence is known. The largest DNA fragment obtained from this PCR reaction was subjected to nested-PCR and resulted in an 1872bp fragment which was subsequently sequenced (see attachment 1).

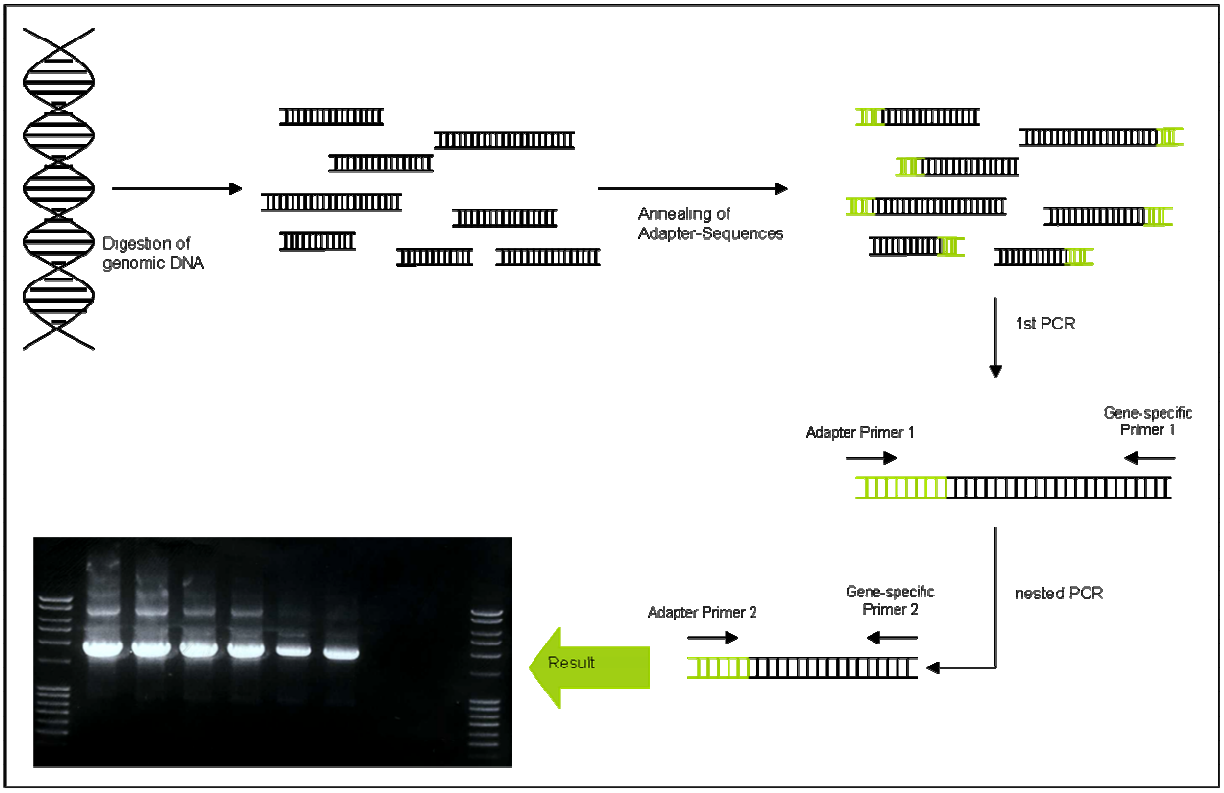


Figure 10: Schematic Work-flow scheme of the Clontech GenomeWalker Kit. The library was constructed using extracted genomic DNA from rabbit skin, followed by digestion, annealing of adapter sequences, and nested PCR.

The rabbit MMP-9 promoter sequence was aligned to the human MMP-9 promoter (Attachment 2). The N-terminal ~500bp and the proximal ~800bp showed a similarity of 76 and 77% whereas the intermediate ~600bp yielded only 13% homology (Figure 11). Furthermore, the CA-repeat located between -154 and -109bp in the human that is also found in the mouse promoter was not present in the rabbit promoter sequence.

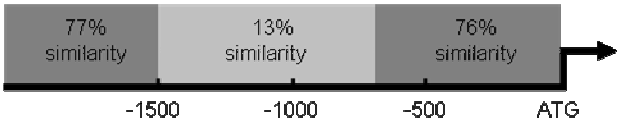


Figure 11: Schematic alignment of the rabbit and human MMP-9 promoter. The rabbit MMP-9 promoter sequence was aligned to the human promoter sequence using Clustal W. The similarities between separate parts of the promoter are illustrated here.

3.1.2 Regulatory parts of the rabbit MMP-9 promoter

The promoter then was cloned into the reporter-gene vector pCAT3 Basic for further characterization. This vector expresses the gene for chloramphenicol acetyltransferase (CAT) upon activation of the promoter cloned in front of it. To identify the minimal promoter fragment required for activation, the promoter sequence was continously shortended by 300bp off the 5'-end (Figure 12) and all of these deletion constructs were also cloned into the reporter-gene vector pCAT3 Basic.

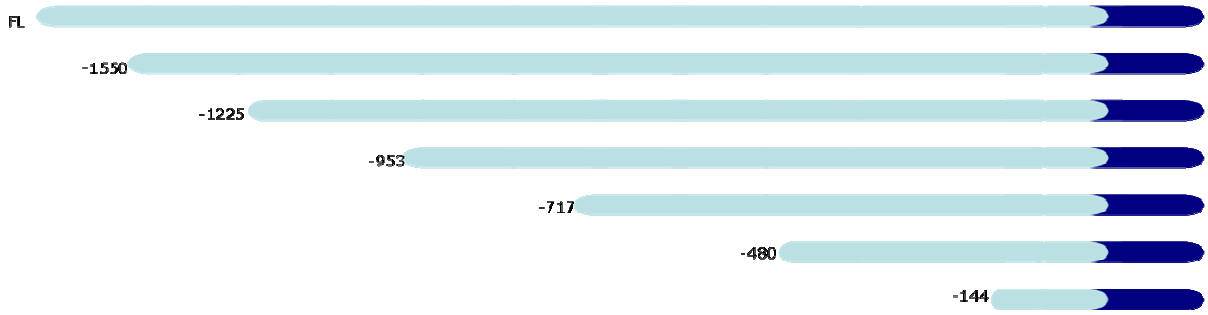


Figure 12: Schematic illustration of rabbit MMP-9 promoter deletion constructs. Deletion mutants were constructed with lengths of 1550bp, 1225bp, 953bp, 717bp, 480bp, and 144bp. The constructs are illustrated here with relative lengths. The dark blue part indicates the start of the coding region.

In order to test the functionality of the constructs, the MMP-9 promoter was activated using the tumor-inducer PMA, and the activation was analyzed by CAT-ELISA. Beforehand, the ability of PMA to induce MMP-9 expression *in-vitro* and the optimal amount of PMA required for induction was determined by addition of the reagent to the cells in serum-free medium and subsequent gelatin zymography of the obtained supernatants. It was hence shown that PMA is able to induce the expression of MMP-9 in Rab-9 cells and determined that the best concentration of PMA for activation experiments is 50nM (Figure 13). Therefore, all further experiments requiring PMA-induction were carried out using this concentration.

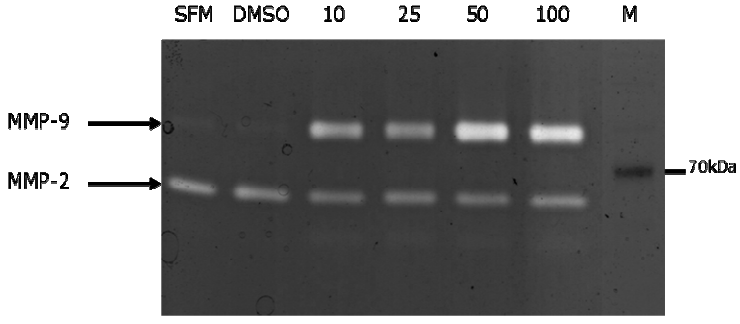


Figure 13: PMA-induced MMP-9 expression in Rab-9 cells. Expression of MMP-9 was induced by addition of different concentrations of PMA to the cells. Supernatants were collected 24 hours after treatment, concentrated, equalized for protein content and subjected to gelatin zymography. Concentrations of PMA were 10, 25, 50, and 100nM as indicated by labelling of lanes.

The CAT-ELISA results of PMA-stimulated rabbit promoter deletion constructs showed an average activation of 48-to 65-fold ±10 of the promoter constructs which were 717bp or longer. PMA was unable to induce activation of the promoter for constructs 493bp and 144bp in length (Figure 14a). The PMA solvent DMSO was added to replicas of the transfection and served as a negative control. No activation could be detected upon CAT-ELISA with these lysates (Figure 14b).

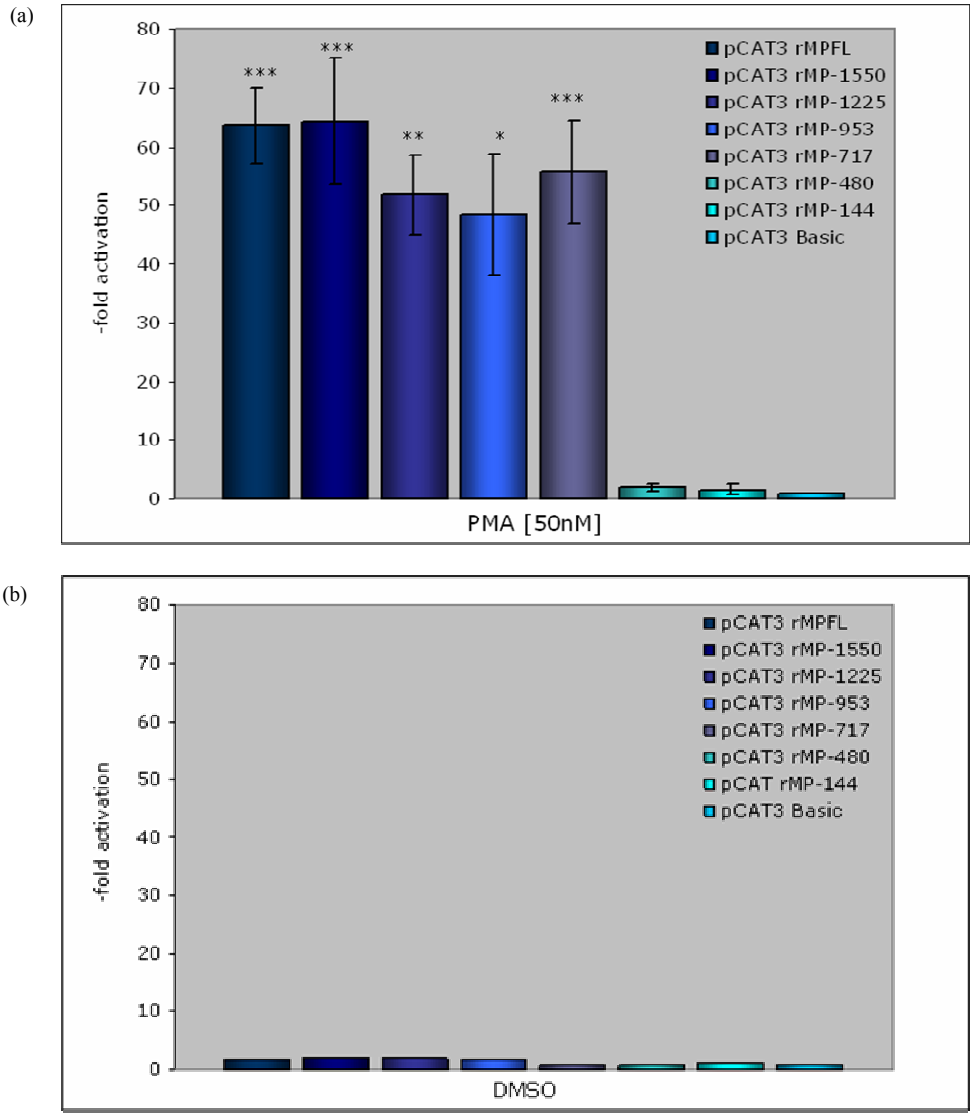


Figure 14: Reporter-gene assays of rabbit MMP-9 promoter deletion constructs stimulated with PMA. CAT-expression in reporter-gene transfected cell lysates was detected by CAT-ELISA after stimulation with PMA (a) or DMSO, which served as negative control for PMA (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

Activation of the promoter deletion constructs by CRPV E2 showed a difference when compared to PMA-stimulation. While promoter constructs of 1872bp, 1550bp, 1225bp,

and 717bp length showed activation levels to be 2.5- to 3.7-fold, and constructs with less than 717bp could not be activated as was previously seen for PMA, there was a significant decrease in activation levels for the 953bp promoter fragment which showed an activation of only 1.7-fold ± 0.3 (Figure 15). This led to the suggestion of a repressor element to be situated between -953bp and -717bp of the promoter which was further investigated later in this study.

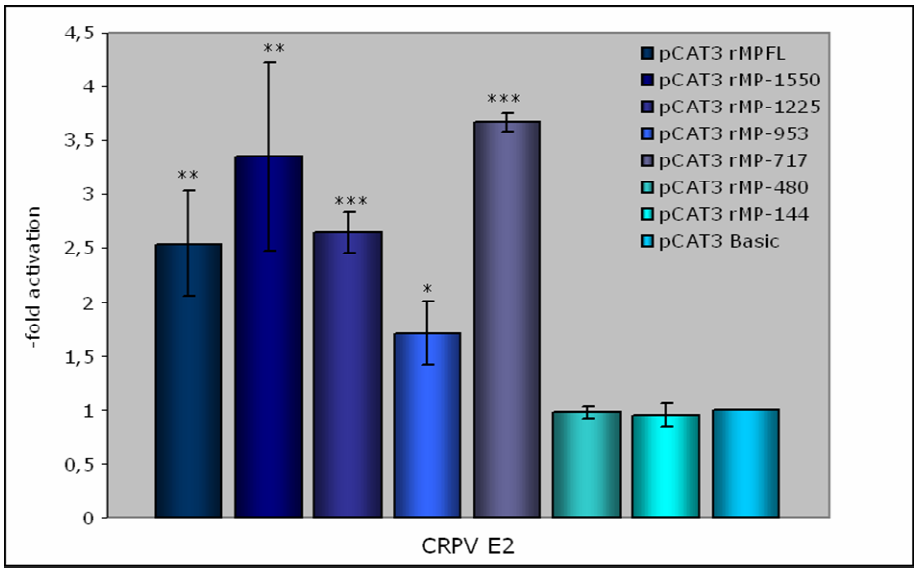


Figure 15: Reporter-gene assays of rabbit MMP-9 promoter deletion constructs induced by co-transfection with CRPV E2. CAT-expression was observed after co-transfection of Rab-9 cells with CRPV E2 and promoter deletion constructs. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

The induction of MMP-9 expression after transient transfection of Rab-9 rabbit cells with CRPV E2 was also investigated on mRNA and protein level. Expression of MMP-9 on protein level was determined by gelatin zymography which yielded a perceptible difference between the empty vector control and the CRPV E2-transfected cells (Figure 16a). The expression of MMP-9 mRNA was investigated using semi-quantitative RT-PCR of transcribed cDNA obtained from rabbit cells after transfection with empty vector or CRPV E2, respectively (Figure 16b).

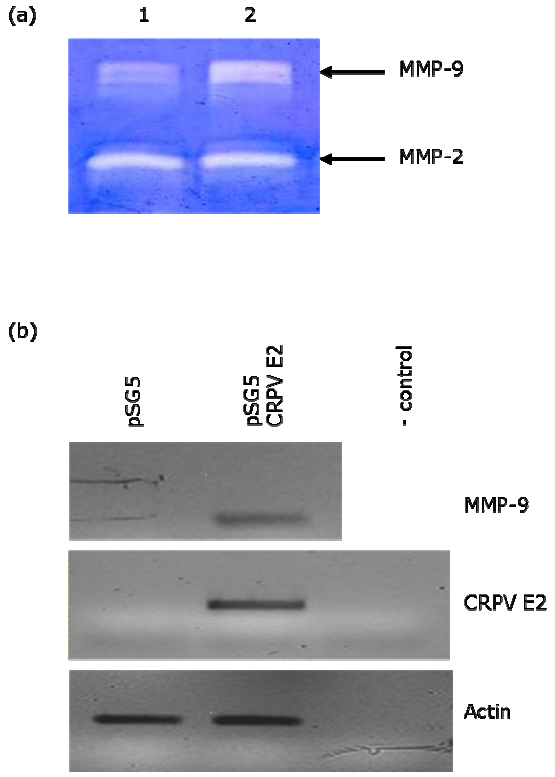


Figure 16: Induction of MMP-9 expression on protein and mRNA level. Supernatants of cells transfected with either pSG5 alone (1) or pSG5 CRPV E2 (2) were subjected to gelatin zymography. The level of MMP-2 serves as a loading control (a). mRNA isolated from transiently transfected cells was transcribed to cDNA and quantitative PCR was conducted at 60°C (70°C for MMP-9) for 36 cycles. H_2O served as negative control (b).

The transcription-factor binding sites (TFBSs) within the MMP-9 promoter were then identified using the programs TFSEARCH and TESS. The thresholds were set to 85% similarity. The results of the programs were examined and TFBSs outlined by both programs were combined in a table (Table 7).

Table 7: List of transcription factor binding sites and their positions.

Transcription factor	Position in the rabbit promoter	Transcription factor	Position in the human promoter
TATA	-47 to -42	TATA	-48 to -44
SP1	-67 to -76	SP1	-64 to -73
AP-1	-103 to -97	AP-1	-98 to -92
NFkB	-134 to -124	CA-repeat	-154 to -109
SP1	-185 to -176		
		NFkB	-350 to -341
		SP1	-504 to -495
NFkB	-469 to -461		
AP-1	-480 to -474	AP-1	-556 to -550
PEA3	-487 to -482	PEA3	-563 to -558
NFkB	-547 to -538	NFkB	-623 to -614
		NFkB	-636 to -628
NFkB	-703 to -695		
		E2	-1120 to -1109
SP1	-985 to -976		
		AP-1	-1485 to -1475
		AP-1	-1681 to -1673
		TATA	-1800 to -1787

As the transcription factor AP-1 has been shown to play a role in the transcriptional activation of MMP-9 (Sato and Seiki, 1993), the two AP-1 binding sites found in the rabbit MMP-9 promoter sequence were each deleted individually by site-directed mutagenesis. Subsequent reporter-gene assays of cells which were transfected with the mutant promoter constructs and stimulated with PMA showed a highly significant decrease of almost 100% in the activation of the reporter gene upon deletion of the distal AP-1 binding site (pCAT3 rMP-1550 AP-1d mt) (Figure 17a). Deletion of the proximal binding site (pCAT3 rMP-1550 AP-1p mt) led to a decrease of 45 ± 5%, which was also significant. Parallel reporter-gene assays of cells co-transfected with the mutant rabbit promoter constructs and CRPV E2 also yielded a significant reduction of reporter-gene activation, however, in this case the reduction of promoter activation was equal for both AP-1 sites and yielded 40% of wild-type activity (Figure 17b).

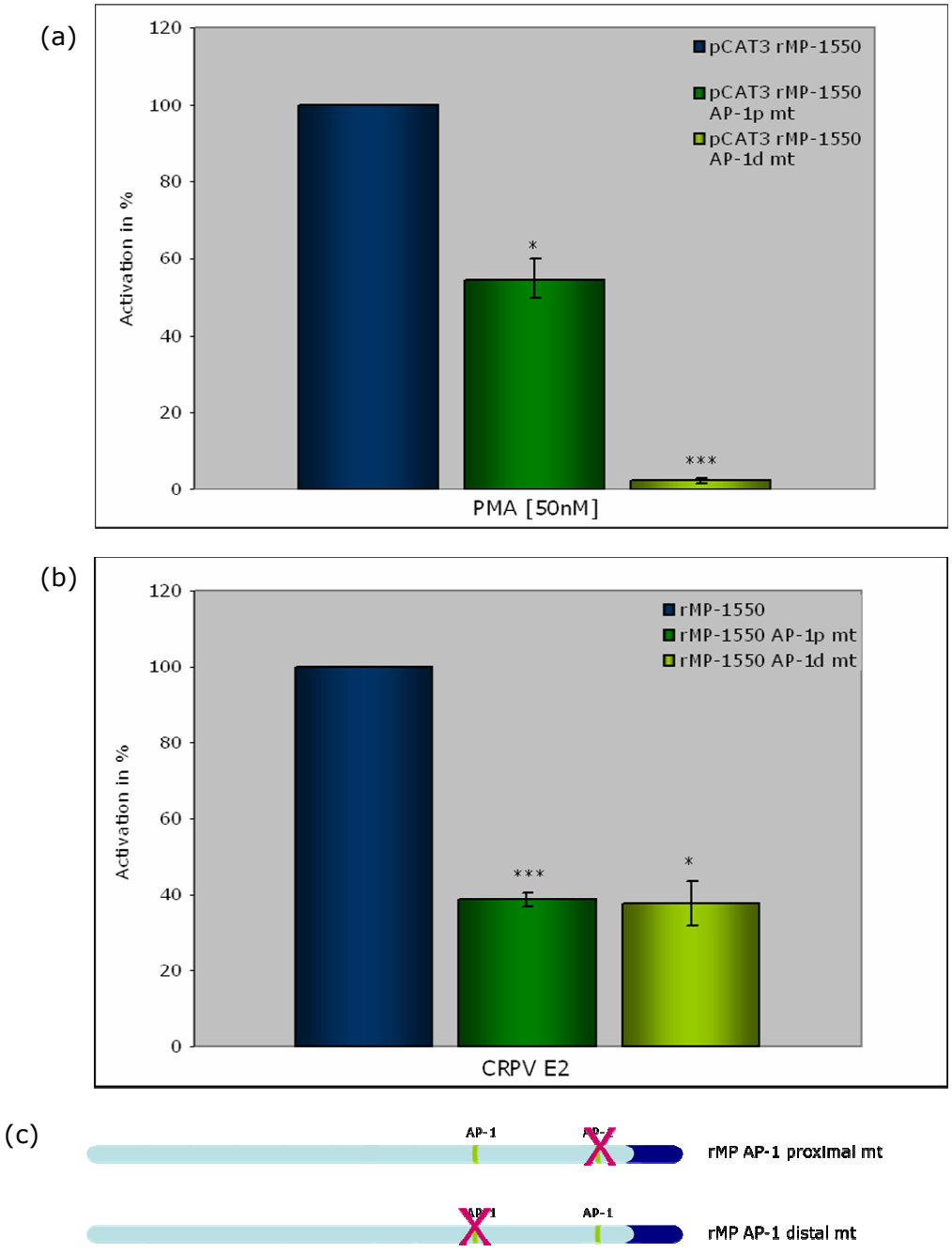


Figure 17: Effect of the AP-1 binding sites within the rabbit MMP-9 promoter on activation. The relative activation of the MMP-9 promoter constructs containing mutations in either of the putative AP-1 transcription factor binding sites was investigated after induction by PMA (a) or co-transfection with CRPV E2 (b). The activation of the wild-type MMP-9 promoter was set to 100%. The schematic illustration shows the AP-1 binding sites mutated within the context of the promoter (light blue). The dark blue part signifies the start of the coding region (c). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the wild-type promoter. Bars show mean value ± SD from three independent experiments in triplicate.

Besides the transcription factor complex AP-1, we examined the role of the ets transcription factor which has been shown to be of importance in tumorigenesis. There

are two ets binding sites (termed PEA3) located within the promoter sequence of -717 and -480bp, and both were deleted individually to investigate the involvement of ets in promoter activation. It could be seen from reporter-gene assays conducted with these mutants that neither of the ets binding sites had an influence on CRPV E2-mediated activation (Figure 18).

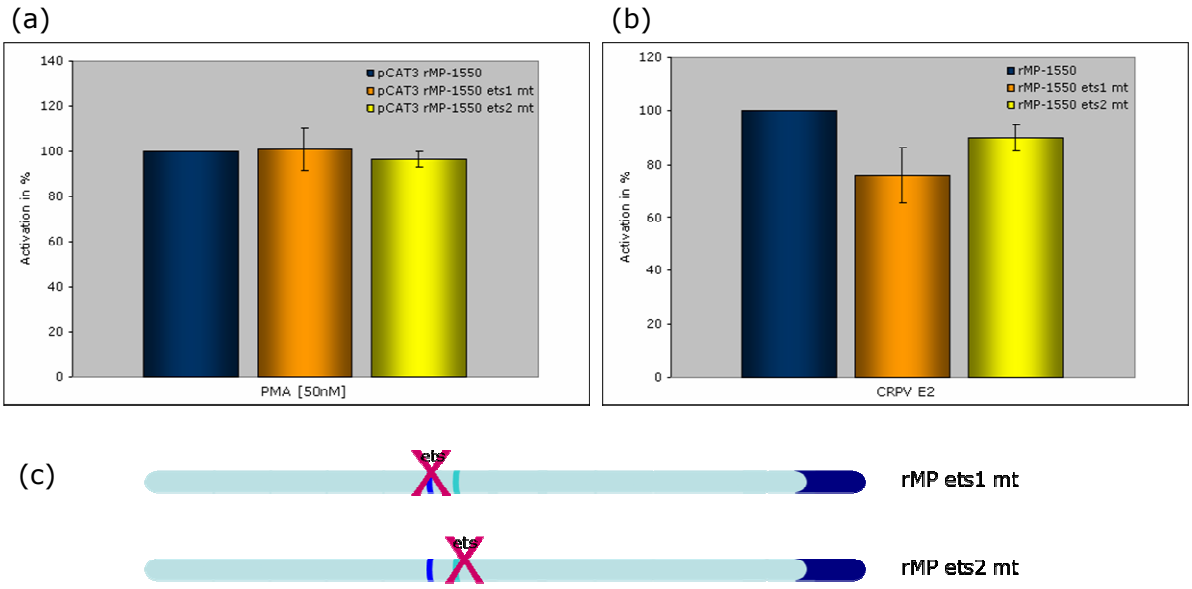
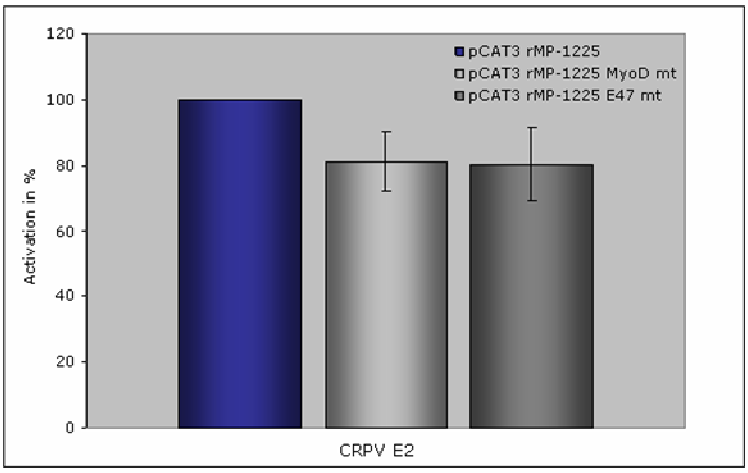


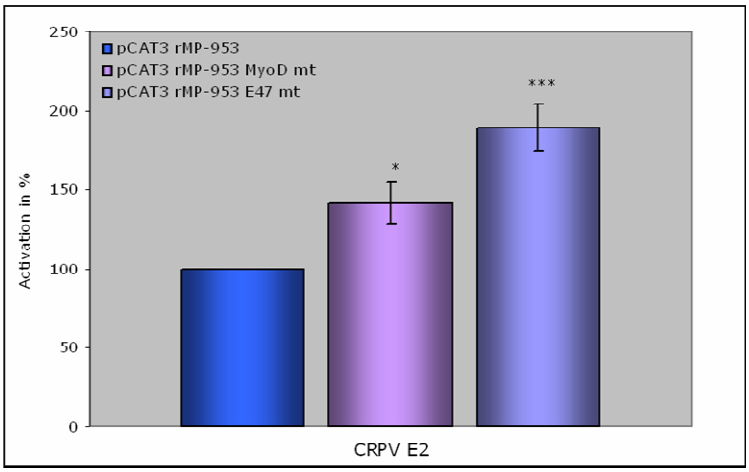
Figure 18: Effect of the ets binding sites within the rabbit MMP-9 promoter on activation. The relative activation of the MMP-9 promoter constructs containing mutations in either of the putative ets transcription factor binding sites was investigated after induction by PMA (a) or co-transfection with CRPV E2 (b). The activation of the wild-type MMP-9 promoter was set to 100%. The schematic illustration shows the ets binding sites mutated within the context of the promoter (light blue). The dark blue part signifies the start of the coding region (c). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the wild-type promoter. Bars show mean value ± SD from three independent experiments in triplicate.

In order to prove the possibility for a repressor element binding within the promoter region between -953bp and -717bp, the respective ~250bp were more closely examined for the presence of potential repressive elements. Two candidates, an E47 and a MyoD binding site have been identified, which have been shown to act as repressors in other studies (REF). After mutation of these sites in the context of both, the 1225bp and the 953bp promoter, the constructs were again used in reporter-gene assays. It could be observed that while mutation of either of the binding sites within the 1225bp promoter did not lead to a significant change in activation by E2 (Figure 19a), mutation of the E47 binding site within the 953bp fragment resulted in a substantial increase in activation by about 85% ± 26% (Figure 19b), which raised activation levels almost to the level of the 1225bp promoter construct (Figure 19c).

(a)



(b)



(c)

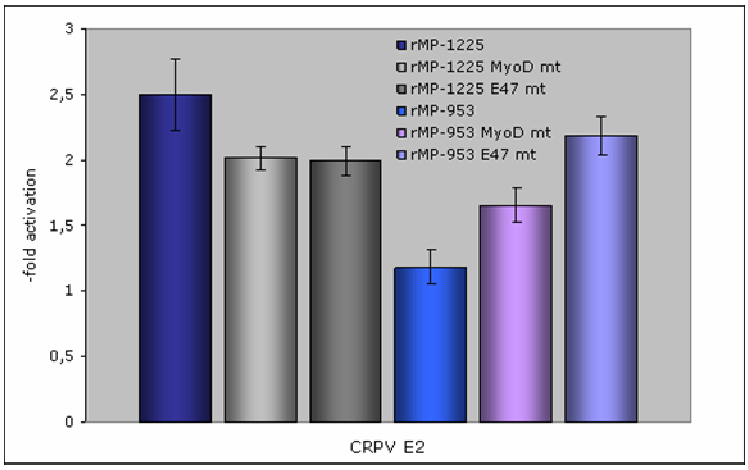


Figure 19: Effects of MyoD and E47 binding sites in the -1225 and -953 promoter deletion constructs. The effect of CRPV E2-induction on promoter constructs containing mutations introduced into the -1225 (a) and -953bp (b) promoters was studied by CAT-ELISA. Wild-type promoters were set to 100%, activation of mutants is given relative to the wild-types. (c) shows the fold activation of -1225 and -953bp promoters and mutants to allow for visualization of the effect of the -953bp promoter mutants compared to the activation level of the wild-type -1225bp promoter.
Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control (a), (b). Bars show mean value ± SD from three independent experiments in triplicate.

To show the comparability of the rabbit model to the human system, analogous experiments were conducted to the ones described above, utilizing the human MMP-9 promoter. Firstly, the ideal concentration of PMA to induce MMP-9 expression in C33A human cervical cancer keratinocytes has been determined by gelatin zymography. The results showed that here, a concentration of 25 nM yielded the best induction (Figure 20). For better comparison, 50 nM were used here as was for induction of rabbit cells.

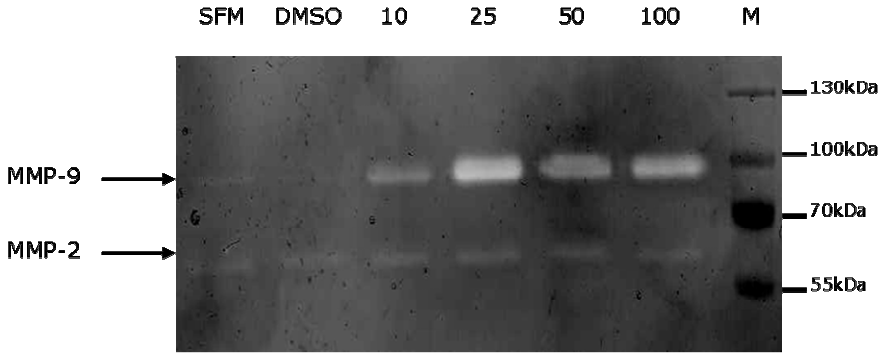


Figure 20: PMA-induced MMP-9 expression in C33A cells. Expression of MMP-9 was induced by addition of different concentrations of PMA to the cells. Supernatants were collected 24 hours after treatment, concentrated, equalized for protein content and subjected to gelatin zymography. Concentrations of PMA were 10, 25, 50, and 100nM as indicated by labelling of lanes.

A full length fragment of the human MMP-9 promoter which corresponded in the alignment to the full length rabbit MMP-9 promoter construct was cloned into the same reporter-gene vector (pCAT3 Basic). Three 5'-deletion constructs spanning similar regions as the constructs of the rabbit MMP-9 promoter were constructed (Figure 21).

Figure 21: Illustration of the human MMP-9 promoter deletion constructs. Human deletion fragments were constructed which cover similar regions as the rabbit MMP-9 promoter deletion mutants. They are depicted here in relative lengths with the dark blue parts indicating the beginning of the coding region.

Reporter-gene assays carried out with all human MMP-9 promoter constructs showed an activation of the reporter-gene of 3.3 – 4.3-fold when induced with PMA (Figure 22a). Induction with HPV16 E2 led to a 2.5 – to 2.9-fold activation of promoter fragments of 1261bp and larger while for the 829bp promoter fragment an induction of 1.3-fold ± 0.2 could be observed (Figure 22b). A small but perceptible increase in the amount of MMP-9 mRNA in pCMV-HA HPV16 E2 transfected cells could be detected by semi-quantitative RT-PCR (Figure 23).

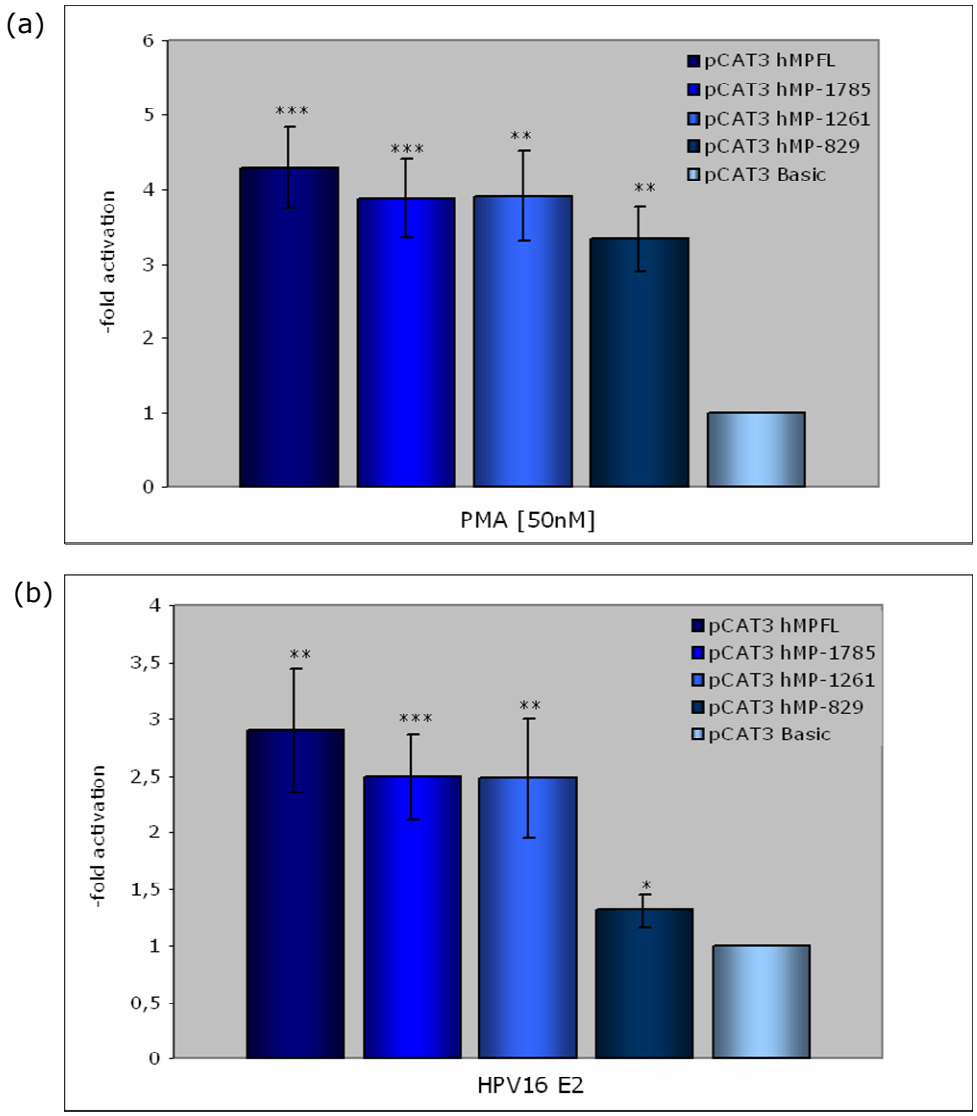


Figure 22: Activation of the human MMP-9 promoter by PMA and HPV16 E2. Activation of the human promoter constructs was determined by CAT-ELISA of cell lysates prepared 48 hours after transfection with either promoter deletion constructs induced by PMA (a) or co-transfected with HPV16 E2 (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

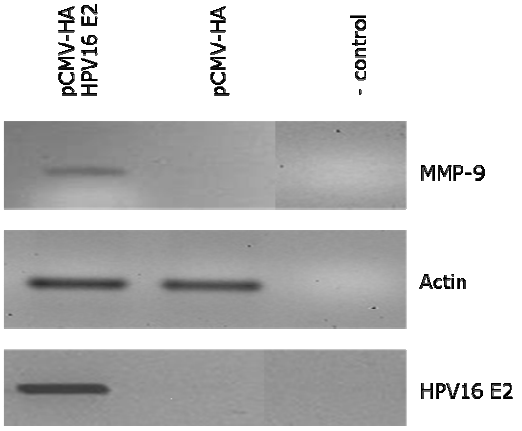


Figure 23: Induction of MMP-9 mRNA expression by HPV16 E2. MMP-9 expression was induced by transient transfection of C33A cells with pCMV-HA HPV16 E2. The level of mRNA was detected by semi-quantitative RT-PCR at 36 cycles, H_2O served as negative control.

Of special interest in conjunction with the activation of the human full length MMP-9 promoter with HPV16 E2 was the presence of an E2-binding site at -1120 to -1109bp. This E2-binding site was deleted by site-directed mutagenesis as has been desribed for binding sites for AP-1 and ets before. It can be seen from the results of subsequent reporter-gene assays that this E2BS has no influence on the activation of the human MMP-9 promoter by E2 (Figure 24).

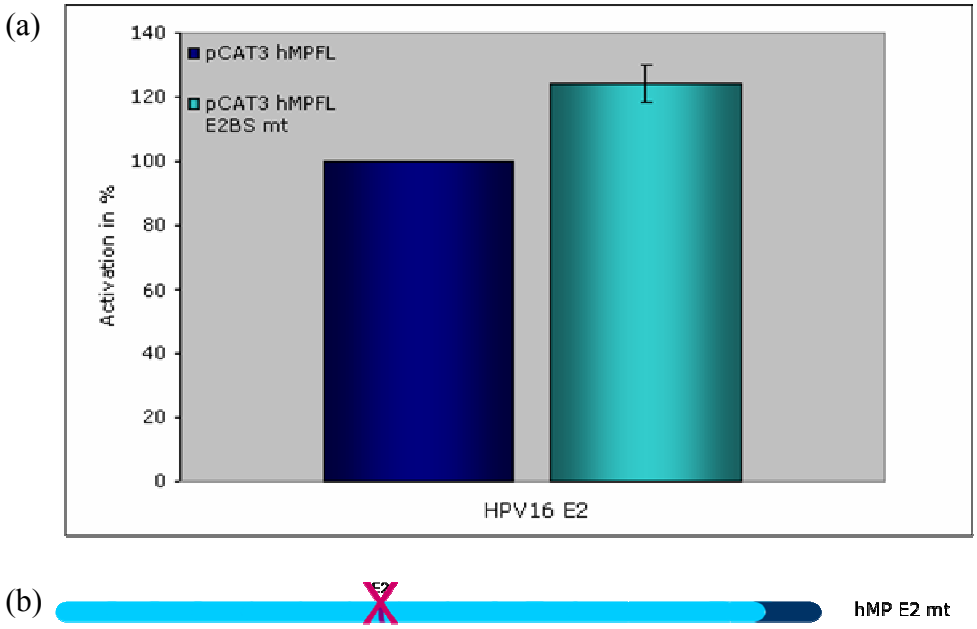


Figure 24: Effect of the E2-binding site on promoter activation. The relative activation of the MMP-9 promoter constructs containing mutations in the putative E2 binding site was investigated after co-transfection with CRPV E2. The activation of the wild-type MMP-9 promoter was set to 100% (a). The schematic illustration shows the E2 binding site mutated within the context of the full length promoter (light blue). The dark blue part signifies the start of the coding region (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the wild-type promoter. Bars show mean value ± SD from three independent experiments in triplicate.

Analogous to the experiments conducted with the rabbit promoter, the role of AP-1 was of interest in the activation of human promoter as well. The AP-1 binding sites found in the human promoter are homologous to the ones in the rabbit promoter in both, position and sequence. It has been shown previously in studies by Behren et al. (2005) that the proximal AP-1 binding site in the human promoter is of importance in E2-mediated activation. Therefore, an AP-1 decoy was used in this study to confirm the involvement of the AP-1 complex in promoter activation. A decoy is a synthetic oligonucleotide that resembles the transcription factor consensus sequences and hence competes for binding of the transcription factor with consensus sequences in target genes. Co-transfection of

the AP-1 decoy with the human MMP-9 promoter and HPV16 E2 resulted in a decrease in activation by 40 ± 4% (Figure 25) as compared to the control decoy.

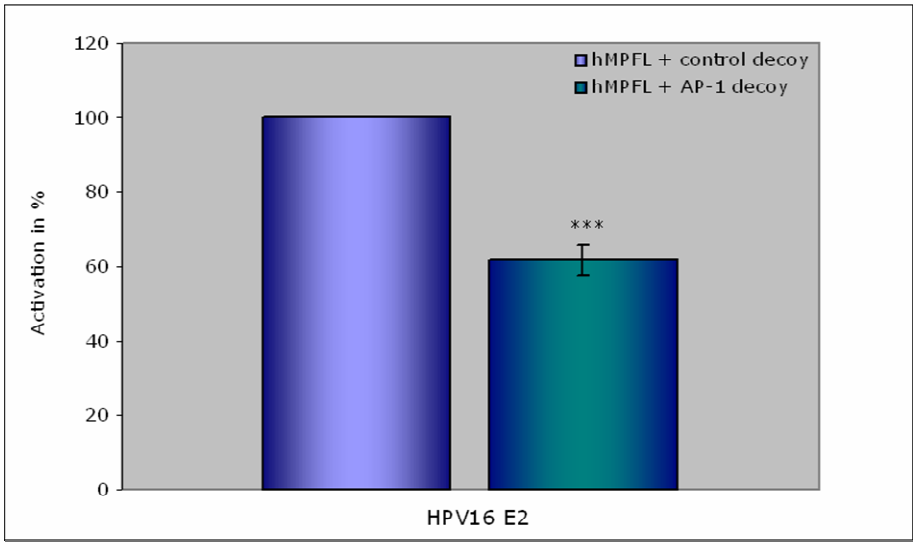


Figure 25: Influence of the transcription factor AP-1 in HPV16 E2-mediated MMP-9 promoter activation. Cells transfected with the full length MMP-9 promoter, HPV16 E2, and either AP-1 decoy or control decoy were lysed and the effect of the decoy on promoter activation was investigated. The result of the control decoy experiment was set to 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the control decoy. Bars show mean value ± SD from three independent experiments in triplicate.

3.1.3 Involvement of c-Jun in E2-mediated promoter activation

It has been described in the introduction that the transcription factor AP-1 is a dimeric complex which consists of members of the jun- and fos- families. To determine the involvement of the often in cancer upregulated component c-Jun in the complex required for activation of the MMP-9 promoter by PMA, CRPV E2, and HPV16 E2, the transactivation-deficient c-jun mutant TAM67 (REF) was used for co-transfection with the respective promoters and papillomavirus E2 proteins or PMA. PMA-mediated activation of the promoter was shown to be decreased to a level of 15% ± 2% as compared to the empty vector control (Figure 26a). CRPV E2-mediated promoter activation was decreased to 22% ± 2% (Figure 26b), and the activation of the promoter by HPV16 E2 was lowered to 46% ± 4% when compared to the empty vector control (Figure 26c).

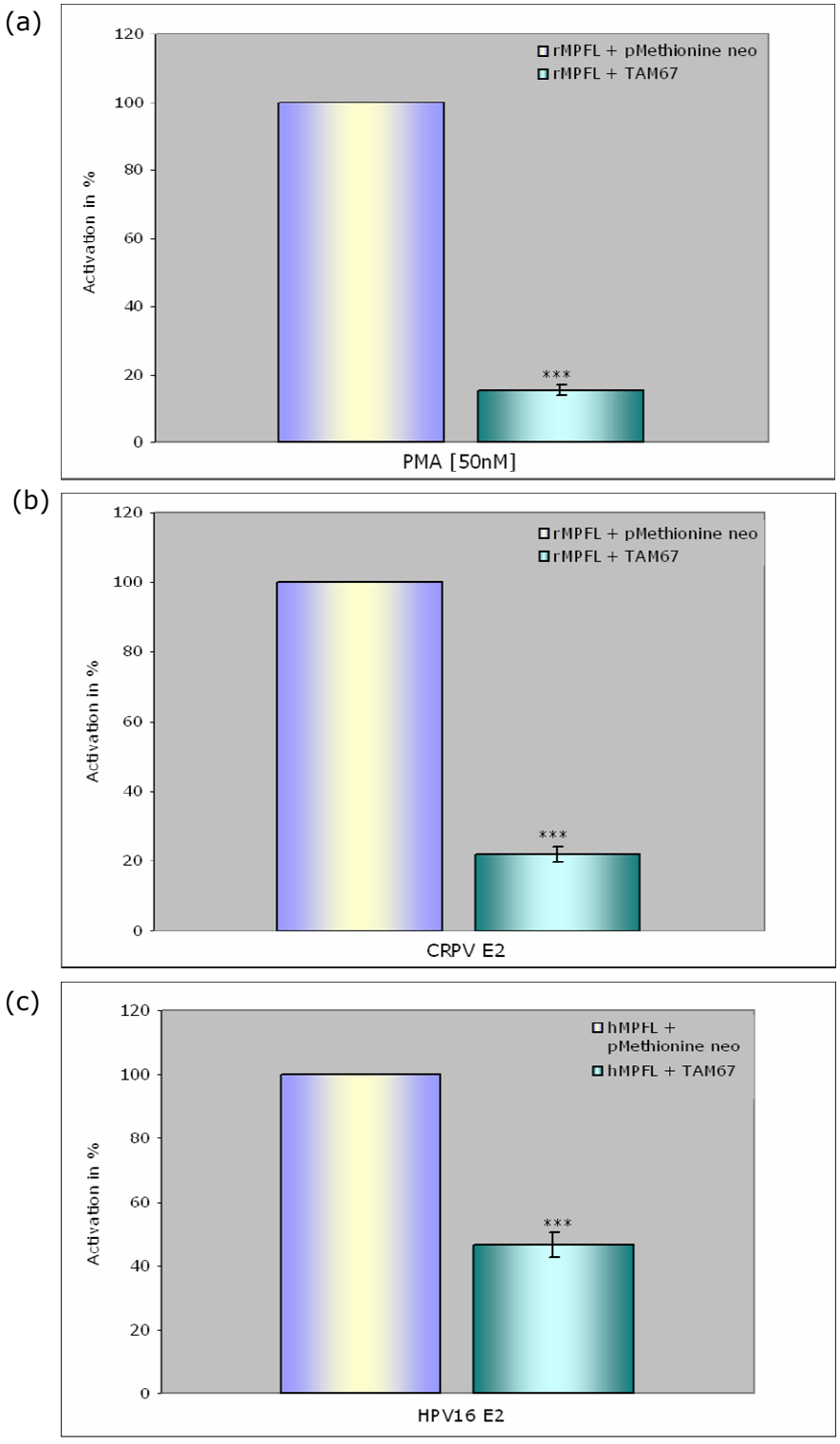


Figure 26: Effect of TAM67 on promoter activation. Rabbit cells were transfected with full length rabbit MMP-9 promoter, and either TAM67 expressing vector or empty vector control. Cells were induced either by addition of PMA (a), or co-transfection with CRPV E2 (b). Human keratinocytes were transfected with full length human MMP-9 promoter and TAM67-vector or control vector, and induced with HPV16 E2 (c). The effect of the transactivation-deficient c-jun construct TAM67 on promoter activation was investigated. The activation of the empty vector control was set to 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

3.2 SIGNAL-TRANSDUCTION PATHWAYS IN E2-MEDIATED MMP-9 PROMOTER ACTIVATION

AP-1 is mainly regulated by the activity of MAP-kinases and MMP-9 induction has been found to rely on the ERK and the p38 pathway. To determine the MAP-Kinase signal-transduction cascades involved in mediating the activation of MMP-9, chemical inhibitors were used. SB203580 is a pyridinyl imidazole that specifically inhibits p38 by binding withing its ATP-pocket. SP600125 specifically inhibits c-jun N-terminal protein kinase (JNK), and PD098059 ablates the phosphorylation of ERK by MEK. Firstly, the functionality of the inhibitors was determined. For the SAP-Kinases p38 and JNK cells were pre-treated with the respective inhibitor for 24 hours and kinases were activated by addition of 10µg/ml anisomycin for 30 minutes. Cells were then lysed and lysates investigated for the appropriate products using Western blotting, and for p38 in addition by using a PathScan p38 ELISA (Figure 27a,c,d). For PD098059, cells were also pre-treated with the inhibitor for 24 hours and subsequently ERK was activated by addition of 50nM PMA. Cell lysates were obtained and equalized for protein amount and analyzed via Western blotting (Figure 27b).

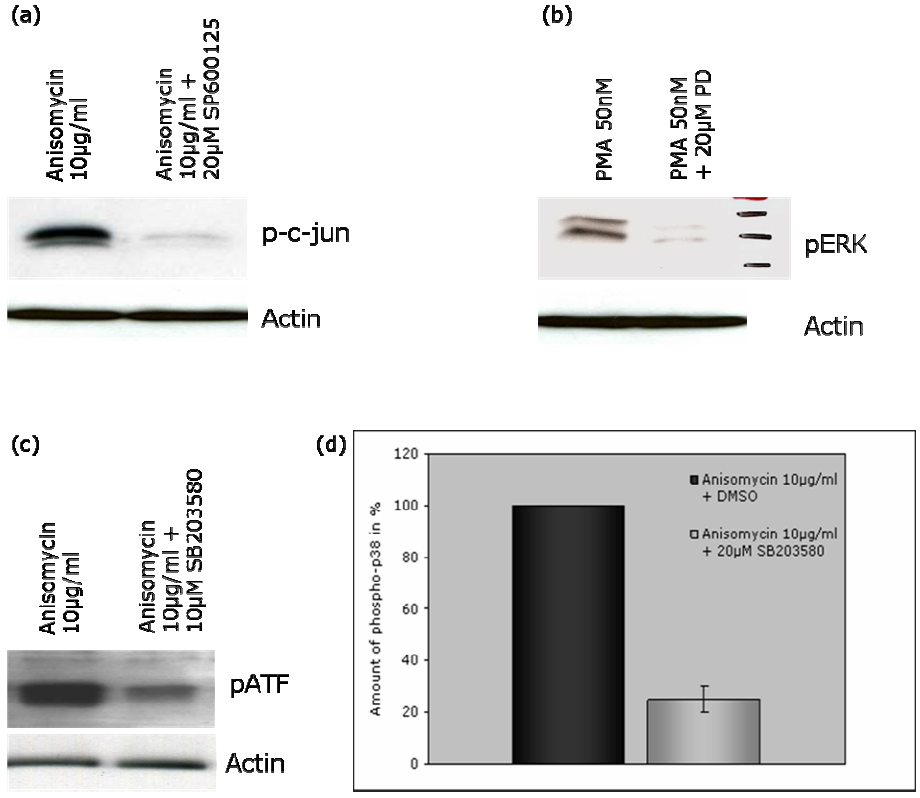


Figure 27: Inhibitor controls. The phosphorylation states of downstream targets of the MAP-Kinases JNK (a), ERK (b), and p38 (c, d) were determined after induction with anisomycin (JNK, p38) or PMA (ERK) and treatment with the respective inhibitors by western blotting or ELISA.

As the controls confirmed the functionalities of the respective inhibitors, these were then used in reporter-gene assays with CRPV E2 (Figure 28) and HPV16 E2 (Figure 29). It was found that in both systems, use of the MEK inhibitor PD098059 led to a highly significant decrease in activation potential of the papillomavirus E2 proteins of about 70% for CRPV E2 and 60% for HPV16 E2. The p38 inhibitor showed little effect in the rabbit system and led to a decrease in promoter activation of about 20% in the human system. SP600125 had no effect on the E2-mediated activation of the promoter in either system.

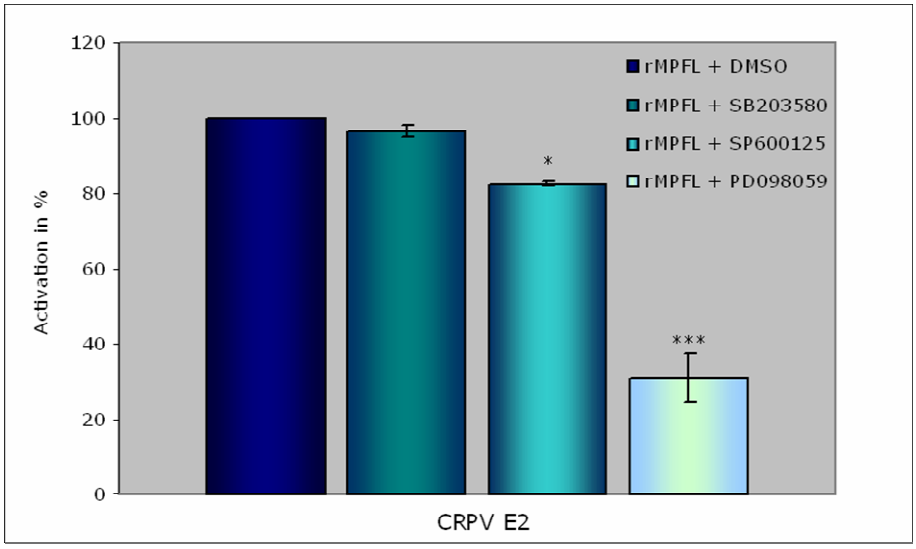


Figure 28: Effect of chemical inhibitors on CRPV E2-induced activation of the rabbit MMP-9 promoter. Cells were transfected with full length MMP-9 promoter constructs and CRPV E2 and treated with chemical inhibitors of the MAP-kinases ERK (PD), JNK (SP), and p38 (SB). Treatment with the inhibitor solvent DMSO served as negative control and was set to 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the DMSO-treated control. Bars show mean value ± SD from three independent experiments in triplicate.

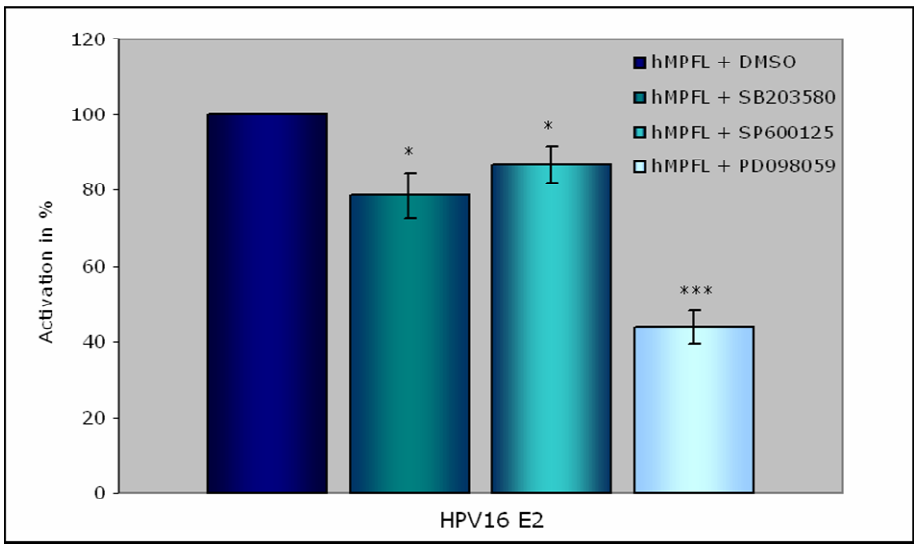


Figure 29: Effect of chemical inhibitors on HPV16 E2-induced activation of the human MMP-9 promoter. Cells were transfected with full length MMP-9 promoter constructs and HPV16 E2 and treated with chemical inhibitors of the MAP-kinases ERK (PD), JNK (SP), and p38 (SB). Treatment with the inhibitor solvent DMSO served as negative control and was set to 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the DMSO-treated control. Bars show mean value ± SD from three independent experiments in triplicate.

The phosphorylation state of the respective MAP-kinase in transfected cells was thus investigated more closely by western blotting. It could be seen that transfection with E2 led to an elevated level of pERK and its downstream-target pELK (Figure 30).

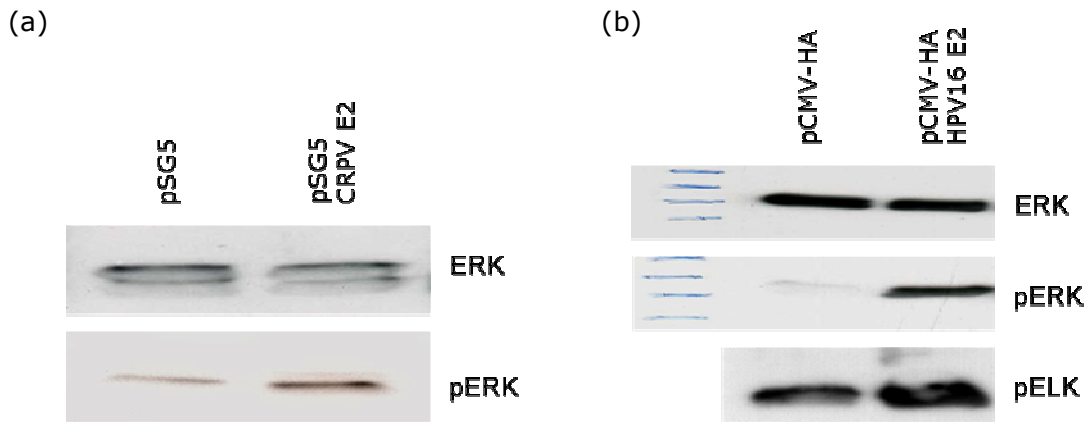


Figure 30: Phosphorylation levels of ERK and its downstream target ELK in Papillomavirus E2 transfected cells. Phoshorylation levels werde detected by western blotting of cell lysates obtained after transfection with CRPV E2 (a) or HPV16 E2 (b) or their respective empty vectors.

The impact of the inhibitors on the transcriptional activation of MMP-9 was also investigated in the human system on the mRNA level. Using semi-quantitative RT-PCR, a

differential expression of MMP-9 was detected in cells transiently transfected with either pCMV-HA HPV16 E2 or the empty vector and treated with the inhibitors described above (Figure 31).

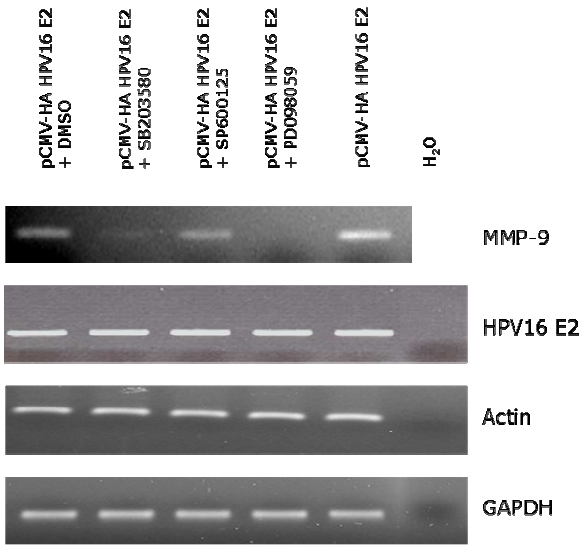


Figure 31: mRNA levels of transiently HPV16 E2 transfected human keratinocytes after treatment with chemical inhibitors. MMP-9 expression was induced by transient transfection of C33A cells with pCMV-HA HPV16 E2. Cells were treated with chemical inhibitors to investigate their effect on mRNA expression. The level of mRNA was detected by semi-quantitative RT-PCR at 36 cycles, H_2O served as negative control.

Further, human cells were transiently co-transfected with the human MMP-9 promoter construct, HPV16 E2 and dominant negative mutants of ERK1 and ERK2. Subsequent reporter-gene assays led to the observation that co-expression of both of these deletion mutants had a similar effect and resulted in a reduction of activation of about 50% as compared to the empty vector control (Figure 32).

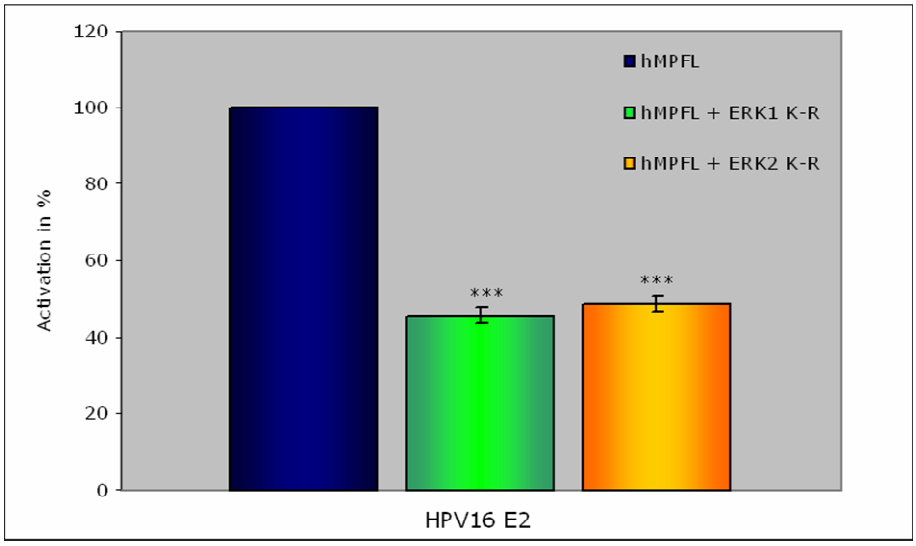


Figure 32: Effect of dominant-negative mutants of ERK1, ERK2, and MEKK1 on human MMP-9 promoter activation. Cells were transfected with the full length human promoter construct, HPV16 E2, and dominant-negative mutants of ERK1, ERK2, and MEKK1. Results are given in % as relative to the empty vector control. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

3.3 ACTIVATION OF THE MMP-9 PROMOTER BY EARLY PROTEINS E6 AND E7

The early proteins E6 and E7 have been shown to be the transforming proteins in HPVs. Most importantly for the present study, E7 has been decribed to interact with the subunits of the transcription factor AP-1. Hence it was of interest, whether E6 and E7 also have the ability to induce the activation of the MMP-9 promoter.

The reporter-gene vector previously constructed and described was used for transient co-transfection with expression-vectors carrying the cDNA for either CRPV E2, CRPV E6, or CRPV E7 or HPV16 E2, HPV16 E6, or HPV16E7. Reporter-gene assays carried out in the rabbit system showed an activation of 9-fold ± 2 for CRPV E6, and an induction of 4-fold ± 0.4 for CRPV E7 (Figure 35a). On the contrary, neither HPV16 E6 nor HPV16 E7 was able to induce the expression of the reporter-gene above the level of the empty vector control (Figure 35b).

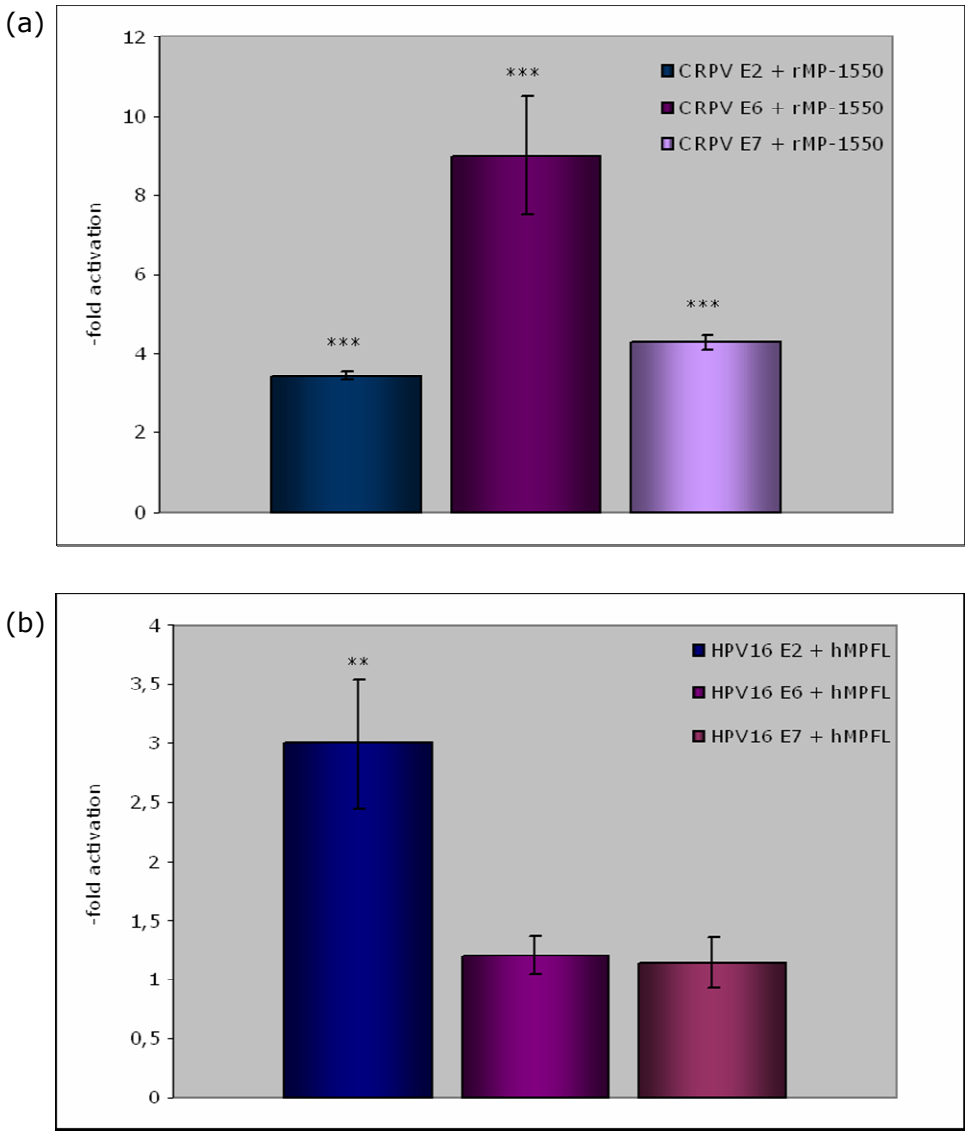


Figure 35: Activation of the MMP-9 prmomoter constructs by papillomavirus early proteins. Rab-9 cells were transfected with the rabbit MMP-9 promoter and CRPV E2, E6, or E7 (a). Human keratinocytes were transfected with the full length human MMP-9 promoter and HPV16 E2, E6, or E7, respectively (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

3.4 ACTIVATION OF THE UROKINASE-PLASMINOGEN ACTIVATOR (UPA)

Besides MMP-9, there are many other proteases upregulated by similar mechanisms during tumorigenesis. It has been shown that the urokinase-plasminogen activator (uPA), is often upregulated in parallel to MMP-9 in cancers. Furthermore, uPA, when bound to its receptor uPAR, converts plasminogen to plasmin which is then able to cleave the pro-form of MMP-9 and thereby activates it. We therefore investigated whether any of the early proteins E2, E6, or E7 of HPV16 has an impact on the expression of uPA.

The level of uPA mRNA was investigated using semi-quantitative RT-PCR. An increase in uPA expression could be detected in transiently HPV16 E2 transfected cells when compared to the empty vector control (Figure 36).

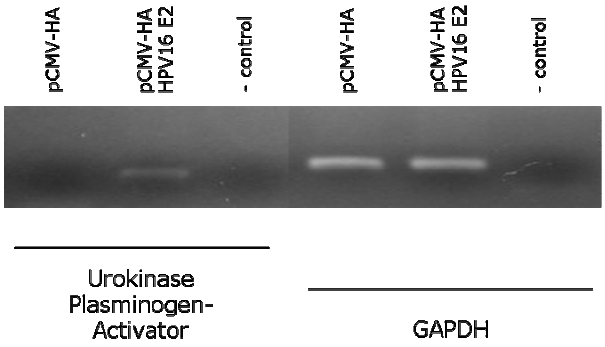


Figure 36: Induction of uPA mRNA expression by HPV16 E2. Urokinase expression was induced by transient transfection of C33A cells with pCMV-HA HPV16 E2 or empty vector control. The level of mRNA was detected by semi-quantitative RT-PCR at 36 cycles, H_2O served as negative control.

As uPA has been previously been described to be activated by MAP-Kinases, the uPA mRNA levels of transiently HPV16 E2 transfected cells treated with chemical inhibitors of the MAPKs p38, JNK, and ERK were investigated by semi-quantitative RT-PCR. It could be observed that the amount of uPA mRNA was diminished upon treatment with (Figure 37).

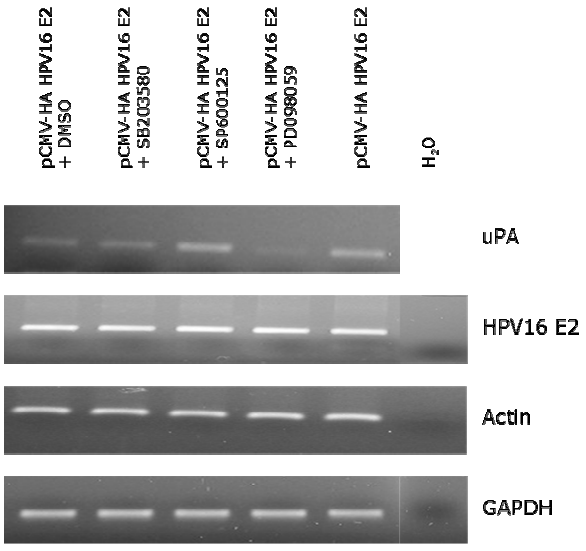


Figure 37: uPA mRNA levels of transiently HPV16 E2 transfected human keratinocytes after treatment with chemical inhibitors. Urokinase expression was induced by transient transfection of C33A cells with pCMV-HA HPV16 E2 or empty vector control. Cells were treated with chemical inhibitors for 48 hours to investigate their effect on uPA mRNA expression. The level of uPA mRNA was detected by semi-quantitative RT-PCR at 36 cycles, H_2O served as negative control.

Using an uPA promoter constructs of 2345bp, reporter-gene assays were thus carried out. It was found that HPV16 E2 was able to activate the uPA-promoter 4-fold, whereas both, transfection with HPV16 E6 and HPV16 E7 resulted in a 3-fold activation (Figure 38).

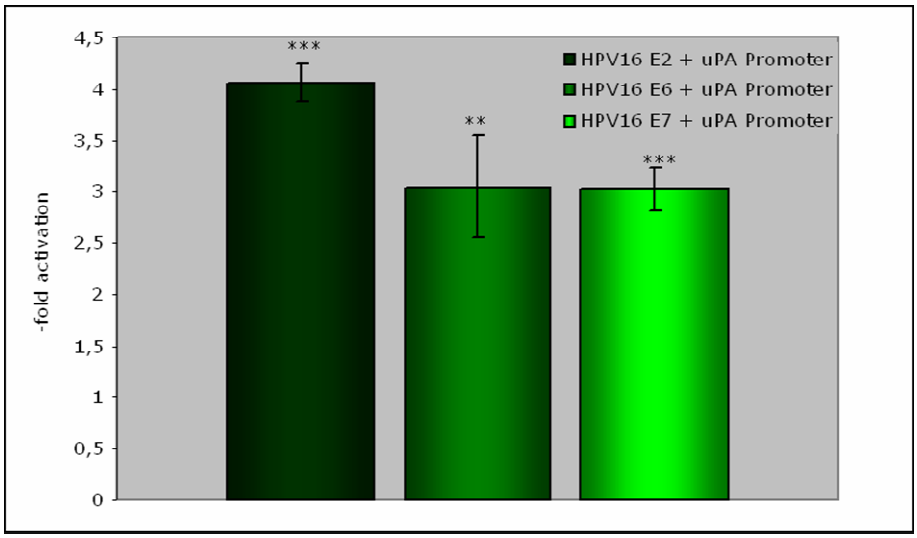


Figure 38: Activation of the uPA promoter by HPV early proteins E2, E6, and E7. Human keratinocytes were transfected with the urokinase-plasminogen activator promoter and HPV16 E2, E6, or E7, respectively (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

3.5 LOCALIZATION STUDIES

It has been reported by Blachon et al. (2005) that high-risk human papillomavirus E2 as opposed to low-risk HPV is able shuttle between nucleus and cytoplasm. The study shows an influence of the cytoplasmic localization on the induction of apoptosis by high-risk HPV. It was hence of interest for the present work if a difference in localization of the E2 protein has an impact on the induction of MMP-9 expression.

3.5.1 Construction of eGFP-tagged HPV16 E2 and localization studies

In order to carry out localization studies with both, HPV16 E2 and HPV6b E2, the proteins were tagged with eGFP to allow for easy visualization. The proteins also obtained a HA-tag at the N- and a KT3-tag at the C-terminus to determine the integrity of the proteins (Figure 39).

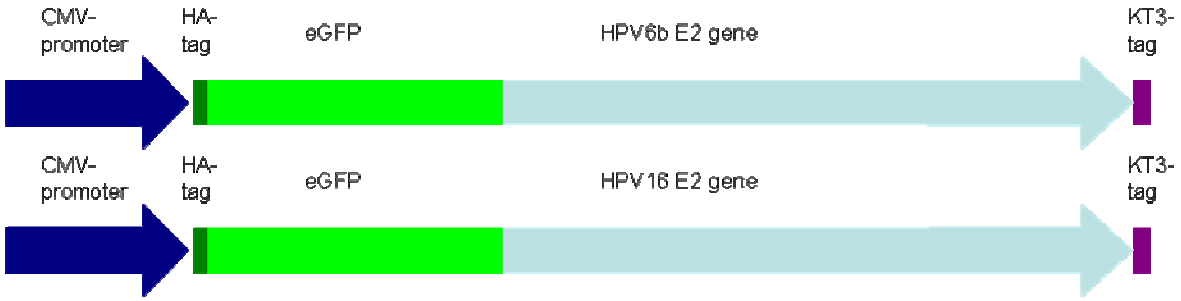


Figure 39: Schematic illustration of the tagged E2 proteins cloned into pCMV-HA. The CMV-promoter of the vector is geiven in dark blue, the HP-tag is also present in the vector and given here in dark green. eGFP is cloned in-frame with the HA-tag and depicted in light green, followed by the HPV E2 cDNA in light blue and the KT3-tag in purple.

Localization studies revealed that while eGFP-HPV6b E2 was strictly nuclear, eGFP-HPV16 E2 was present in the nucleus as well as within the cytoplasm (Figure 40). Addition of leptomycin B an inhibitor of exportin-1 receptor (CRM1)-dependent nuclear export resulted in solely nuclear localization of eGFP-HPV16 E2 (Figure 41).

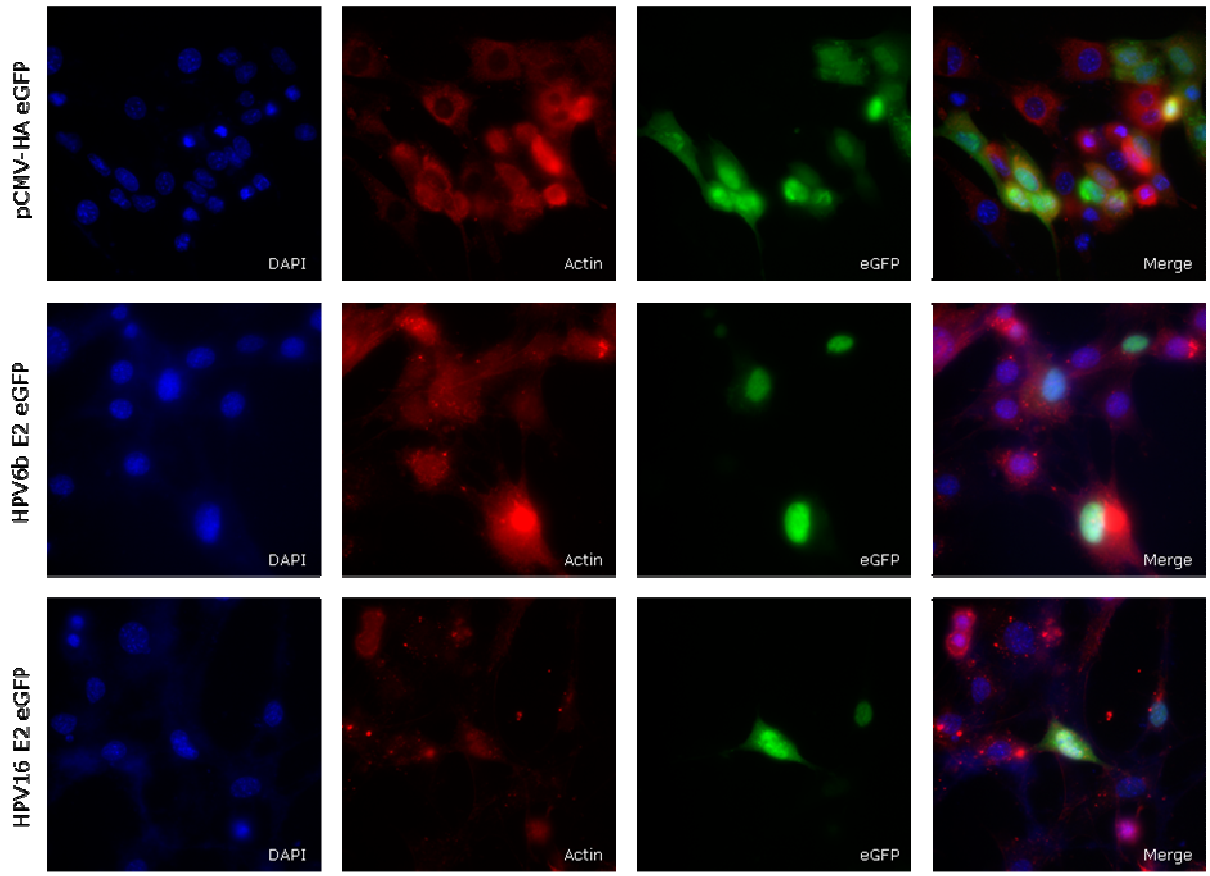


Figure 40: Immunofluorescence of fibroblasts transfected with the eGFP-tagged proteins. Cells were stained with DAPI (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence.

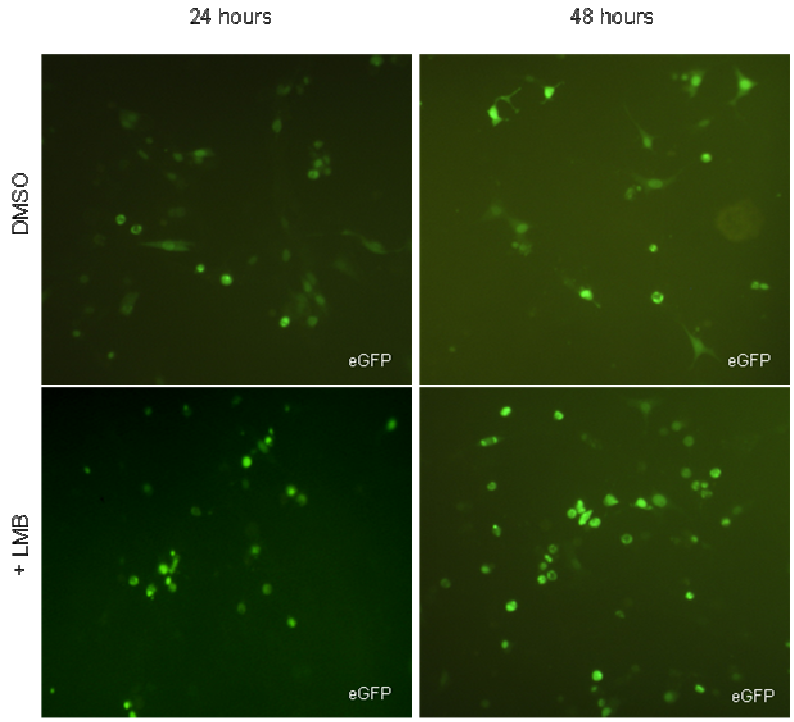


Figure 41: Localization of HPV16 E2eGFP protein after treatment with leptomycin B. Localization of the eGFP-tagged proteins was determined via eGFP autofluorescence.

The ability of the eGFP-tagged HPV16 E2 to induce the acitvation of the MMP-9 promoter upon co-transfection was also determined and compared to the untagged HPV16 E2. It could be observed that although activation was less than that of wt-E2, the difference was significant (Figure 42). Hence further investigation of the effects of the mutant E2 proteins on promoter activation was carried out using proteins without eGFP-tag.

To investigate the regulatory sequences impacting the localization of HPV16 E2 as compared to HPV6b E2, the respective protein sequences of HPV16 E2 and HPV6b E2 have been compared. It was found that HPV16 E2 contains only one nuclear localization sequence (NLS) as compared to HPV6b E2, but also shows a nuclear export sequence (NES) which is not found in the low-risk type. Hence, different approaches were used to investigate the potential role of E2 localization in MMP-9 promoter activation. Firstly, either one of the two NLS found in HPV6b E2 were introduced to HPV16 E2, resulting in the mutant constructs HPV16 E2 NLS1 and HPV16 E2 NLS2. Secondly, the NES found in HPV16 E2 was mutated (HPV16 E2 NESmt) or deleted (HPV16 E2 NESdel). Using immunofluorescence and the eGFP-tagged E2 mutants, the localization of the mutant proteins was determined.

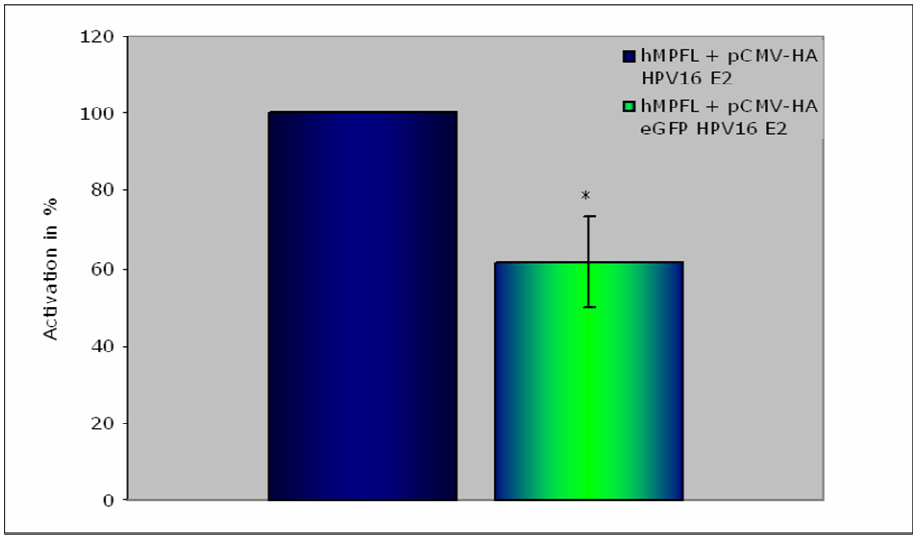


Figure 42: Effect of the eGFP-tag on activation of the human MMP-9 promoter. Cells were transfected with the full length human MMP-9 promoter construct and either HPV16 E2 or eGFP-tagged HPV16 E2. Wild-type E2 was set to 100% and relative activation was determined for the eGFP-tagged protein. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to untagged E2. Bars show mean value ± SD from three independent experiments in triplicate.

3.5.2 Impact of additional NLS sites on localization

Immunofluorescence of the NLS mutant HPV16 E2 eGFP fusion proteins (Figure 43) revealed that both of the mutants containing an additional NLS site were localized solely within the nucleus (Figure 44).

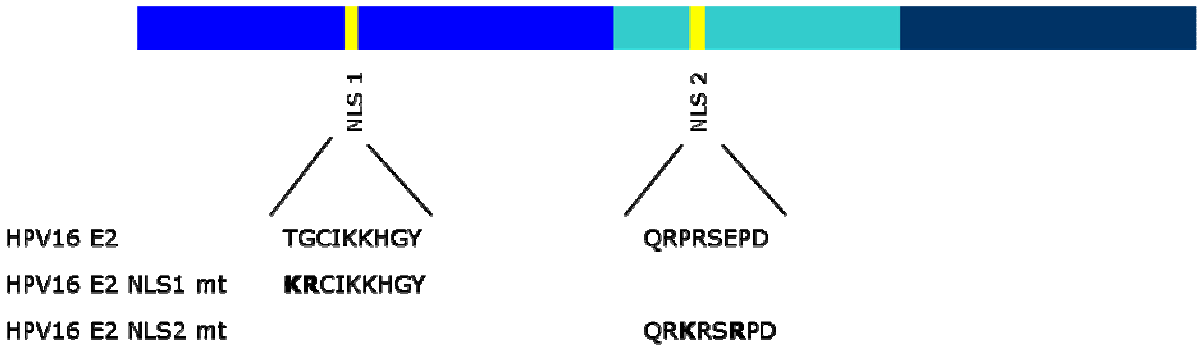


Figure 43: Illustration of the mutations introduced into the E2 protein. Two NLS sites found in low-risk HPV types but absent from high-risk types were separately introduced into HPV16 E2. The original sequences are given in this illustration, the amino acid changes are indicated in bold underneath.

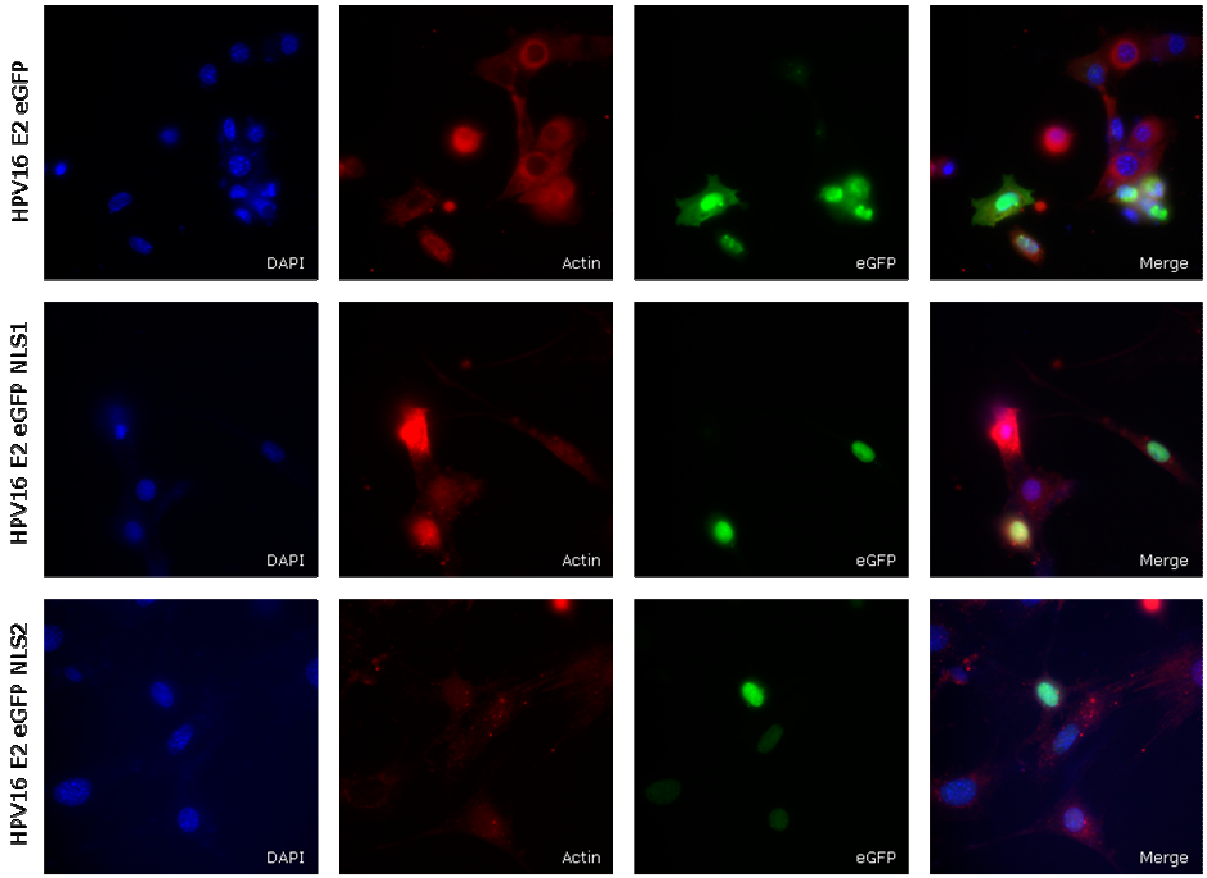


Figure 44: Immunofluorescence of NLS mutants. Cells were stained with DAPI (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence.

In reporter-gene assays, the ability of the mutant proteins to activate the MMP-9 promoter was investigated. Here, the NLS mutant constructs showed no significant decrease in their activation potential of the MMP-9 promoter (Figure 45).

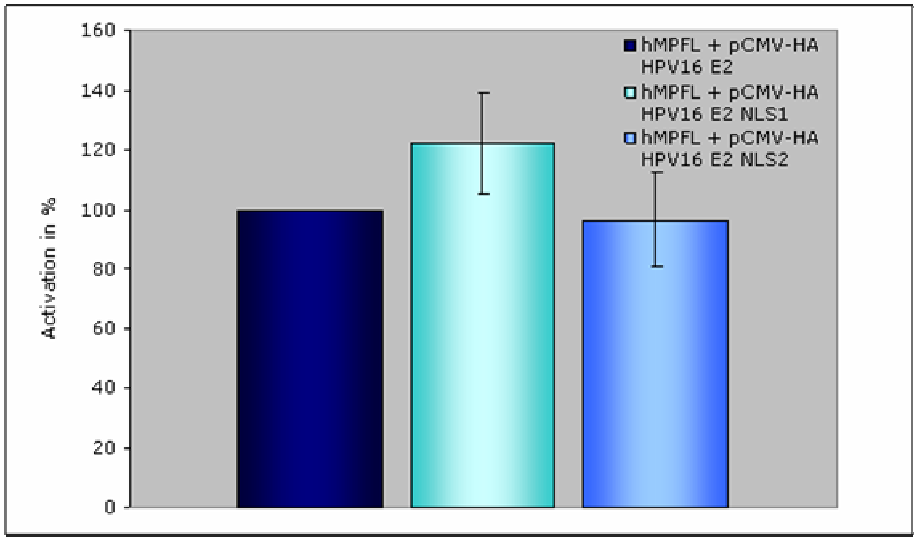


Figure 45: Effect of the introduced NLS sites on activation of the MMP-9 promoter. Cells were transfected with the full length human MMP-9 Promoter and HPV E2 or the HPV16 E2 NLS mutants. Promoter activation by wild-type E2 was considered to be 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to wild-type E2. Bars show mean value ± SD from three independent experiments in triplicate.

3.5.3 Influence of the NES site on protein localization

The nuclear export sequence (NES) found in high-risk HPV types such as HPV16 was mutated in a way that NetNES 1.1, a program detecting NES sequences, did no longer recognize an export signal. A second mutant was constructed in which the whole NES sequence was deleted (Figure 46).

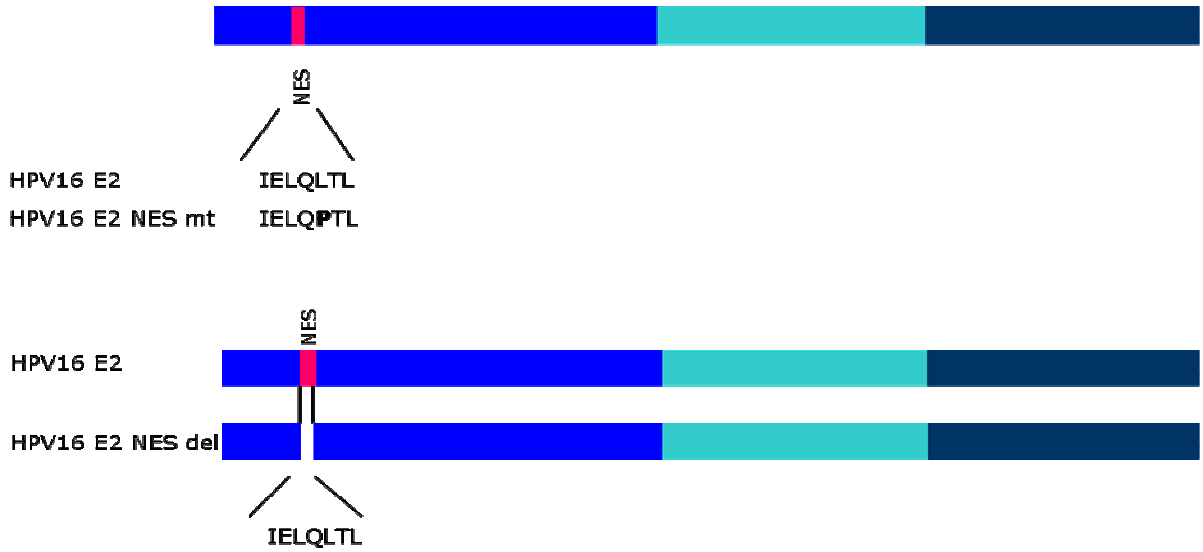


Figure 46: Illustration of the mutations introduced to the NES sites. The NES sites present only in high-risk HPV types was mutated (NES mt) or deleted (NES del) in HPV16 E2. The original sequences are given for HPV16 E2 NES mt, the amino acid changes are indicated in bold underneath. The deleted amino acids in HPV16 E2 NES del are indicated.

The HPV16 E2 NESmt construct was still present in both, cytoplasm and nucleus, however, presence in the nucleus was far more frequent than with the wild-type construct. The HPV16 E2 NESdel was found to be solely nuclear (Figure 47).

Deletion of the complete NES sequence resulted in an insignificant decrease of promoter activation, however, the HPV16 E2 NESmt construct showed a significant decrease in activation potential (Figure 48). A similar decrease in promoter activation has previously been described for the transactivation-deficient HPV31 E2 mutant I73A in a study by Behren et al. (2005) and was confirmed here and which is of nuclear location as deterined by immunofluorescence (Figure 49).

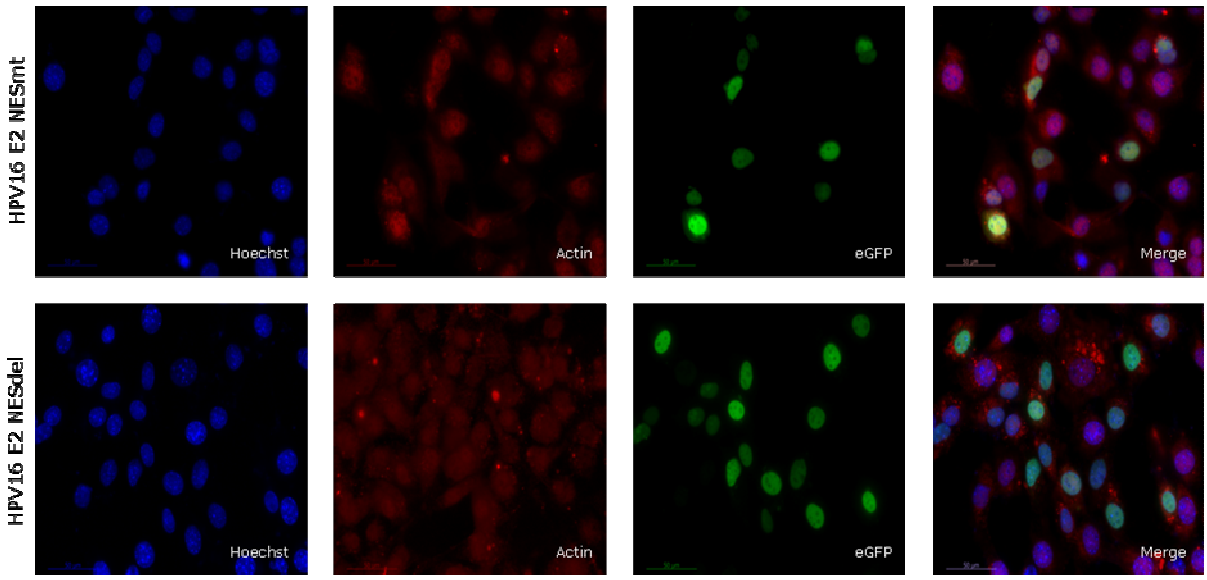


Figure 47: Immunofluorescence of NES mutant E2 proteins. Cells were stained with Hoechst 33342 (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence.

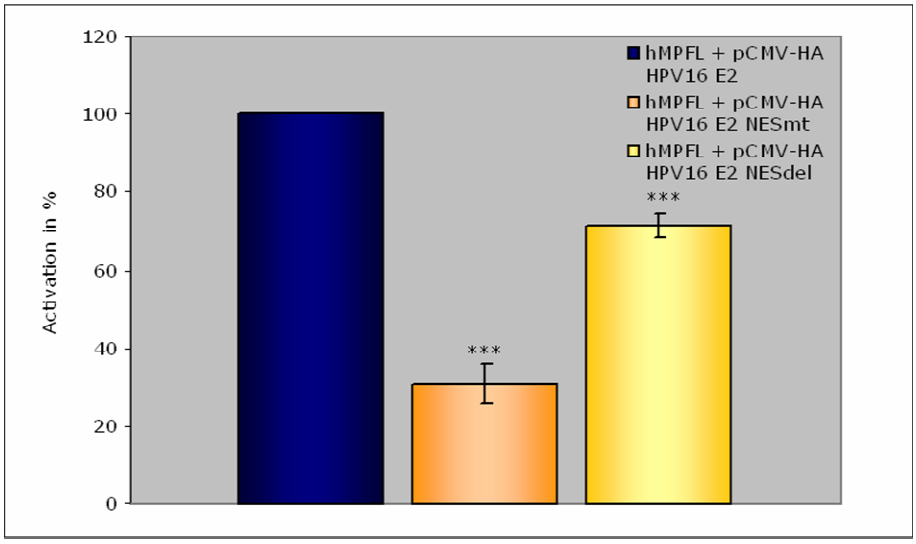
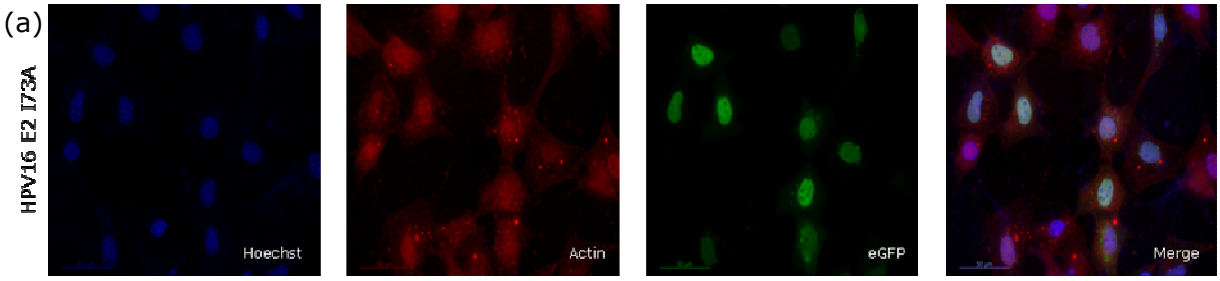


Figure 48: Effect of mutations in the NES site on MMP-9 promoter activation. Cells were transfected with the full length human MMP-9 Promoter and HPV E2 or the HPV16 E2 NES mutants. Promoter activation by wild-type E2 was considered to be 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to wild-type E2. Bars show mean value ± SD from three independent experiments in triplicate.



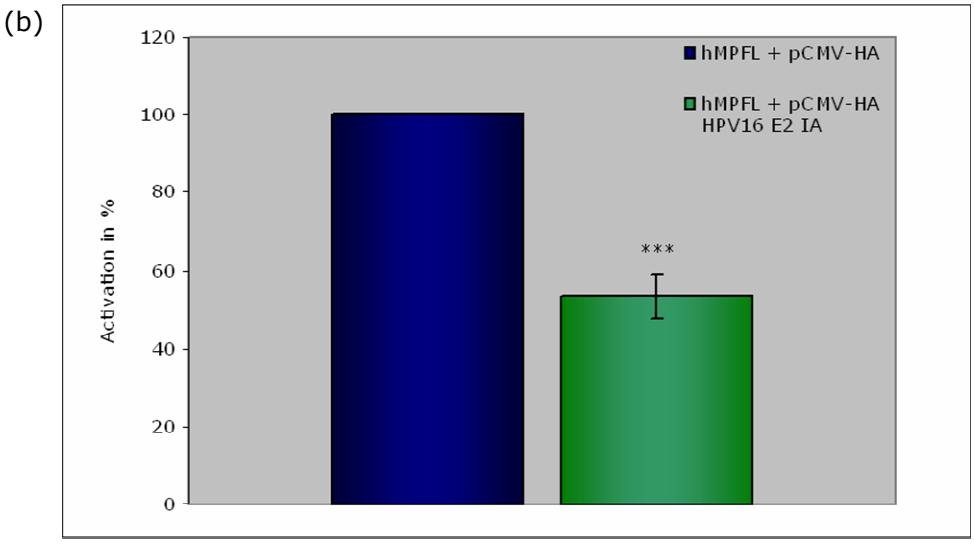


Figure 49: Transactivation-deficient E2 mutant I73A. Cells were stained with Hoechst 33342 (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence (a). Cells were transfected with the full length human MMP-9 Promoter and HPV E2 or the HPV16 E2 I73A mutant. Promoter activation by wild-type E2 was considered to be 100% (b). Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to wild-type E2. Bars show mean value ± SD from three independent experiments in triplicate.

Hence, it was interesting to investigate whether the E2 protein of HPV6b which, as previously described, remains strictly within the nucleus, is also able to activate the human MMP-9 promoter in reporter-gene assays. As it turns out, HPV6b E2 induces a 4.2-fold activation on the promoter (Figure 50) and is therefore a potent activator of the MMP-9 promoter.

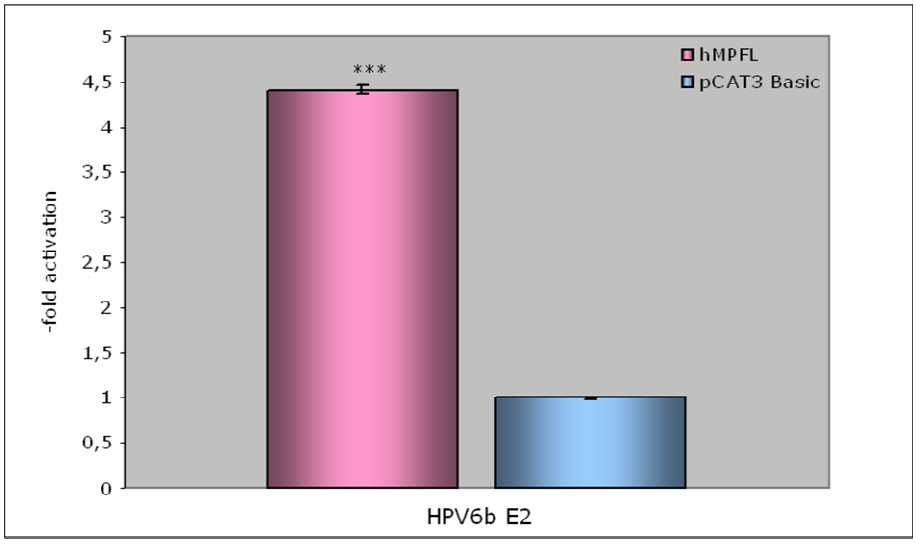


Figure 50: Activation of the human MMP-9 promoter by HPV6b E2. Cells were transfected with HPV6b E2 and the human MMP-9 promoter and lysates obtained were subjected to CAT-ELISA. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to the empty vector control. Bars show mean value ± SD from three independent experiments in triplicate.

3.5.4 The NLS deletion mutant

The results obtained for the E2 protein of low risk type HPV6b suggest that the E2 proteins interact intranuclearly with proteins that then lead to activation of the MMP-9 promoter. To investigate this hypothesis, a mutant was created in which the only NLS found in HPV16 E2 is rendered non-functional (Figure 51a) resulting in a mainly cytoplasmic localization of the protein (Figure 51b,c). When this mutant was used in reporter-gene assays with the human MMP-9 promoter, a CAT-expression of only 40% of that of wildtype HPV16 E2 was observed which is significantly lower than wildtype HPV16 E2 activation (Figure 52).

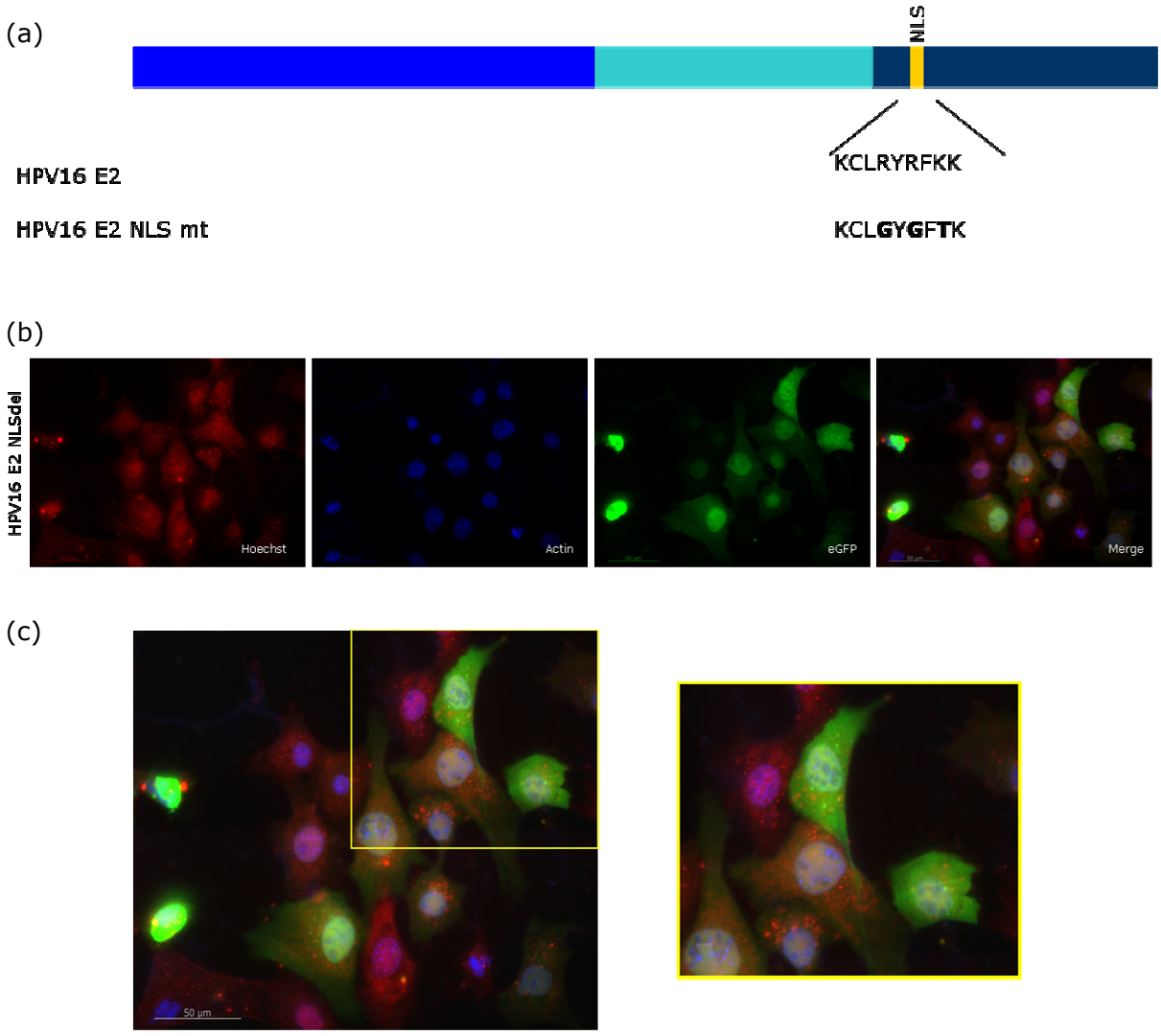


Figure 51: NLS mutant construct and localization. The NLS site found in all HPV types was deleted from HPV16 E2 by site-directed mutagenesis. The original sequences are given in this illustration, the amino acid changes are indicated in bold underneath (a). Cells were stained with Hoechst 33342 (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence (b). Enlargement of the merged picture shown in b (c).

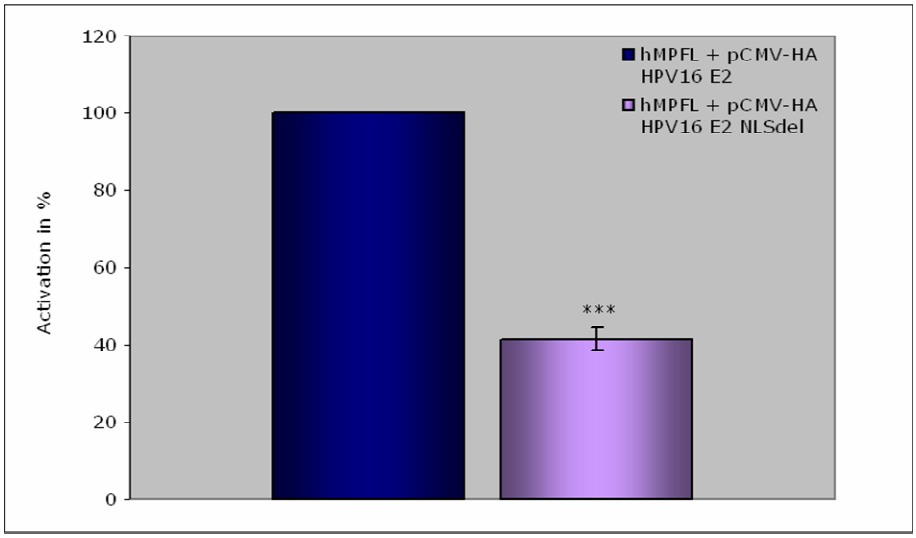
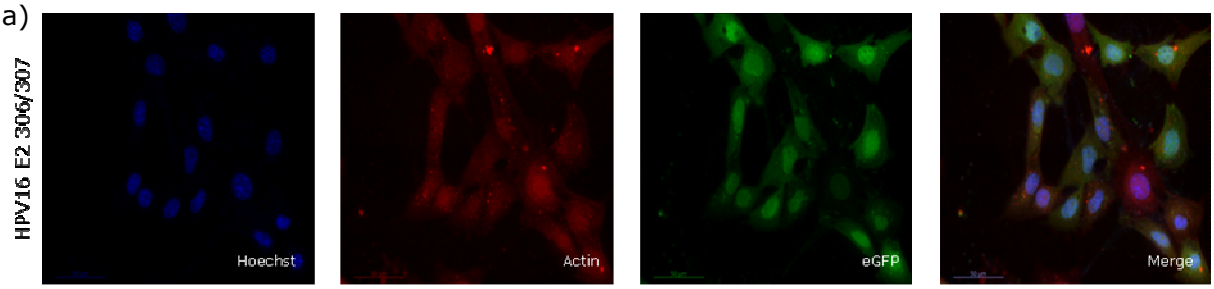


Figure 52: Effect of HPV16 E2 NLS deletion on MMP-9 promoter activation. Cells were transfected with the full length human MMP-9 Promoter and HPV E2 or the HPV16 E2 NLS deletion mutant. Promoter activation by wild-type E2 was considered to be 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to wild-type E2. Bars show mean value ± SD from three independent experiments in triplicate.

A mutation previously described to be deficient in DNA-binding is located within the same region as the NLS sequence found in HPV16 E2 is the 306/307 mutation described for HPV31 in Behren et al., 2005. This mutation was introduced into the HPV16 E2 protein and it could be observed that this mutant had a similar effect on promoter activation as was described for HPV31 E2 protein in the above study. Immunofluorescence of the eGFP-tagged mutant showed a distribution of the protein throughout the cell but more frequently within the cytoplasm as was previously observed for wild-type E2 (Figure 53).

(a)



(b)

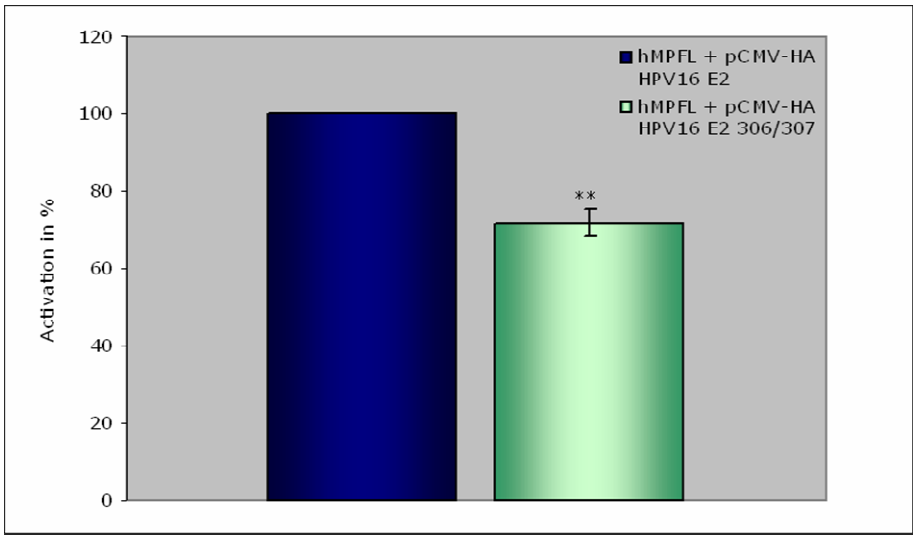


Figure 53: DNA-binding deficient E2 mutant 306/307. Cells were stained with Hoechst 33342 (DNA) and actin was detected with an anti-actin primary and Cy3-labelled anti-rabbit secondary antibody. GFP was detected by autofluorescence (a). Cells were transfected with the full length human MMP-9 Promoter and HPV E2 or the HPV16 E2 306/307 mutant. Promoter activation by wild-type E2 was considered to be 100%. Statistics were done by Students T-Test *, P≤0.05; **, P≤0.01; ***, P≤0.005 as compared to wild-type E2. Bars show mean value ± SD from three independent experiments in triplicate.

4 DISCUSSION

4.1 INDUCTION OF MMP-9 PROMOTER ACTIVATION BY PAPILLOMAVIRUS E2 OCCURS VIA TWO AP-1 BINDING SITES

The expression of proteases has been linked to the initiation and progression of cancer. Overexpression of matrix-metalloproteinase 9 has been associated with many cancers including tumors of the skin (O'Grady *et al*., 2007), cervix (da Silva Cardeal *et al*., 2006), colorectal (Mook *et al*., 2004), and head and neck region (Pacheco *et al*., 2002). In the rabbit animal model for HPV-induced skin tumorigenesis, an enhanced expression of MMP-9 could be observed in papillomas and carcinomas as opposed to healthy skin (Behren *et al*., 2005). To investigate the mechanism involved in the activation of the rabbit MMP-9 promoter, the sequence of the rabbit MMP-9 promoter had first to be investigated. Therefore, in this study, a 1872bp fragment of the rabbit MMP-9 promoter has been isolated and sequenced. Alignment of the obtained rabbit promoter sequence to the sequence of the human MMP-9 promoter resulted in the interesting observation that the highest similarities between the two promoters are found within the proximal 800bp and the distal 500bp, separated by a region of low similarity of about 600bp.

Although the proximal region of the MMP-9 promoter seems highly conserved among species, the CA-repeat which is present within the human and also the mouse MMP-9 promoter, is absent in the rabbit. The length of the CA-repeat in the human MMP-9 promoter has been associated with several diseases such as mutiple sclerosis (Nelissen *et al*., 2000; Fiotti *et al*., 2004), intracranial and abdominal aortic aneurysms (Shimajiri *et al*., 1999; Yoon *et al*., 1999), and cancer (Huang *et al*., 2003). It has been descibed in the latter study that a shortening of the microsatellite CA-repeat results in a decrease in MMP-9 promoter activation and is accompanied by a reduction of invasion in matrigel assays. However, the absense of the CA-repeat seems to have no effect on the regulation of the rabbit promoter.

The highly homologous C-terminal region of the promoter has been described to be sufficient for rabbit and human MMP-9 promoter activation *in-vitro* (Fini *et al*., 1994; REF) and is characterized by the presence of two sequence and position conserved AP-1 binding motifs. A deletion of the proximal AP-1 binding site in the human promoter has been described to eliminate basal as well as PMA-induced promoter activity in a variety of oncogenically-transformed cell lines (Sato and Seiki, 1993). This result, however, seems dependent on cell type, as the inactivation of this AP-1 binding motif led to only a partial decrease of promoter activation in fibroblasts (Fini *et al*., 1994). In this study, it was confirmed that PMA-induced activation of the rabbit MMP-9 promoter was completely abolished when the proximal AP-1 binding site was mutated. Upon MMP-9 induction by

CRPV E2 it could be observed that this mutation resulted in only a partial decrease in promoter activation of approximately 60%, a finding that was also obtained when the distal AP-1 binding site was deleted. As the rabbit experiments were carried out in Rab-9 cells, which are a fibroblast cell line, this may be the reason why activation of the MMP-9 promoter was only partially reduced while previous studies using the truncated human MMP-9 promoter in rabbit epithelial cells showed a complete decrease in activation (Behren *et al*., 2005). We could demonstrate that the rabbit -480bp deletion construct was neither activated by PMA nor by CRPV E2. This promoter fragment does still contain both AP-1 binding sites, however, the distal AP-1 consensus sequence was at the very end of the promoter fragment. It has been described that an NFkB consensus element and an SP1-like element in the human MMP-9 promoter cooperate with the proximal AP-1 motif in determining the response to PMA (Sato and Seiki, 1993). In the rabbit promoter, however, the corresponding NFkB binding-site is located upstream of the distal AP-1 binding site (Fini *et al*., 1994). This may consequently explain the inability of PMA to induce the activation of the -480bp rabbit MMP-9 promoter construct.
An inhibitory effect was observed when the rabbit -953bp promoter construct was induced by CRPV E2. This effect was hypothezised to be due to a repressive element binding to the fragment between -717bp and -953bp in the rabbit promoter or steric changes in the promoter region induced by the absense of an element which would be present upstream of -953bp. The repressive effect was abolished upon elongation of the promoter fragment to -1225bp. To further investigate this inhibitory effect, the transcription factor binding sites positioned between -717bp and -953bp were determined. The binding sites of two transcription factors, MyoD and E47 (Chu et al., 1997; Bolós et al., 2003), which have been described to have repressive effects have been chosen and mutated within the context of both, the -953bp and the -1225bp promoter. It could be observed that upon mutation of the E47 binding site, the activation of the -953bp promoter fragment increased to the level detected for the -1225bp and the -717bp promoter. The deletion of the E47 binding site in the context of the -1225bp promoter fragment showed no significant change in activation as compared to the wild-type promoter. Therefore, it can be suggested that the repression observed within the -953bp promoter is an artifical repression which is caused by the truncation of the promoter and that E47 binding itself has no effect on the full length promoter.

4.2 THE AP-1 COMPLEX COMPONENT C-JUN PLAYS AN IMPORTANT ROLE IN PAPILLOMAVIRUS E2-INDUCED MMP-9 PROMOTER ACITVATION

Abbarent expression of c-Jun has been shown to be involved in several human cancers such as myeloid leukemia (Rangatia, *et al*., 2003), Hodgkin lymphoma (Mathas *et al*.,

2002), and prostate cancer. In head and neck tumors, c-jun expression has been shown to correlate with the the expression of the matrix-metalloproteinases 1 and 9 (Pacheco *et al*., 2002). c-Jun negative ras-transformed cells lack many characteristics of ras transformation including the ability to induce tumors in nude mice (Johnson *et al*., 1996). Furthermore, ectopical expression of c-Jun in breast cancer cells resulted in a change in AP-1 composition and subsequent conversion into a more aggressive cell line and additionally also an increase in MMP-9 (Smith *et al.,* 1999). Co-transfection of cells with the transactivation-deficient c-jun mutant TAM67 leads to the integration of the TAM67 into newly forming AP-1 complexes which compete with functional AP-1 complexes for binding to the AP-1 binding motifs. Upon binding of a complex containing TAM67, transcription of the respective gene cannot progress and is abolished (Grant *et al*., 1996). TAM67 expression has already shown the importance of c-Jun in the growth of non-small cell lung cancer cells (Shimizu *et al*., 2008). Furthermore, TAM67 can abrogate an oncogenic phenotype in Epstein-Barr-Virus (EBV) late-membrane protein-1 (LMP-1) positive nasopharyngeal carcinoma (Jin *et al*., 2007) and inhibits breast cancer growth *in-vivo* and *in-vitro* (Liu *et al*., 2002). Most interestingly for this study, TAM67 has been shown to block HPV16 E7-enhanced tumor progression in mice (Young *et al*., 2002). Here, using co-transfection of TAM67, we were able to observe a highly significant inhibition of MMP-9 promoter activation by PMA as well as by CRPV and HPV16 E2. It can hence be concluded that c-Jun is a member of the transcription-factor complex binding to the MMP-9 promoters which is also confirmed by the above mentioned finding by Smith *et al*. (1999) that an overexpression of c-Jun results in an increase in MMP-9 expression. Furthermore, the highly significant ihnhibiton detected is probably due to the fact that TAM67 may also inhibit NFkB. The significance of AP-1 in the activation of the promoter by E2 was confirmed and taken together with the above mentioned finding that c-Jun is involved in HPV16 E7-mediated tumor progression (Young *et al*., 2002), AP-1 is worth to be considered as a target for the prevention of HPV-induced cancers.

4.3 THE MAP-KINASE ERK IS INVOLVED IN E2-MEDIATED MMP-9 PROMOTER ACTIVATION

An enhanced activation of ERK has been described in prostate (Zayzafoon *et al*., 2003), ovarian (Al-Ayoubi *et al*., 2008), and cervical cancers (Branca *et al*., 2004) among others. Its activation is further known to be of importance in the phosphorylation and regualtion of c-Jun. It has often been described that the human MMP-9 promoter is regulated by the activity of the mitogen-activated protein kinases. Most often this regulation is a result of the MAP-kinases ability to induce transcription of members of the transcription factor complex AP-1. To investigate the role of the MAP-kinase members JNK, p38, and ERK, in

the papillomavirus E2-mediated activation of the MMP-9 promoters, chemical inhibitors were added to the cells. In subsequent reporter-gene assays, a highly significant decrease in promoter activation could be detected in cells treated with PD098059. As it has previously been shown by Behren *et al*. (2005), this inhibitor was able to block CRPV E2-induced invasion in rabbit epithelial cells. Western blot analysis of the phosphorylation states of ERK in this study yielded phosphorylation of ERK and its downstream-target ELK in both, rabbit and human cell lines after transfection with the respective papillomavirus E2 proteins. It has been shown that several viral proteins are involved in the regulation of MAPK pathways. The E5 protein of HPV16 is commonly known to activate ERK through its upregulation of EGF-receptor signalling (Crusius *et al*., 1997; Crusius *et al*., 2000; Cartin and Alonso, 2003). The Karposi's Sarcoma-Associated Herpesvirus (KSHV) induces AP-1 by activation of primarily ERK and JNK, but to a low degree also by p38 (Xie *et al*., 2005) in contrast to the Hepatitis B Virus HBx protein, which has been described to induce and activate AP-1 by via ERK and JNK (Benn *et al*., 1996). The LMP-1 protein of EBV, which is also known to upregulate MMP-9, has been shown to influence a variety of cellular transcription factors such as NFkB and AP-1 (Yoshizaki *et al*., 1998).

4.4 THE E6 AND E7 PROTEINS OF CRPV BUT NOT HPV16 INDUCE MMP-9 PROMOTER ACTIVATION

The E7 protein of human papillomavirus has been desribed to interact with several members of the AP-1 complex. Therefore, it may be possible that this protein hence influences the expression of AP-1 dependent genes such as MMP-9. Furthermore, it is plausible to suggest that the oncogene E6, which has also been shown to interact with cellular genes, is also involved in the induction of proteases to promote tumor progression, especially as the E2 ORF has been shown to be disrupted after integration of the HPV genome in late stages of carcinogenesis. Co-transfection of the human and rabbit MMP-9 promoter with the respective early proteins E6 and E7 yielded a difference in activation capacity between the HPV and CRPV proteins. While the HPV proteins did not induce promoter activation, the CRPV E7 protein resulted in an activation comparable to that of E2. CRPV E6 resulted in a 9-fold activation. Previous investigation of the function of the CRPV E6 protein by Ganzenmueller *et al*. (2008) has already shown that the protein is different to that of HPV as it does neither bind E6Ap nor induce degradation of p53. Here, we were able to describe another difference between HPV and CRPV E6. However, the importance of HPV E2 in the induction of human MMP-9 expression in infected cells has thus been made even clearer. The question that remain to answer is how MMP-9 remains upregulated even after integration of the HPV genome into the host cell DNA. As this integration event occurrs only in the late stages of infection it is possible

that the activation of the ERK cascade results in an encironment which is favorable for the development of lesions and enhanced cellular proliferation. Upon induction of this process, the activation of ERK may continue in an autocrine way and thus influence the expression of viral (Chan *et al*., 1990) and cellular genes that are regualted by AP-1.

4.5 THE EARLY PROTEINS E2, E6, AND E7 OF HPV16 ALL AFFECT THE ACTIVATION OF THE HUMAN UPA PROMOTER

It has been determined in several studies, that the uPA/uPAR system is involved in the establishment and progression of tumors. Upon binding to the receptor, uPA cleaves plasminogen to plasmin, which in turn is able to activate several classes of proteins including members of the matrix-metalloproteinases. Furthermore, uPA expression has been desribed to be acivated by similar means as MMP-9. The uPA promoter has been previously characterized and the transcription-factor AP-1 has been described to be of importance in the activation of uPA. Futhermore, binding of uPA to uPAR leads to an activation of signal-transduction pathways including ERK. As the mechanisms of MMP-9 and uPA induction are similar and uPA has been shown to be of importance in activating MMP-9, it was interesting to determine whether the expression of HPV16 E2, has an effect on uPA expression or promoter activation. Experiments with the rabbit uPA promoter could not be carried out due to the fact that it is yet unavailable. In human keratinocytes, mRNA levels of uPA were detected after transient transfection with HPV16 E2 and an increase in the mRNA level of uPA could be detected, which decreased upon addition of the ERK inhibitor PD098059 to the transfected cells. Additionally, in reporter-gene assays an involvement of the HPV16 early proteins E6 and E7 in uPA promoter activation could be observed. This may result in a continued activation of MMP-9 secreted by surrounding stromal cells by plasmin in late stages of tumorigenesis, when the viral genome becomes integrated. The continued, E2-independent expression of uPA may thus lead to the maintenance of a growth-favorable microenvironment.

4.6 THE ROLE OF HPV16 E2 LOCALIZATION IN MMP-9 PROMOTER ACTIVATION

In a study by Blachon *et al*. (2005) it was shown that E2 of high-risk HPV-types is located within the nucleus and cytoplasm of an infected cell. It is able to move freely between the two compartments due to the presence of a nuclear export signal (NES) and a nuclear localization sequence (NLS). Low-risk HPV E2 on the other hand is present solely within the nucleus. As opposed to high-risk E2, low-risk E2 proteins contain three NLSs but no NES. In their study, Blachon *et al*. (2005) associated the localization of the proteins with the induction of apoptosis as can be detected in high-risk HPV infection. Using HPV16 E2

mutants, we set out to investigate the role of E2 localization on the protein's ability to activate the human MMP-9 promoter. To confirm the localization of the constructed mutants within the cell, the E2 protein and mutants were tagged with enhanced green fluorescent protein (eGFP). eGFP fluorescence can be detected under fluorescent light in the microscope and localization of the proteins can hence be determined. Introduction of either of the two additional NLS sites identified in low-risk HPV E2 into the HPV16 E2 sequence resulted in a mainly nuclear localization of the protein. Activation of the human MMP-9 promoter as detected by reporter-gene assays was similar to that achieved by wild-type HPV16 E2. Mutation as well as deletion of the nuclear export sequence also resulted in a mainly nuclear localization of the E2 protein.In reporter-gene assays, however, the E2 mutant containing only a mutation within the NES resulted in a highly significant decrease in MMP-9 promoter activation as opposed to the NES deletion mutant which retained the activation capacity of wild-type E2. A study by … *et al*. has shown that a mutation of the amino acid at position 73 in the E2 sequence leads to a transactivation-deficient protein, which has a reduced ability to induce papillomas in the context of the viral genome *in-vivo* (Jeckel *et al*., 2002). The I73A mutant has been shown to have a reduced capacity to activate the truncated 670bp human MMP-9 promoter *in-vitro* (Behren *et al*., 2005). Reporter-gene assays carried out with the described mutant and a full length human MMP-9 promoter resulted in a decrease in activation as previously described. In contrast to the NES mutant, this protein showed a nuclear and cytoplasmic distribution in the cell. As both, the amino acid exchanges in the NES mutant and the isoleucin at position 73 are located within close proximitiy to each other in the transactivation domain of the E2 protein, it may be that the decrease in promoter activation observed with the NES mutant is a result of a transactivation deficiency. This will have to be subject to further investigations. As the nuclearly localized proteins were able to activate the MMP-9 promoter, it was interesting to investigate whether the low-risk HPV6b E2 protein, is able to induce an activation of the promoter as well. As this was shown to be the case, it can be hypothezised that the E2 protein induced MMP-9 expression in HPV-infected cells via ERK and AP-1-mediated promoter activation by interaction with a nuclear mediator of unknown identity. Two methods were available in order to prove this theory. It is known that the nuclear import of the E2 protein occurs via the exportin-1 receptor (CRM1) which can be inhibited by addition of actinomycin D. However, the import of MAP-kinases into the nucleus occurrs by a similar mechanism and hence inhibition of nuclear import would have led to unclear results in the reporter-gene assays. Therefore, an NLS deletion mutant was constructed which was to be localized within only the cytoplasm of the cells. Immunofluorescence of the created mutant confirmed a mainly cytoplasmic localization of the protein. Reporter-gene assays showed a significant decrease in the activation potential of the cytoplasmic E2 mutant. As the

mutation introduced is not located within the transactivation domain of the E2 protein, but within the DNA-binding domain, this decrease in activation cannot be accounted for by a loss in transactivation efficiency. Furthermore, as activation of the promoter cannot be due to direct binding of the E2 protein to the MMP-9 promoter, as no consensus sequence was detected, this decrease in activation must be a result of the change in E2 localization. Interestingly, when investigated by immunofluorescence, a previously described DNA-binding deficient E2 mutant (E2 306/307), which has also been shown to have a lowered promoter activation potential, was also found to by of mainly cytoplasmic localization.

In this study, we have been able to show that binding of the transcription-factor AP-1 to the MMP-9 promoter is important for the activation of the promoter by papillomavirus E2. Futhermore, by using ERK1 and -2 dominant negative mutants it was determined that both ERK proteins effect the activation of the promoter. Using chemical inhibitors, we ruled out that the JNK and the p38 pathway contribute largely to the activation of the MMP-9 promoter by papillomavirus E2. However, MEK1, an activator of ERK was shown to be involved in the activation of ERK and thus of the MMP-9 promoter.
Taking all of the results obtained within the course of this study together, part of the mechanism can be proposed by which the activation of the MMP-9 promoter is induced by the papillomavirus E2 protein. It can be concluded that the E2 protein binds intracellularly to either another protein by protein-protein interaction or to the promoter of a yet unknown gene whose expression is thereby induced. In either way, signalling occurrs within the cytoplasm or via a receptor and ultimately leads to the phosphorylation of ERK, a step which involves activation of MEK1. ERK then leads to the activation of an AP-1 complex in which c-Jun plays a major role. The resulting AP-1 complex binds to promoters of tumor-progression promoting genes such as MMP-9 and uPA and initiates gene expression (Figure 54).
As the proposed mechanism is still incomplete and contains a lot of unknown factors, it will have to be subject to further investigation.

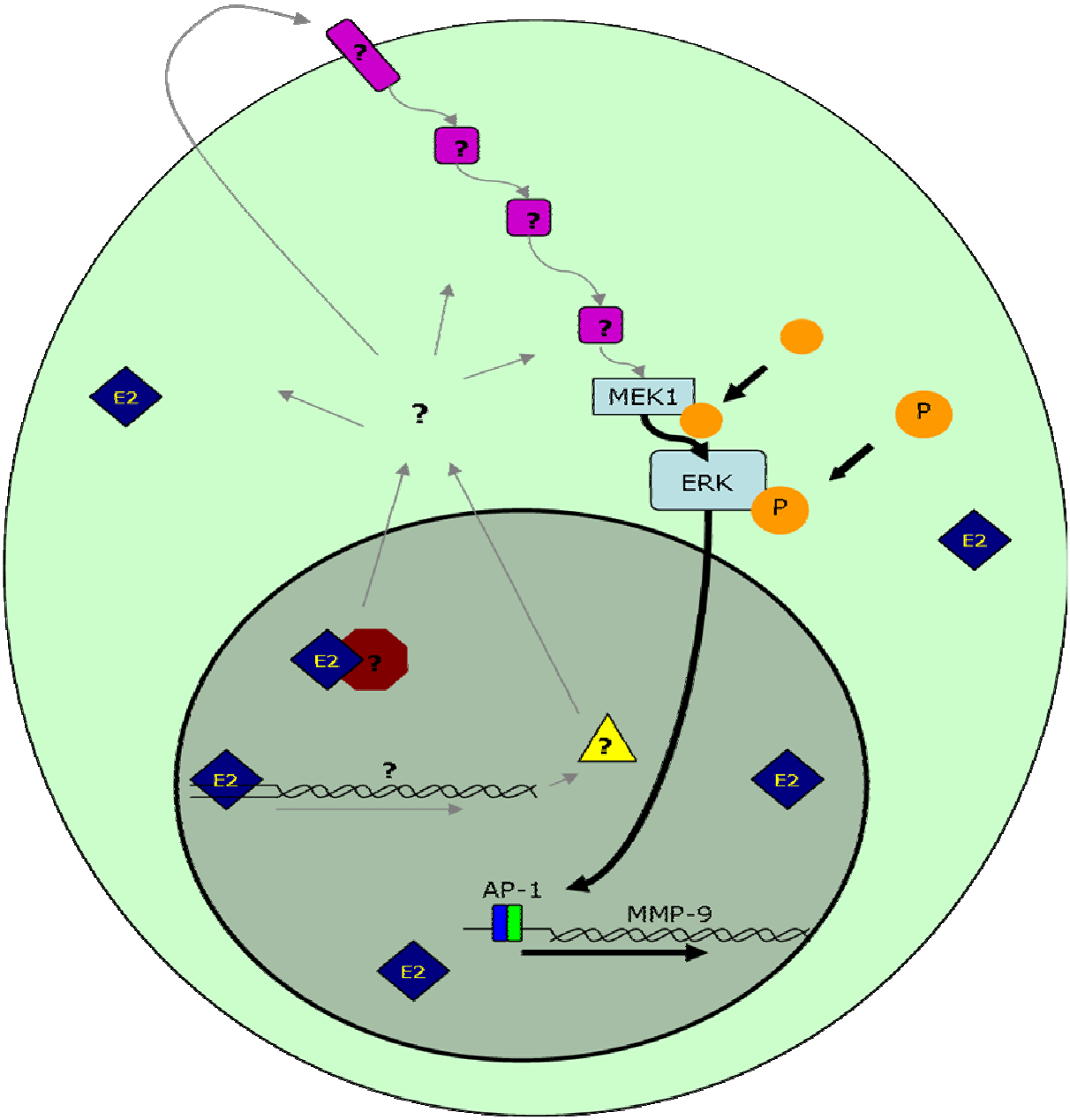


Figure 54: Schematic illustration of proposed mechanisn of E2-mediated MMP-9 promoter activation.

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Attachment 1:

Sequence of the rabbit MMP-9 promoter

```
TCCAACCCAT TCGCCTATAC CTGGTCCTCT CAGTTCCCTG TAAACAGTAA -1804
TACAAATCCA AGGCTTCAGA ACCAGGCAGC CCGAGTTCTG TGTGCCAACC -1754
TGCTGTGTGA CCTCTGGCAA GTTCCTTAGC TGTGCTGAGC CTGTTCTTCC -1704
TCTGAGAAAC CAACCTCATA GACAGTAAGG ATGAGACGAG AGGCTCACGG -1654
AGAGCTTATC ACAGTGTTGG ACCATACAGT AAGTCTCAAA ACACTGCCTT -1604
CTGATGGCTC AGAAGGGAGT GACTTTGCCC AAGGTTACAG AACTGGAACT -1554
GGCAGAGCTG GGACGGAGAT CCAGGACTGT GTGACCCCAG AGCAGGTGCT -1504
CACTAATTAG TGGAGCTTAG AGCTTCCGCT TTCCTATCCA GGACGTCAGC -1454
TGTCTCCATC GCCATGGAGT CATTTATCAG AAAAGCACAG CTGGTGCCCG -1404
GCACATAGTA GGCTCTTTAA ACATAGCGTG TGTTCTTTCC AGGCAAGGCA -1354
GCCGGCCGTG AGCCTGTCCT ACGCTGGCTG CCCGGGAGGC AGGGGGAAAG -1304
GAAGCTGCTG GTTGTTGGGA GAAGCGGTGA GGACTGGGGC GGAAGCTTTT -1254
GTGAGCAGGG CTGGGGAACG GGGAGGCCAG CTGAGGAATC CAGGACCCAG -1204
GAAGGGGAAG GCGTGGACTC TAGTCACACA GCAGCTCAGA GCCCAAGCCT -1154
TCCGCCTCCT GGTTTTAGGT TGGATTCATG ATCTTGGCCT TGACAGTGAA -1104
AAGCAGTCAC TGATGTTTTA ACAACAGCAC CCACCATGCT AAGCATTCTG -1054
TATGTTTATT TTCATATCAA TTTAGGGACA AAGGGATGAT TTAAAAAAAT -1004
CTATTTATTC AAAACCAGGG GGCGGAGAGG GGGAGAGAAT GCCAGCTACC -954
TTGTACTAGT AGTTCACTCT GCAAATACTT GCAACAGCCA GGTGCCGGGC -904
CAAGAGCTTA TAATTCCATC TGCGTCTCCC ACGCAGGTGG CAGGGACTCA -854
AGTACTTGAG CCGTCACCTG CTGCCTCCCA GGGTGACACC AGCAGGAAGC -804
TGGGTGAGAA GTAGAGCTGG GGCTGGAACC CAGGCCCTCC AGTGCAGGAT -754
GCAAGCATCC TAAGCAGTGT TTTAGTCACA GCATCCAACA CCTGCCGGGG -704
GGGGGGGGTG CTATTTTTTG ACAGCCGAGC AAAGAAGTCC CCAGTGGTGA -654
GGCCGTGTGC CCGAGGCCCT CAGGGAGCAC CGTGAGGCTG TCTGCTGAGC -604
TCTCCCCATC ACTGCCCTGA AGATTCAGCC TGTCGGAGAC AGGGGGTTAC -554
CCCGGTGGAA TTCCCCAAAT CCTGCCTCAG AGAGCCCACT CCTTCCGCCC -504
AGCTGGAGCC GGGAGGAGGA AGCTGAGTCA GAGGAGGGCT TTCCAGGAGG -454
GAGGACGTAG CCTGCGGGGA GAGCCTCAGG AGGGGGTGTC ACAGAGTCAA -404
GGGTGGGCCT GGGGTGGCAC TCAGGAAGGG GGTCCCAGGG CGTCCAACAT -354
CCTTTCGCTG AGCTGACCAC TTGGGACCTC CTACCCCGTC CCTCTCCCTC -304
CTGTTCCCAC AAACGCTGCA GTTTGCAAAA CCCAACCGCT CCCCTGAGGG -254
CCTGTGGTTT CCTGTGGGTC TGGGGTCCTG CCTGACTCGG CAGCGGGGAC -204
TGCAGGCGGG TGGGGGGAGA GGCGGGGGGA GAGGAGGTGG TGAAAATCTG -154
TTCTTTGCCT TCTCATGCTG GGGCTGCCCC CTTCCCCCCT GCACCGGCCC -104
TGAGTCAGGC ACTTGCCTGC AGGAAGAGGG GCGGGGTCAC GACTCAGGAG -54
TGCCTCTTTA AAGCCCCCGC TGCCGTCGTG CGCCAGACGC TGCTCCCCTC -4
ACCATGAGCC CCAGACAGCC CC                                +18
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Attachment 2:

Clustal W alignment of the rabbit and human MMP-9 promoters

```
NZW             ----------------TCCAACCCATTCGC--CTATACCTGG-TCCTCTCAGTTCCCTGT -1812
hum             CTGCCCCTGTCACCGCATCCACCTACCCACTTCTATACCTGGGTCATCACAGTTCCCTGT -2222
                                * *** *  * *  ********** ** ** ***********

NZW             AAACAGTAATACAAATCCAAGGCTTCAGAACCAGGCAGCCCG-------------AGTTC -1765
hum             AAATGGTAATAAAGATGAAAAGCTTCAGAGCCAGGCAGTTCTGGGCTTGAACACTAGTTC -2162
                ***  ****** * **  ** ******** ********  *              *****

NZW             TGTGTGCCAACCTGCTGTGTGACCTCTGGCAAGTTCCTTAGCTGTGCTGAGCCTG---TT -1708
hum             TGTGGATTAACTCGCTCTGTGATCACAGGCAAATTCCTTAACTCT-CTGAGCCTTAGTTT -2103
                ****    ***  *** ***** * * ***** ******* ** * ********    **

NZW             CTTCCTCTGAGAA-------------------ACCAACCTCATAGACAGTAAGGATGAGA -1667
hum             CCCCCTCTGAAAACAGGAGGGATACTCATTAAACTTACCTTACAGGTGGTGAGGATGAAA -2043
                *  ******* **                   **  **** * **   ** ******* *

NZW             CGAGAGGCTCACGGAGAGCTTATCACAGTGTTGGACCATACAGTAAGTCTCAAAA----C -1611
hum             CGAGAGGCTTATAGAGAACTTATTACGGTGCTTGAC---ACAGTAAATCTCAAAAAATGC -1986
                ********* *  **** ***** ** *** * ***   ******* ********    *

NZW             ACTGCC-TTCTGATGGCTCAGAAG-GGAGTGACTTTGCCCAAGGTTACAGAACTGGAAC- -1554
hum             ATTATTATTATTATGGTTCAGAGGTAAAGTGACTT-GCCCAAGGTCACATAGCTGGAAAA -1927
                * *    ** * **** ***** *   ******** ********* *** * ******

NZW             TGGCAGAGCTGGGACGGAGATCCAGGACTGTGTGACCCCAGAGCAGGTGCTCACTAATTA -1494
hum             TGGCAGAGCCGGGATGGAAATCCAGGACTTCGTGACTGCAAAGCAGATGTTCATTGGTTA -1867
                ********* **** *** **********  *****  ** ***** ** *** *  ***

NZW             GTGGAGCTTAGAGCTTCCGCTTTCCTATCCAGGACGTCAGCTGTCTCCATCGCCATGGAG -1434
hum             GTGAACTTTAGAACTTCAACTTTTCTGTAAAGGAAGTTAATTATCTCCATC-TCACAGTC -1808
                *** *  ***** ****  **** ** *  **** ** *  * ********  **  *

NZW             TCATTTATCAGAAAAGCACAGCTGGTGCCCGGCACATAGTAGGCTCTTTAAACATAGCGT -1374
hum             TCATTTATTAGATAAGCATATAAAATGCCTGGCACATAGTAGGCCCTTTAAATACAGCTT -1748
                ******** *** ***** *     **** ************** ******* * *** *

NZW             GTGTTCTTTCCAGGCAAGGCAGCCGGCCGTGAGCCTGTCCT------ACGCTGGCTGCCC -1320
hum             ATTGGGCCGGGCGCCATGGCTCATGCCCGTAATCCTAGCACTTTGGGAGGCCAGGTGGGC -1688
                 *          * ** ***    * **** * ***  *        * **  * **  *

NZW             GG-------GAGGCAGGGG---GAAAGGAAGCTGCTGGTTGTTGGGAGANGCGGTGAGGA -1270
hum             AGATCACTTGAGTCAGAAGTTCGAAACCAGCCTGGTCAACGTAGTGAAACCCCATCTCTA -1628
                 *       *** ***  *   ****  *  *** *    ** * ** *  *  *    *

NZW             CTGGGG---CGGAAGCTTTTGTGAGCAGGGCTGGGGAACG-------------------G -1232
hum             CTAAAAATACAAAAAATTTAGCCAGGCGTGGTGGCGCACGCCTATAATACCAGCTACTCG -1568
                **       *  **  *** *  **  * * *** * ***                    *

NZW             GGAGGCCA--GCTGAGGAAT--CCAGGACCCAGGAAGGGGAAGGCG---TGGACTCTAGT -1179
hum             GGAGGCTGAGGCAGGAGAATTGCTTGAACCCGGGAGGCAGATGTTGCAGTGAGCCGAGAT -1508
                ******    ** *  ****  *  * **** *** *  ** *  *   **  *     *

NZW             CACACAGCAGCTCAG-AGCCCAAGC------------CTTCCGCCTCCTGGTTTTAGGTT -1132
hum             CACGCCACTGCACTCCAGCCTGGGTGACAGAGTGATACTACACCCCCCAAAAATAAAATA -1448
                *** *  * ** *   ****   *             ** *  ** **     * *  *

NZW             GGATTCATGATCTTGGCCTTGACAGTGAAAAGCAG-----TCACTGAT---GTTTTAACA -1080
hum             AAATAAATAAATACAACTTTTTGAGTTGTTAGCAGGTTTTTCCCAAATAGGGCTTTGAAG -1388
                  **  ** *      * **   ***    *****     ** *  **   * *** *

NZW             ACAGCA---------CCCACC--ATGCT---------------AAGCATTCTGTATGTTT -1046
hum             AAGGTGAATATAGACCCTGCCCGATGCCGGCTGGCTAGGAAGAAAGGAGTGAGGGAGGCT -1328
                *  *           **  **  ****                *** * *  *   *  *
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NZW             ATTTTCATATCAATTTAGGGACAAAGGGATGATTTAAA----AAAAT-----CTATTTAT -995
hum             GCTGGTGTGGGAGGCTTGGGAGGGAGGCTTGGCATAAGTGTGATAATTGGGGCTGGAGAT -1268
                  *    *   *   * ****   ***  **   ***     * ***     **    **

NZW             TCAAAACCAGGGGGCGGAGAGGGGGA---GAGAATGCC------AGCTACCTTGTACTAG -944
hum             TTGGCTGCATGGAGCAGGGCTGGAGAACTGAAAGGGCTCCTATAGATTATTTTCCCCCAT -1208
                *      ** ** ** * *  ** **   ** *  **          **  **   * *

NZW             ----TAGTTCACTCTGCAAATA------------CTTGCAACAG--------------CC -914
hum             ATCCTGCCCCAATTTGCAGTTGAAGAATCCTAAGCTGACAAAGGGGAAGGCATTTACTCC -1148
                    *    ** * ****  *             **  ***  *             **

NZW             AGGTGCCG----GGCCAAGAGCTTA--------------TAATTCCAT------------ -884
hum             AGGTTACACTGCAGCTTAGAGCCCAATAACCTGGTTTGGTGATTCCAAGTTAGAATCATG -1088
                ****  *      **  *****  *              * ******

NZW             -------------------CTGCGTCTCCCACGCAGGTG-GCAGGGACTCAAGTA----- -849
hum             GTCTTTTGGCAGGGTCTCGCTCTGTTGCCCAGGCTGGAGTGCAGTGACATAATCATGGCT -1028
                                   **  **  **** ** ** * **** ***  **  *

NZW             --CTTGAGCCGTCACCTGCT----------------------------GCCTCCCAGGG -820
hum             CACTGTATCCTTGACCTTCTTTCTGGGCTCAAGCAATCCTCCCACCTCGGCCTCCCAAAG -968
                  **  * ** * **** **                                ********  *

NZW             TGACACCA--GCAGGAA-----------GCTGGG---TGAGAAGTAGAGCTGGGGCTGG- -777
hum             TGCTAAGATTACAGGAATGAGCCACCATACCTGGCCCTGAATCTTGGGTCTTGGCCTTAG -908
                **  *  *   ******           *  **   ***    * *  ** ** **

NZW             ------AACCCAG-----GCCCTCCAGTGCAGGAT-GCAA------GCATCCTAAGCAGT -735
hum             TAATTAAAACCAATCACCACCATCCGTTGCGGACTTACAACCTACAGTGTTCTAAACATT -848
                      ** ***       ** ***  *** *  *  ***      *  * **** ** *

NZW             GTTTTAGTC--ACAGCATCCAACACCTGC-------CGGGGGGGGGGGGTGC-------- -692
hum             TTATATGTTTGATCTCATTTAATCCTCACATCAATTTAGGGACAAAGAGCCCCCCACCCC -788
                 * *  **   *   ***  **  *   *         ***     * *  *

NZW             -----TATTTTTTGACAGCCGAGCAAAGAAGTCCCCAGTGGTGAGGCCGTGTGCCCGAGG -637
hum             CCGTTTTTTTTTTTTACAGCTGAGGAAACAC-TTCAAAGTGGTAAGAC-ATTTGCCCGAGG -730
                     * ****** ***** *** *** *  * *  ****** ** *  * *********

NZW             CCCTCAGGGAGCACCGTGAGGC--TGTCTGCTGAGCTCT---------------CCCCAT -594
hum             TCCTGAAGGAAGAGAGTAAAGCCATGTCTGCTGTTTTCTAGAGGCTGCTACTGTCCCCTT -670
                 *** * ***  *  ** * **  *********   ***               **** *

NZW             CACTGCCCTGAAGATTCAGCCTGTCGGAGACAGGGGGTTACCCCGGTGGAATTCCCCAAA -534
hum             TACTGCCCTGAAGATTCAGCCTGCGGAAGACAGGGGGTTGCCCCAGTGGAATTCCCCAG- -611
                 **********************  * ************ **** *************

NZW             TCCTGCCTCAGAGAGCCCACTCCTTCCGCCC--AGCTGGAGCCGGGAGGAGGAAGCTGAG -476
hum             CCTTGCCTAGCAGAGCCCATTCCTTCCGCCCCCAGATGAAGCAGGGAG-AGGAAGCTGAG -552
                 * *****   ******** ***********  ** ** *** ***** ***********

NZW             TCAGAGGAGGGCTTTCCAGGAGGGA------GGACGTANCCTN-----CGGGGAGAGCCT -427
hum             TCA-AAGAAGGCTGTCAGGGAGGGAAAAAGAGGACAGAGCCTGGAGTGTGGGGAGGGGTT -493
                *** * ** **** **  *******      ****  * ***       ****** *  *

NZW             CAGG-----------------AGGGGGTGTCACAGA--GTCAAGGGTGGGCCTGGGGTG -387
hum             TGGGGAGGATATCTGACCTGGGAGGGGGTGTTGCAAAAGGCCAAGGATGGGCCAGGGGGA -433
                  **                 *********  ** *  * ***** ****** ****

NZW             GCACTCA----GGAAGGGGGTCCCAGGGCGTCCAACATC-CTTTNGNTGAGCTGACCACT -332
hum             TCATTAGTTTCAGAAAGAAGTCTCAGGGAGTCTTCCATCACTTTCCCTTGGCTGACCACT -373
                 ** *       *** *  *** ***** ***   **** ****   *  **********

NZW             TGGGAC-------------------------CTCCTACCCCGTCCCTCTCCCTCCT---- -301
hum             GGAGGCTTTCAGACCAAGGGATGGGGGATCCCTCCAGCTTCATCCCCCTCCCTCCCTTTC -313
                 * * *                         ****  *  * **** ********
```

```
NZW             -----GTTCCCACAAACGCTGCAGTTTGCAAAACCCAACCGCTCCCCTGAGGGCCTGTGG -246
hum             ATACAGTTCCCACAAGCTCTGCAGTTTGCAAAACCCTACCCCTCCCCTGAGGGCCTGCGG -253
                     ********** * ****************** *** *************** **

NZW             TTTCCTGTGGGTCTGGGGTCCTGCCTGACTCGGCAGCGGGGACTGCAGGCGGGTGGGGGG -186
hum             TTTCCTGCGGGTCTGGGGTCTTGCCTGACTTGGCAGTGGAGACTGCGGGCAGTGGAGAGA -193
                ******* ************ ********* ***** ** ****** *** *  * * *

NZW             AGAGGCGGGGGGAGAGGAGGTGGTGAAAATCTGTTCTTT---------GCCTTCTCATGC -135
hum             GGAGGAGGTGGTGTAAGCCCTTTCTCATGCTGGTGCTGCCACACACACACACACACACAC -133
                 **** ** **   * *   *     *     ** **            *   * **  *

NZW             TGGGGCTGCCCCCTTCCCCCCTGCACC---GGCCCTGAGTCAGGCACTTGCCTGCAGGAA -78
hum             ACACACACACACACACACACACACACCCTGACCCCTGAGTCAG-CACTTGCCTGTCA-AG -75
                     *   * *   * * *   ****     *********** **********    *

NZW             GAGGGGCGGGGTCACGACTCAGGAGTGCCTCTTTAAAGCCCCCGCTGCCGTCG-TGC-GC -20
hum             GAGGGGTGGGGTCAC-----AGGAGCGCCTCCTTAAAGCCCCCACAACAGCAGCTGCAGT -20
                ****** ********     ***** ***** ********** *  * *  * *** *

NZW             CAGACGCTGCTCCCCTCACCATGAGCCCCAGACAGCCCC +19
hum             CAGACACCTCTGCCCTCACCATGAGCCTCTGGCAGCCCC +19
                ***** *  ** *************** * * *******
```

Attachment 3:

Alignment of the amino acid sequences of the HPV16E2 mutants used in this study

```
HPV16E2          METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2NESmt          METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2NESdel         METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2I73A           METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2NLS1           METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2NLS2           METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2306/307        METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60
E2NLSdel         METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60

HPV16E2          TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120
E2NESmt          TLAVSKNKALQAIELQPTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120
E2NESdel         TLAVSKNKALQA-------ETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 113
E2I73A           TLAVSKNKALQAAELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120
E2NLS1           TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPKRCIKKHGYTVEVQF 120
E2NLS2           TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120
E2306/307        TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120
E2NLSdel         TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVLTAPTGCIKKHGYTVEVQF 120

HPV16E2          DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2NESmt          DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2NESdel         DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 173
E2I73A           DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2NLS1           DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2NLS2           DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2306/307        DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180
E2NLSdel         DGDICNTMHYTNWTHIYICEEASVTVVEGQVDYYGLYYVHEGIRTYFVQFKDDAEKYSKN 180

HPV16E2          KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240
E2NESmt          KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240
E2NESdel         KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 233
E2I73A           KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240
E2NLS1           KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240
E2NLS2           KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRK 240
E2306/307        KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240
E2NLSdel         KVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRP 240

HPV16E2          RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2NESmt          RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2NESdel         RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 293
E2I73A           RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2NLS1           RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2NLS2           RSRPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2306/307        RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300
E2NLSdel         RSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCL 300

HPV16E2          RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2NESmt          RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2NESdel         RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 353
E2I73A           RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2NLS1           RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2NLS2           RYRFKKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2306/307        RYRFAAHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
E2NLSdel         GYGFTKHCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLSQVKIPKTITVSTG 360
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HPV16E2	FMSI 364
E2NESmt	FMSI 364
E2NESdel	FMSI 357
E2I73A	FMSI 364
E2NLS1	FMSI 364
E2NLS2	FMSI 364
E2306/307	FMSI 364
E2NLSdel	FMSI 364

AFFIDAVIT

I hereby declare that I have drawn up this thesis autonomously and without illicit assistance.
No supplemental materials or references other than the ones specified have been used.

Heidelberg, September 2008

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Sabrina Mühlen