Scuola Internazionale Superiore di Studi Avanzati - Trieste

p300/CBP TRANSCRIPTIONAL CO-ACTIVATORS: A COMMON CELLULAR TARGET OF HPV E6 AND E7 ONCOPROTEINS

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Rodzicom moim, przyjaciołom i tym wszystkim

kochanym ludziom, którzy w cieżkich chwilach

wspierali mnie dobrym słowem

Poświęcam

Agnieszka

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ABBREVIATIONS

aa	amino acid(s)
Ad	Adenovirus, Adenoviral
BPV	Bovine Papilloma Virus
BRK	Baby Rat Kidney
CAT	Chloramphenicol Acetyltransferase
CBP	CREB Binding Protein
CDK	Cyclin-Dependent Kinase
CIN	Cervical Intraepithelial Neoplasia
CKII	Casein Kinase II
CRE	cAMP Responsive Element
CREB	CRE binding protein
CRPV	Cotton-Tail Rabbit Papilloma Virus
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminedetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FCS	Foetal Calf Serum
GST	Glutathione-S-Transferase
GM-CSF	Granulocyte Macrophage Colony Stimulation Factor
h	Hour
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase Complex
HECT	Homologous to E6-AP Carboxyl-Terminus
HFK	Human Foreskin Keratinocytes
HIV	Human Immunodeficiency Virus
HMG	High Mobility Group
HPV	Human Papilloma Virus
HTLV	Human T-Leukemia Virus
IPTG	Isopropyl-thiogalactopyranoside
ivt	*In vitro* Transcribed and Translated

kb	kilo base(s)
kDa	kilo Daltons
LT	Large T antigen
MHC	Major Histocompatibility Complex
nm	Nanometer
NURD	Nucleosome Remodelling Histone Deacetylase
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PKA	Protein Kinase A
PMSF	Phenylmethylsulfonyl fluoride
POD	PML oncogenic domain
PV	Papilloma Virus
Py	Polyoma
REF	Rat Embryo Fibroblast
SV40	Simian Virus 40
TF	Transcription Factor(s)
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
URR	Upstream Regulatory Region
WB	Western Blot
wt	Wild Type

SUMMARY

E6 and E7 are the two major oncoproteins encoded by the human papillomaviruses (HPV). During HPV infection they need to play multiple roles, interfering with several cellular pathways in order to create a favourable environment for viral replication, by neutralising the cellular surveillance controls that are turned on as a result of unscheduled DNA synthesis. These requirements are more difficult for the high-risk HPV types, which begin to replicate their genomes in differentiating cells, and as a result, the activities of the E6 and E7 proteins have more pronounced effects on their cellular target proteins.

In this thesis, the data demonstrating that the p300 transcriptional co-activator is a common target of E6 and E7 proteins, will be presented. From these studies, it seems likely that both viral proteins, although binding within the same domains of p300, target apparently different functions of p300.

In the first part of the thesis, the interaction between the E6 proteins from different PV types has been analyzed. All PV E6 tested (both human and animal in origin) showed similar levels of interaction with p300, suggesting that this interaction plays a fundamental role in the PV life cycle and is a common requirement for viral replication. However, only high-risk mucosal HPV E6s were able to restore the function of an Ad E1a mutant, deficient in p300 binding and transformation in BRK transformation assays. It can be speculated that transcriptional inhibition of p300 is a prerequisite for cellular transformation mediated by high-risk mucosal E6s, hence only high-risk E6s were able to repress p300 transcriptional activity, while low-risk E6s appeared to have only marginal effects (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999).

In the second part of the study, the interaction between E7 and p300 was also investigated. Both low- and high-risk E7 proteins showed significant levels of interaction with p300. As a consequence of this interaction, the high-risk E7 protein was able to block the p300 co-activation function. There are at least two mechanisms involved in this repression: first - inhibition of protein-protein interactions and second - inhibition of HAT activity of p300. Whilst the first one allows the competition between the E7 protein and different cellular proteins for the limiting amounts of p300 within cell, the second allows E7 to manipulate the acetylation state of histones, which may lead to the subsequent alteration of chromatin structure and perturbation in gene expression. In addition, E7 may also block the acetylation of other target proteins of p300.

Interaction of both E6 and E7 with a common cellular target, the p300 transcriptional co-activator, may thus provide a means for interfering with the control of key biological processes, such as cell cycle progression, differentiation and apoptosis.

1. INTRODUCTION

1.1. Human Papilloma Viruses (HPV)

Human Papillomaviruses (HPV) are small, non-enveloped icosahedral particles ~52-55nm diameter. There are 72 capsomers (60 hexameric + 12 pentameric) arranged on a T = 7 lattice (Figure 1). Despite the great variety of PV types both of human and animal origin, all the members of this group share a remarkably conserved genome. The Papillomavirus genome consists of circular, ds DNA of around 8kbp in size comprising approximately eight open reading frames (ORF), which are transcribed as polycistronic messages from a single DNA strand (Figure 1). The HPV genome can be divided into 3 domains: a non-coding upstream regulatory region (URR), where regulatory sequences required for viral replication and transcription are concentrated; an early region (E) encoding E6, E7, E1, E2, E4 and E5 proteins, and a late region (L) encoding the two capsid proteins L1 and L2.

The early proteins E1 and E2 are necessary for viral DNA replication and bind as a complex to sequences around the origin of replication (FRATTINI and LAIMINS 1994a; FRATTINI and LAIMINS 1994b; MOHR *et al.* 1990; USTAV *et al.* 1991). E4 is expressed as a fusion protein with five amino acids from the N-terminus of E1 (E1^E4) and has been suggested to be involved in the alteration of the cytoskeleton network (DOORBAR *et al.* 1991). The function of the E5 gene is largely unknown, but appears to encode a membrane protein with a weak transforming activity (LEPTAK *et al.* 1991; PIM *et al.* 1992). E6 and E7 are the main transforming proteins of the high-risk HPV types and function by modulating the activities of cellular proteins that regulate the cell cycle (MUNGER *et al.* 1989b; PHELPS *et al.* 1988; SCHEFFNER *et al.* 1990; WERNESS *et al.* 1990). Two late genes L1 and L2 encode capsid
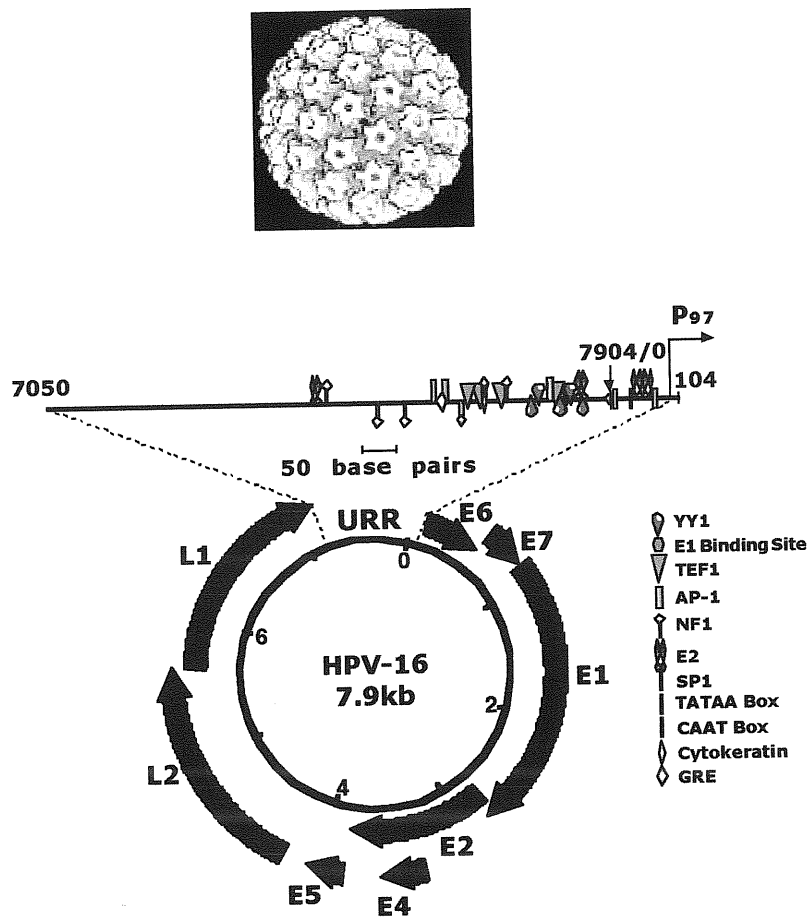
Figure 1.
Viral capsid (upper panel) and genome organisation of Human Papillomavirus type 16 (lower panel). Viral Open Readings Frames (ORFs) are shown: E: early proteins, L: late proteins. The Upstream Regulatory Region (URR) is shown together with the selection of cellular and viral transcription factors binding sites.

proteins. The L1 protein forms pentamers (capsomers), which are able to assemble into virus-like particles (VLPs) even in the absence of L2 (KIRNBAUER *et al.* 1993). The minor capsid protein L2 is thought to bind the viral DNA and stabilizes the viral particles.

The non-transcribed upstream regulatory region (URR) which covers about 10% of the genome, contains enhancers and cis-acting elements essential for the transcription and replication of viral DNA. The transcription of viral genes is tightly regulated in infected cells. The regulation of viral gene expression is complex and is controlled by the viral E2 protein (MCBRIDE *et al.* 1991; STUBENRAUCH *et al.* 1998), and cellular transcription factors such as AP-1, NF-1, SP-1, Oct1, YY1 and glucocorticoid receptor, which act on different enhancer elements within the URR (BAUKNECHT *et al.* 1992; BUTZ and HOPPE-SEYLER 1993; GLOSS and BERNARD 1990; GLOSS *et al.* 1987; HOPPE-SEYLER *et al.* 1991; THIERRY *et al.* 1992; TUREK 1994).

1.2. Epidemiology of HPV infection and association with disease

It is well established that chronic infection of cervical epithelium by HPV is necessary for the development of cervical cancer. HPV DNA has been demonstrated in more than 99.7% of cervical cancer biopsy specimens, with the high-risk HPV-16 and HPV-18 viruses being most prevalent. Cervical cancer is the third most common cancer worldwide and, for women, the second most common after breast cancer, accounting for 6% of all malignancies in women. Around 232 000 women die from invasive cervical cancer every year, with 80% of the cases occurring in developing countries, where it is the leading cause of cancer-related death among women (PARKIN *et al.* 2001; PISANI *et al.* 2002).
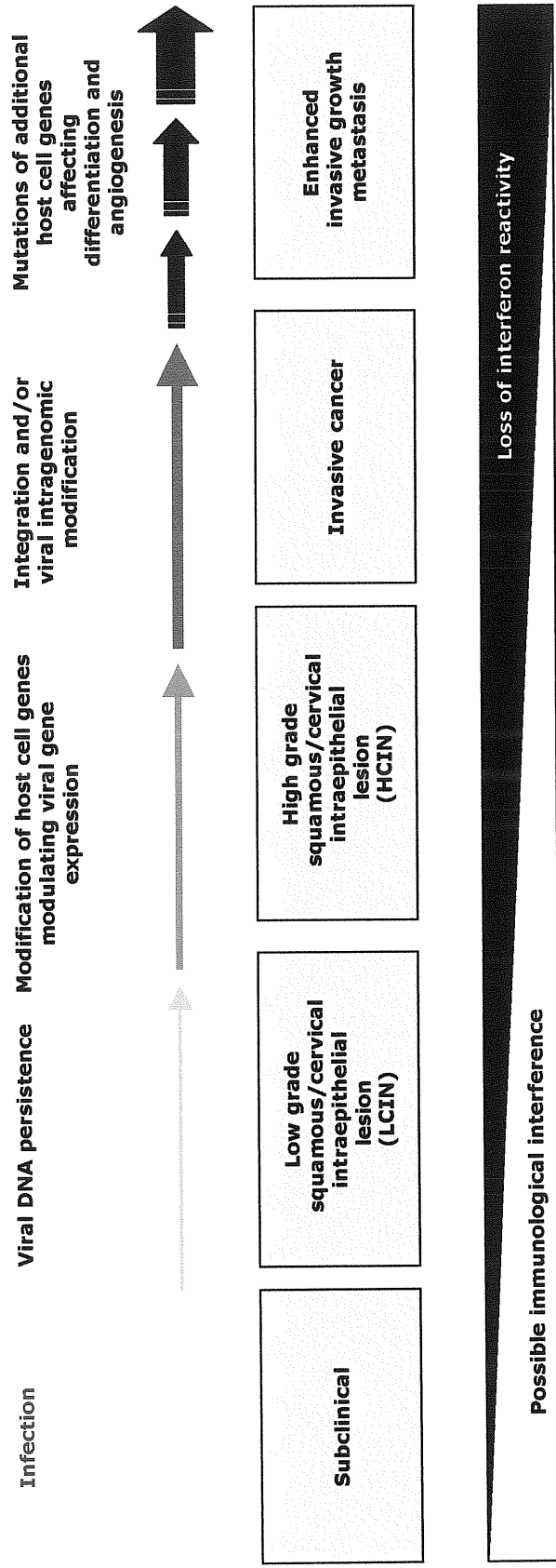
Figure 2. Tentative scheme of Papillomavirus-induced pathogenesis resulting in cervical cancer.
From H. zur Hausen, 1999, *Semin Cancer Biol;* **9**:405-411

Of the more than 100 identified types of HPVs, only a small subset specifically infects the genital epithelium (ZUR HAUSEN 2002). Genital papillomavirus infections can be passed between individuals through sexual contact, and represent one of the most common sexually transmitted diseases. Those types associated with malignant lesions are referred as 'high-risk" types. Although HPV-16 is the most prevalent, other frequently found types include HPV-18, 31, 33, and 45 (BOSCH *et al.* 1995; DE VILLIERS 1994; LAIMINS 1993; WALBOOMERS *et al.* 1999; ZUR HAUSEN and DE VILLIERS 1994). It is important to note that not every woman infected with HPV is destined to develop cervical cancer. Whilst HPV infection is quite prevalent, cervical cancer is not (HILDESHEIM *et al.* 1994). Other viral types such as HPV-6 and HPV-11 are regularly found to be associated with benign lesions or occasionally with low-grade dysplasias of the cervix, and therefore defined as low-risk types.

The development of cervical cancer is a multistep process (Figure 2) (OSTOR 1993; ZUR HAUSEN 1999). In the majority of cases, HPV infection does not lead to a clinical manifestation and is cleared by the host immune system in a relatively short time (6-12 months). However, a small percentage of infections induce the development of low- and/or high-grade cervical intraepithelial neoplasias (CIN), which can ultimately progress to an invasive cervical carcinoma (Figure 2).

HPVs are also associated with the development of cutaneous squamous cell carcinoma (SCC), particularly in immuno-compromised individuals, where between 34-50% of patients surviving 15 years after renal transplantation develop skin cancer (LONDON *et al.* 1995).

1.3. Replication and the viral life cycle

HPV infects the dividing basal keratinocytes of the dermal layer (squamous epithelial cells) and its life cycle is absolutely dependent upon keratinocyte differentiation (STUBENRAUCH and LAIMINS 1999) (Figure 3). The receptor for HPV entry into epithelial cells has not been characterized, although the integrin α6-β4 protein complex has been suggested as a candidate receptor (EVANDER *et al.* 1997). This integrin is present on epithelial stem cells (LI *et al.* 1998a), but no functional studies have yet shown it to mediate HPV entry. Furthermore, HPV virions also bind to heparin, a ubiquitous polysaccharide which may provide the initial attachment event, which is then followed by association with a receptor complex and internalisation (JOYCE *et al.* 1999).

There are two phases of viral genome replication:

- Plasmid replication (or maintenance)
- Vegetative Replication

Plasmid Replication - Cells located in the lower layers of the stratified epithelium divide producing daughter cells, which withdraw from the cell cycle, migrate away from the basal layer and become committed to differentiation. Following entry into the cell, the viral genome is established as an extrachromosomal plasmid in the cell nucleus. The viral DNA is amplified to between 50 and 400 copies/diploid genome and this occurs in synchrony with cellular DNA replication (STUBENRAUCH and LAIMINS 1999) As infected cells divide, the viral DNA is distributed equally amongst the daughter cells: while one cell migrates away and initiates a differentiation program, the other continues to divide thereby providing a reservoir of viral DNA. The viral production is restricted to differentiating cells and the

stratum coroneum	Release of infectious virus
stratum granulosum	Viral capsid production and assembly
stratum spinosum	Viral early gene expression Induction of viral major late promoter and Viral DNA amplification
stratum basale	Infection of stem cell (?) Viral early gene expression0 Maintenance of viral copy number
basal membrane	
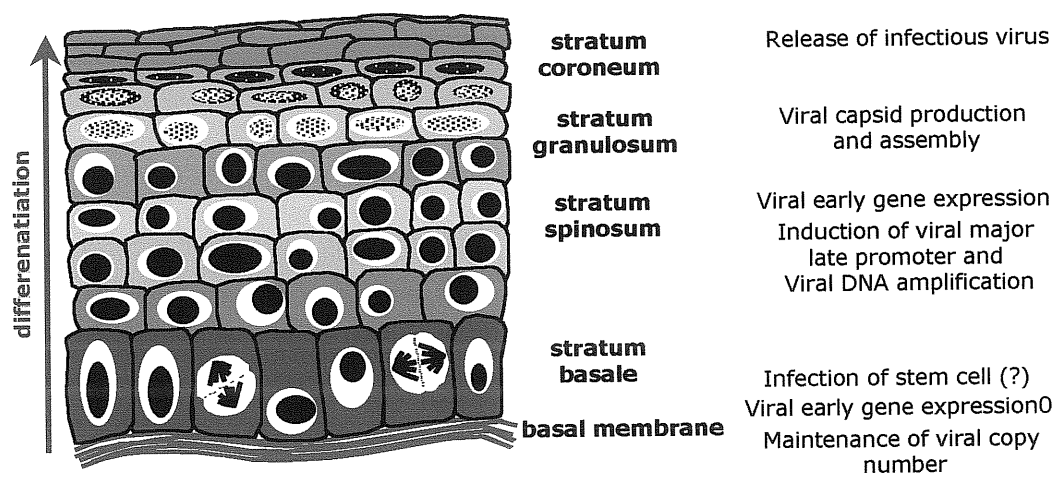
Figure 3.
Differentiation dependent functions in HPV-infected epithelial cells.

infection can persist in the basal layers for several years (STUBENRAUCH and

LAIMINS 1999). E1 and E2 are the first viral genes to be transcribed.

HPVE2

E2 protein is a major regulator of viral gene expression (BOUVARD *et*

al. 1994b; CRIPE *et al.* 1987; PHELPS and HOWLEY 1987). The E2

proteins are relatively conserved among Papillomaviruses. The N-

terminal domain, consisting of approximately 200 amino acids, is

required for transcriptional transactivation function, viral replication

function and association with the E1 protein. This N-terminal domain

is followed by the variable hinge region, which is a flexible domain

joining N- and C- terminal domains. The C-terminal domain, of about

85 amino acids, is necessary for dimerisation and site-specific

binding to DNA (GIRI and YANIV 1988). E2 regulation of viral gene

expression occurs at multiple levels. E2 is also alternatively spliced,

expressing a full-length activator and an N-terminally truncated

repressor form. The relative ratio between the full-length activator

form and the shorter (lacking the activation domain), repressor form,

has a major impact upon the levels of viral gene expression (LAMBERT

et al. 1987). However, the expression of the full-length

transactivator form also appears to be critical. At low levels of

expression, when only distal E2 binding sites are occupied, E2

protein functions as a transactivator of the viral promoter, whereas

increased intracellular levels of E2 result in occupation of all four E2

binding sites and repression of HPV transcription (BOUVARD *et al.*

1994b; USHIKAI *et al.* 1994).

E2 binds as a dimer, with high affinity for the specific E2 recognition sites

(ACC(N)6GGT) (BEDROSIAN and BASTIA 1990) within the viral URR. At the

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same time, recruitment of E1 allows formation of a multimeric complex that facilitates stable binding of E1 to an A/T-rich sequence within the viral origin of replication (SANDERS and STENLUND 1998; SEDMAN and STENLUND 1995). E1 possess helicase and ATPase activities (HUGHES and ROMANOS 1993; YANG *et al.* 1993) and also recruits a number of cellular DNA replication enzymes to the viral ori (BONNE-ANDREA *et al.* 1995; MASTERSON *et al.* 1998; PARK *et al.* 1994). Transient replication of HPV genomes requires only E1 (GOPALAKRISHNAN and KHAN 1994) although it is stimulated by the E2 protein. However, stable maintenance in normal keratinocytes also requires E6 and E7 expression (DEL VECCHIO *et al.* 1992; THOMAS *et al.* 1999), suggesting a role for the E6 and E7 proteins in maintaining the episomal state of the HPV genome. E7 targets a number of cell cycle regulatory proteins, thereby stimulating G1/S transition and DNA synthesis. The host cell's normal response to such unscheduled induction of proliferation would be, however, to trigger apoptosis and/or growth arrest. To overcome these obstacles, the high-risk E6 protein targets a variety of cellular proteins involved in regulating these mechanisms (discussed below).

Vegetative Replication - occurs in terminally differentiated cells in the epidermis. In normal epithelia, differentiation is coupled to cell cycle exit. The replicative phase of high-risk HPV infection is confined to more differentiated cells, that have already exited the cell cycle and are non-permissive for DNA synthesis (DOORBAR *et al.* 1997). In terminally differentiated cells (or growth-arrested cells in culture) control of viral copy number appears to be lost and the DNA is amplified up to very high copy numbers (thousands copies/cell) (LAMBERT 1991).

Expression of HPV late genes is regulated by epithelial differentiation, however our knowledge of how this occurs is rather limited. In one model, the key event for the activation of late viral function is induction of the late promoter by differentiation-specific transcription factors (KUKIMOTO and KANDA 2001). In a second model, the activation of viral replication is an initial event that then leads to titration of transcription factors, which repress late gene expression in basal cells (STUBENRAUCH *et al.* 1996). In the third model, transcription of E1 and E2 switches to a late promoter, which is not repressed by E2 (KLUMPP and LAIMINS 1999). In a recently proposed model, restriction of the translation of the late viral transcripts to differentiated keratinocytes is achieved by differential codon usage, which matches the specific composition of the tRNA population in these cells (ZHOU *et al.* 1999).

It seems likely that the switch from the early to late promoter results from a combination of different events, the net result being increased E1 and E2 expression and subsequent amplification of the viral genomes. The major late transcripts also encode an E1^E4 fusion protein (NASSERI *et al.* 1987), which is thought to facilitate capsid release by perturbing the organisation of the cytokeratin matrix (DOORBAR *et al.* 1991), as well as E5 whose function is also related to mitogenic stimulation. The capsid proteins, L1 and L2, are translated from mRNAs that also encode E1^E4 upstream (DOORBAR *et al.* 1990) and viral assembly then occurs as cells reach the epithelial surface and die (STOLER *et al.* 1990), thereby allowing for the release of new infectious virions.

In low-grade cervical lesions the HPV genomes are exclusively episomal. In contrast, in cervical carcinomas, high-risk HPVs genomes are often found integrated into the cellular host DNA (SCHWARZ *et al.* 1985; STOLER *et al.*

1992). HPV DNA integration has been suggested to be an important event in the development of cervical cancer, since this often results in a disruption of the E2 region (BAKER *et al.* 1987; SCHWARZ *et al.* 1985). Integration thus abrogates the inhibitory actions of E2 on the E6 and E7 promoter, resulting in increased steady-state levels as well as stabilization of the viral mRNAs encoding for E6 and E7 (JEON and LAMBERT 1995). These observations are consistent with the hypothesis that integration provides a selective advantage to cervical epithelial precursors of cervical carcinoma (JEON *et al.* 1995).

1.4. The HPV oncogenes

Insights into the mechanisms by which HPV infection can result in malignancy come from the observation that three proteins encoded by high-risk genital HPVs, E6, E7 and E5, are key oncoproteins. The functions of these proteins and their relative contribution towards HPV-induced transformation will be now discussed.

1.4.1. HPV E5

The HPV E5 gene encodes a highly hydrophobic protein of around 80 aa in length (BUBB *et al.* 1988; HALBERT and GALLOWAY 1988) that is localized mainly to the endosomal membranes, Golgi apparatus and to a lesser extent, the plasma membranes (CONRAD *et al.* 1993). It has been shown that the HPV E5 protein stimulates the growth of mouse fibroblasts and can transform them to anchorage-independent growth (VALLE and BANKS 1995), as well as enhancing the frequency of keratinocyte immortalization (STOPPLER *et al.* 1996).

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Most of E5's activity appears to be related to its ability to modulate growth factor signalling pathways. E5 has been shown to enhance proliferation of keratinocytes in an EGF dependent manner (CRUSIUS *et al.* 1997; VALLE and BANKS 1995), and E5 expression results in a ligand-dependent increase in the levels of EGFR phosphorylation (STRAIGHT *et al.* 1993) thereby enhancing mitogenic signalling from EGFRs (CRUSIUS *et al.* 1997; CRUSIUS *et al.* 1998; GU and MATLASHEWSKI 1995; LEECHANACHAI *et al.* 1992). The mechanism by which E5 enhances EGFR signalling is believed to be mediated by its interaction with the 16 kDa subunit of the vacuolar ATPase. This interferes with the acidification of the endosomes (STRAIGHT *et al.* 1995) resulting in a decreased rate of EGFR degradation and enhanced recycling of the EGFR back to the plasma membrane and thus prolonging signalling by the EGFR/EGF complex (STRAIGHT *et al.* 1995).

EGFR-independent pathways have also been implicated in the E5-activated signalling cascades (CRUSIUS *et al.* 2000), for example HPV-16E5 has been shown to modulate the sorbital-dependent activation of MAP kinase p38 and Erk1/2 in human keratinocytes. MAP kinases activation by E5 has been shown to occur via two different pathways: PKC-dependent and PKC-independent (CRUSIUS *et al.* 1997; GU and MATLASHEWSKI 1995). One of the consequences of E5 signalling is stimulation of the AP-1 family proteins such as c-Jun, JunB, c-Fos (CHEN *et al.* 1996a; CHEN *et al.* 1996b), and as a consequence of c-Jun and c-Fos up-regulation, there is an increase in viral gene expression, which is controlled by the URR containing recognition sites for a variety of transcription factors including AP-1 and NF-1. As these transcription factors may activate the HPV enhancer, E5 may consequently increase the expression of early genes E6 and E7, thus contributing to the

early steps of cell transformation (BOUVARD *et al.* 1994a; STOPPLER *et al.* 1996).

HPV E5 has also been found to suppress the expression of p21$^{WAF1/Cip1}$ (TSAO *et al.* 1996), which may promote cell proliferation by activating the CDK4-cyclin D complex leading to subsequent inactivation of the pRb checkpoint control. Thus, E5 most likely augments the transforming activity encoded by E6 and E7 and this is supported by studies that have shown that E5 can enhance E7-induced cell proliferation (VALLE and BANKS 1995).

Insights into the role of E5 in the viral life cycle have been obtained from studies using organotypic raft cultures. Viruses mutated in E5 did not affect the expression of differentiation markers but did reduce expression of late viral proteins. Additionally it has been revealed that during epithelial differentiation a decreased expression of cyclin A and B is observed in E5 mutant cells compared to HPV wild-type cells. This suggests that HPV E5 modifies the differentiation-induced cell cycle exit and supports the ability of HPV-positive keratinocytes to retain proliferative competence. In these studies, E5 was found to have little effect on the levels of the epidermal growth factor receptor (EGFR) or on its phosphorylation status, suggesting a role for high-risk HPV E5 in modulation of late viral functions through activation of proliferative capacity in differentiated cells (FEHRMANN *et al.* 2003). Additionally, it has been shown that E5 plays a subtle role in reprogramming differentiated cells in order to support viral DNA synthesis, a prerequisite for amplification of viral DNA (GENTHER *et al.* 2003). Cells harboring E5 mutant genomes displayed a quantitative reduction in the percentage of suprabasal cells undergoing DNA synthesis, compared to cells containing wt HPV-16 DNA. This reduction in DNA synthesis, however, did not prevent amplification of viral DNA in the differentiated cellular

compartment. Likewise, late viral gene expression and the perturbation of normal keratinocyte differentiation were retained in cells harboring E5 mutant genomes.

Interestingly, during the development of cervical tumours, deletions encompassing the HPV E5 gene frequently occur and it is therefore generally assumed that E5 has no role in the later stages of malignancy. However it is worth noting that, although E5 is frequently deleted, the EGFR genes are often amplified in cervical tumours, possibly compensating for E5 loss (BAUKNECHT *et al.* 1989; KOHLER *et al.* 1989).

1.4.2. HPV E6

1.4.2.1. Structure and general features

The E6 proteins of Human Papillomaviruses are approximately 150 aa long, with an apparent molecular mass of 18 kDa. Figure 4 shows the structure of HPV-18 E6 with the known sites of interaction with some of its cellular target proteins, many of which are also targeted by the transforming proteins of other tumour viruses.

A major characteristic of the E6 structure is the presence of two zinc fingers formed by Cys-X-X-Cys motifs (BARBOSA *et al.* 1989; COLE and DANOS 1987; GROSSMAN and LAIMINS 1989) whose integrity is essential for virtually all the biological functions of E6 (KANDA *et al.* 1991; SHERMAN and SCHLEGEL 1996), and which are highly conserved throughout all HPV types (COLE and DANOS 1987). A hydrophilic N-terminal domain precedes the zinc fingers that are separated by a hydrophobic region. A unique characteristic of the high-risk mucosal HPV E6 proteins is the presence of a PDZ domain-binding motif at their extreme carboxyl-termini (GARDIOL *et al.* 1999;
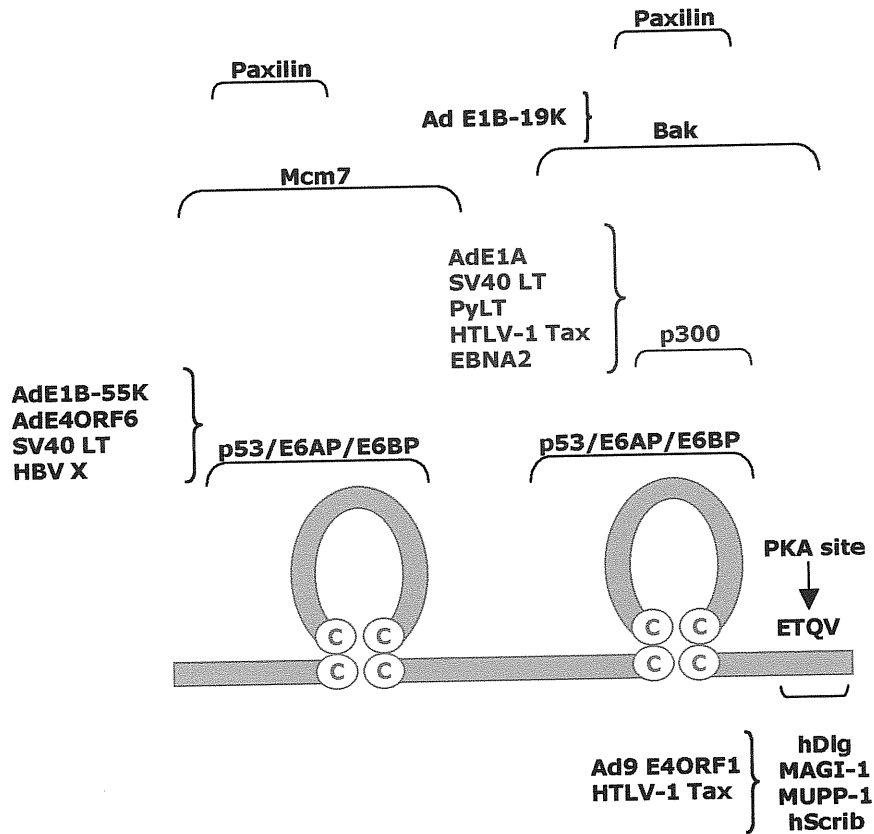
Figure 4. The high-risk HPV E6 protein.
Schematic diagram of E6 showing the two zinc fingers, together with the regions involved in interactions with some of the cellular proteins that are targeted by the oncoproteins of other viruses.

KIYONO *et al.* 1997; LEE *et al.* 1997), the relevance of which will be discussed below.

1.4.2.2. Localization

Due to its low level of expression (ANDROPHY *et al.* 1985; BANKS *et al.* 1987), the subcellular localisation of the endogenous E6 protein has been extremely difficult to determine. Indeed, different studies have reported E6 to be localised either in the nucleus, cytoplasm or membranes (ANDROPHY *et al.* 1985; LIANG *et al.* 1998; SHERMAN and SCHLEGEL 1996). It has been recently shown that upon overexpression in human keratinocytes, the E6 proteins of low-risk HPVs may have different cellular localisation compared to their high-risk counterparts (GUCCIONE *et al.* 2002; SWINDLE *et al.* 1999). HPV-16E6 has been shown to posses a basic nuclear localization signal (NLS) located at the C terminal half of the protein (LE ROUX and MOROIANU 2003). Interaction of E6 with the karyopherin (Kap) α2 adapter allows it to enter the nucleus via a classical Kapα2β1-mediated pathway. Interestingly, HPV-16E6 also interacts via its NLS with both Kapβ1 and Kapβ2 import receptors, which suggest that nuclear entry of high-risk HPV-16E6 oncoprotein occurs via several pathways (LE ROUX and MOROIANU 2003).

1.4.2.3. E6 cellular targets

Many studies have been performed to investigate the mechanisms of action of HPV E6. High-risk E6 proteins interact with a large number of cellular proteins whose functions are associated with the regulation of cell growth and survival. Some of them are listed in Table 1.

In the following section I propose to discuss some of those interactions that appear to be essential for E6's transforming activity.

p53-independent functions of E6

Although the degradation of p53 by HPV E6 is an essential element of viral replication as well as being a major contributing factor in the development of cervical cancer, it is now clear that p53 is not the only important target of E6. Thus, in common with many other viral transforming proteins, HPV E6 is a multifunctional protein and targets a number of different cellular proteins. In the following section I will consider the effects of E6 upon p53-independent pathways.

Apoptosis

Intracellular targeting of the HPV-16E6 protein by E6-binding peptide aptamers resulted in selective, apoptotic elimination of HPV-positive, but not HPV-negative cancer cells (BUTZ *et al.* 2000) suggesting that the anti-apoptotic activity of E6 is required for cancer cell survival. This apoptotic activity is believed to be p53 related, but there is increasing evidence that E6 can also inhibit p53-independent apoptotic pathways, promoted by different stimuli. Indeed, in transgenic mice expressing HPV-16E6 in the ocular lens, E6 was found to prevent apoptosis both in wt and in p53-null animals (PAN and GRIEP 1995), and similarly it has been reported to inhibit drug-induced apoptosis in cells lacking p53 (STELLER *et al.* 1996). Among the newly discovered cellular targets of E6, there are indeed several pro-apoptotic factors. One of them is Bak, whose high levels of expression in the upper epithelial layers (KRAJEWSKI *et al.* 1996) appear to represent a common obstacle for a broad range of HPV types that replicate in differentiating keratinocytes. Indeed, the E6 proteins of both high- and low-risk mucosal HPVs, plus those of high-risk cutaneous types have been shown to inhibit Bak-induced apoptosis (JACKSON *et al.* 2000; THOMAS and

BANKS 1998; THOMAS and BANKS 1999). HPV-18E6 has been shown to stimulate the ubiquitin-dependent degradation of Bak catalysed by E6AP, probably by accelerating a normal cellular process, since Bak appears to be a natural substrate of E6AP even in the absence of E6 (THOMAS and BANKS 1998). Degradation of Bak by HPV-11E6 is less effective, and this correlates with a weaker anti-apoptotic activity of the low-risk mucosal HPV types (THOMAS and BANKS 1999).

Cell polarity

As mentioned above, a unique structural feature of the high-risk mucosal HPV E6 proteins is a highly conserved C-terminal motif. This motif is now known to represent a PDZ (PSD95/Dlg/ZO-1) binding domain, and is absent in all other HPV E6 proteins. Therefore it is now widely thought that this domain may contribute to the carcinogenic activities of the high-risk E6 proteins. Through this domain E6 has been found to interact with several PDZ domain-containing proteins: hDlg (GARDIOL *et al.* 1999; KIYONO *et al.* 1997; LEE *et al.* 1997); hScrib (NAKAGAWA and HUIBREGTSE 2000), MUPP1 (LEE *et al.* 2000b), and MAGI-1, -2, -3 (GLAUNSINGER *et al.* 2000; THOMAS *et al.* 2002) resulting in their ubiquitin-mediated degradation. PDZ domains consist of approximately 90 aa long protein-protein interaction units and are most often found within the cellular proteins located at areas of cell-cell contact, such as synaptic junctions in neurons and tight junctions in epithelial cells. The PDZ proteins are also often involved in the clustering of ion channels, signalling enzymes, and adhesion molecules to specific structures at the membrane-cytoskeleton interface of polarised cells (FANNING and ANDERSON 1999; KIM 1997). Interestingly, loss of the control of cell polarity and cell contact is a characteristic hallmark of metastasis.

Thus, it quite probable that the interaction between high-risk E6 and PDZ containing proteins may account for the contribution of E6 to the later stages of malignancy. Indeed, recent studies in transgenic mice support this hypothesis, since mice expressing E6 mutated in the PDZ binding site fail to display epithelial hyperplasia (NGUYEN *et al.* 2003).

Telomerase activation

Telomerase is a multisubunit enzyme responsible for replicating telomeric DNA at the ends of chromosomes and its activation is a critical step in cellular transformation (ELENBAAS *et al.* 2001; HAHN *et al.* 1999). In most normal somatic cells, telomerase activity is absent, and this results in a gradual loss of telomeres through successive cell divisions. It has been suggested that critically shortened telomeres initiate the natural pathway leading to senescence and cell death (ALLSOPP *et al.* 1995; HARLEY 1991; LIU 1999; REDDEL 1998). In contrast, most tumour cells maintain telomere length and telomerase activity (BRYAN *et al.* 1997; COUNTER *et al.* 1998; KIM *et al.* 1994). Interestingly, the high-risk E6 oncoproteins have been found to be potent activators of telomerase (KLINGELHUTZ *et al.* 1996), and this is believed to be an additional important contributing event towards E6-induced malignancy. Regulation of telomerase activity has been shown to occur primarily through the level of expression of the human telomerase reverse transcriptase (hTert) gene, encoding the telomerase catalytic subunit (COUNTER *et al.* 1998; MEYERSON *et al.* 1997; NAKAYAMA *et al.* 1998). The precise mechanism of telomerase activation by E6 is unknown, however, several studies have demonstrated that E6 can up-regulate the steady-state levels of endogenous hTert in keratinocytes by transcriptional activation of its promoter (GEWIN and GALLOWAY 2001; OH *et al.* 2001;

VELDMAN *et al.* 2001). Interestingly, this mechanism seems to be p53-independent, and may involve the *c-myc* activator (GEWIN and GALLOWAY 2001; OH *et al.* 2001).

De-regulation of transcription

The E6 proteins of both high- and low-risk HPV types have long been known to modulate transcription from many cellular and viral promoters (DESAINTES *et al.* 1992; SEDMAN *et al.* 1991; VELDMAN *et al.* 2001; VELDMAN *et al.* 2003). It is only recently, however, that an understanding of some of the mechanisms by which this occurs has come with the demonstration that E6 interacts with the p300/CBP transcriptional co-activators (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999) and this will be discussed in detail further on. The HPV major transcriptional activator, E2, also interacts with p300/CBP (LEE *et al.* 2000a). This appears to involve a cellular protein, AMF-1/Gps2, which in turn enhances p300 activity but which, intriguingly, is also a target for E6-mediated degradation (DEGENHARDT and SILVERSTEIN 2001; PENG *et al.* 2000). Therefore the interaction between E6 and p300 may represent a means of down-regulating E2 transcriptional activity through a feedback mechanism.

It has been recently reported that both high- and low-risk HPV E6 proteins interfere with the transcriptional activity of the p53 homolog p73 (PARK *et al.* 2001). However, several studies have reported that p73 is unable to bind HPV E6 proteins and is not susceptible to E6-dependent proteolysis (MARIN *et al.* 1998; PARK *et al.* 2001; PRABHU *et al.* 1998), therefore transcriptional interference by E6 would appear to be indirect, possibly as a result of its interaction with p300.

1.4.3. HPV E7

As already discussed, the productive life cycle of HPV occurs in differentiating epithelial cells which, have stopped DNA synthesis and are not inherently capable of supporting viral replication. Several studies have shown that the ultimate effect of E7 expression is to subvert the tight link coupling cellular differentiation with the termination of proliferation in normal epithelium, hence retaining the differentiating keratinocytes in a DNA replication competent state (CHENG *et al.* 1995; WOODWORTH *et al.* 1992). This is achieved through a variety of biological activities, which are responsible for the transforming potential of E7, once its expression becomes uncoupled from the viral life cycle.

1.4.3.1. Structure and general features

E7 proteins of the high-risk HPV types are small, primarily nuclear phospho-proteins of approximately 100 aa in length that dimerise through a zinc-finger motif in the C-terminus. On the basis of homology with Adenovirus E1a (Ad E1a) (PHELPS *et al.* 1989), HPV-16E7 has been divided into 3 domains: conserved domain 1, 2 and 3 (CD1,2,3) (Figure 5A). The amino terminal 37 amino acids have marked homology with conserved regions 1 and 2 (CR1 and CR2) of Ad E1a and with the homologous region found in SV40 large T antigen (BARBOSA *et al.* 1990; PHELPS *et al.* 1991; PHELPS *et al.* 1988). This homology is also reflected in the similarity of the mechanisms used by these DNA tumour virus proteins to alter cell proliferation. Deletions and point mutations within the HPV-16E7 CD1 region and Ad E1a CR1 lead to reduced transforming activity (BANKS *et al.* 1990; PHELPS *et al.* 1992; WATANABE *et al.* 1990). The HPV-16E7 CD2 domain contains an LXCXE motif involved in the interaction with the

A.

B.
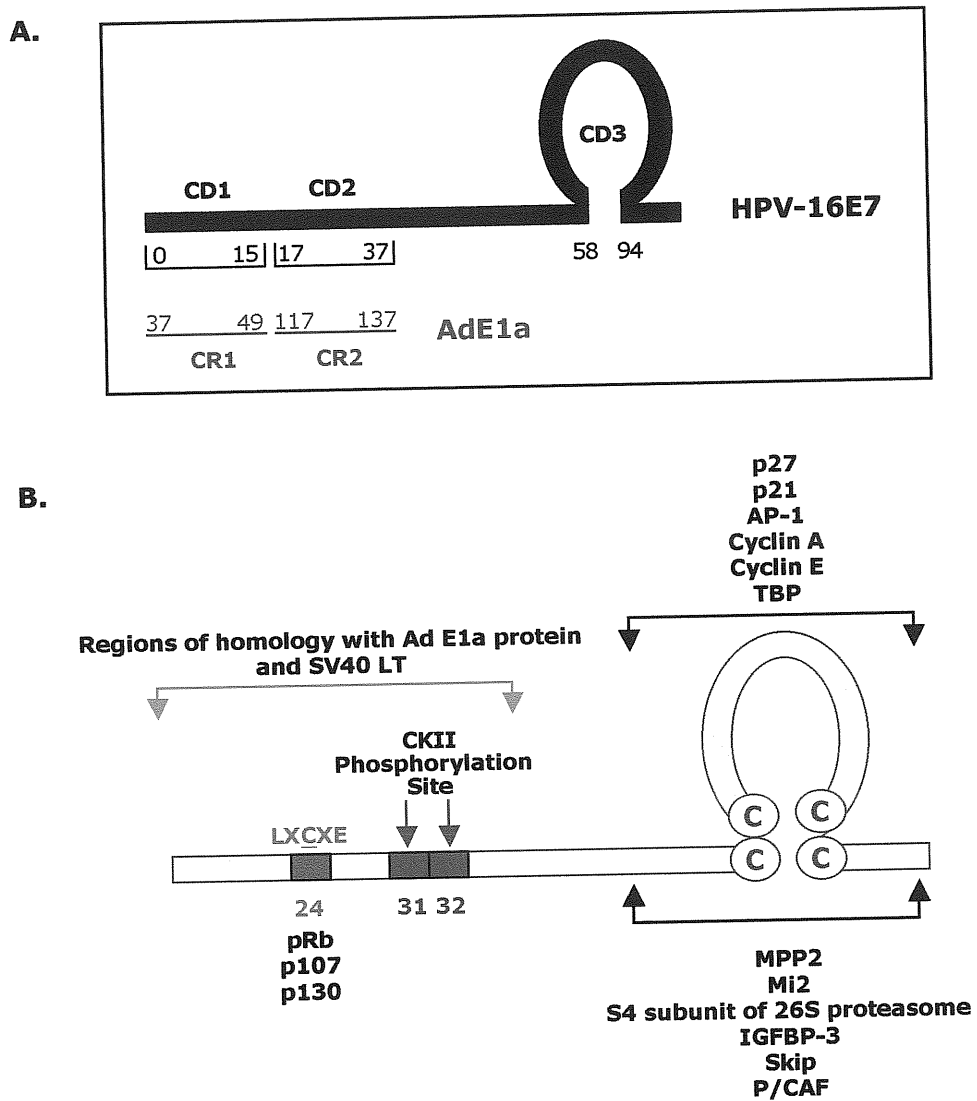
Figure 5. The high-risk HPV E7 protein.
Schematic diagram of E7 showing the regions of homology with Ad E1a (**Panel A**), together with the regions involved in interactions with some of cellular targets. The CKII phosphorylation site is indicated (**Panel B**).

"pocket proteins", which comprises the retinoblastoma gene product (pRB) and related proteins, p107 and p130, (DAVIES *et al.* 1993; DYSON *et al.* 1992; DYSON *et al.* 1989), which are negative cell-cycle regulators involved in the G1/S and G2/M transition. The LXCXE motif within CD2 is not the only one responsible for pRB binding activity – a low affinity binding site was also identified within the C-terminal domain of E7 (PATRICK *et al.* 1994). The CD3 region of E7 consists of two CXXC motifs involved in zinc binding and dimerisation (MCINTYRE *et al.* 1993; ZWERSCHKE *et al.* 1996) and it shows very little homology with Ad E1a CR3. Intriguingly, the HPV E6 proteins contain two tandem copies of this domain and this has led to the speculation that E6 and E7 may have evolved through duplication of an ancestral viral gene.

The E7 protein is normally phosphorylated (SMOTKIN and WETTSTEIN 1987) largely by CKII (BARBOSA *et al.* 1990; FIRZLAFF *et al.* 1989). The target residues necessary for CKII phosphorylation on HPV-16 E7 are serine 31 and serine 32 (BARBOSA *et al.* 1990). Mutation within the CKII phosphrylation site results in greatly reduced transforming activity of E7, although the underlying mechanism is still largely unknown. Surprisingly, the CKII phosphorylation-deficient mutant retains the ability to bind to pRb and this suggest that pRb binding and phosphorylation by CKII are independent activities of the E7 protein (BARBOSA *et al.* 1990; EDMONDS and VOUSDEN 1989).

1.4.3.2. Localization

The E7 protein has been found in a variety of locations within the cell, including cytoplasm (SMOTKIN and WETTSTEIN 1987), nucleus (GREENFIELD *et al.* 1991; SATO *et al.* 1989; SMITH-MCCUNE *et al.* 1999) and nucleoli (ZATSEPINA *et al.* 1997). While high-risk HPV E7 displays diffuse nuclear expression, low-risk E7 has a nuclear punctuate pattern of expression. Additionally, low-risk viral E7 oncoproteins show co-localization with PML, whereas high-risk viral proteins do not (GUCCIONE *et al.* 2002). Different sites of localisation within the cell suggest that E7 may shuttle between different compartments of the cell and this translocation may be related to the state of differentiation of the host cell and regulated by E7 post-translational modifications.

1.4.3.3. E7 cellular targets

E7 exerts its oncogenic function by physical association with a variety of cellular proteins (Figure 5B) which allows it to modulate cellular growth regulatory pathways. Some of the cellular target proteins of HPV E7 are listed in Table 2. It is important to note that a very large number of these proteins are implicated in the regulation of transcription.

Table 2. Cellular targets of HPV E7

E7 target	Cellular function	Result of E7 binding
pRb	Transcriptional repressor G1 arrest	Proteolytic degradation of pRb, derepression of E2F
cyclin A, cyclin E, cdk2	Subunits of cyclin-dependent kinases	Cell cycle progression
p27	CDK inhibitor	Activation of cyclin E/cdk2 kinase and cell cycle progression
p21	CDK inhibitor	Activation of cyclin E/cdk2 kinase and cell cycle progression
AP-1	Transcrptional activator	Increase of AP-1 dependent transcription
TBP	Transcrptional activator	Repression of cellular genes expression
M2-PK α–glucosidase	Gycolytic control enzymes	Expansion of phosphometabolite pools, modulation of carbohydrate metabolism
Skip	Transcriptional factor	Inhibition of transcriptional activity
P/CAF	Transcriptional co-activator HAT activity	Inhibition of transcriptional and HAT activity
S4	Subunit of 26S proteasome	Increased ATPase activity, destabilization of pRb

pRb tumour suppressor and the pocket protein family

The first cellular targets of the HPV E7 proteins to be identified were members of the retionoblastoma (pRb) tumour supressor family (DYSON *et al.* 1989; MUNGER *et al.* 1989b; PHELPS *et al.* 1992). The pRb protein plays an important role in regulating the transition of the cell cycle from G1 into S phase. In normal cells, Rb is hypophosphorylated in early G1 and becomes increasingly phosphorylated as the cells progress towards S phase. Hyperphosphorylation of pRB by the cyclin-dependent kinases (CDKs) and subsequent release of free E2F leads to the increased expression of genes required for DNA synthesis (NEVINS 1992; NEVINS 2001).

The E7 proteins from the high-risk group of HPVs interact with pRB more strongly than those from low-risk HPVs (MUNGER *et al.* 1989b), and this may account in part for differences in their respective transforming activities. However, HPV-1E7 has been shown to interact with pRb as efficiently as HPV-16E7 and to activate E2F-responsive promoters with similar efficiency, yet shown to be transformation negative (CICCOLINI *et al.* 1994; SCHMITT *et al.* 1994).

HPV E7 binds to the "pocket domain" of pRB, p107 and p130, via an LXCXE motif lying in the CD2 region (DYSON *et al.* 1992; MUNGER *et al.* 1989b). This interaction alone is not sufficient for release of free E2F since additional sequences within the C-terminal half of E7 are also required for this interaction (HUANG *et al.* 1993; PATRICK *et al.* 1994). Consistent with this is the fact that E2F does not contain an LXCXE motif and binds to a different region of pRB (DICK *et al.* 2000; HUANG *et al.* 1993; WU *et al.* 1993). This apparent discrepancy has been resolved by studies demonstrating that high-risk HPV E7 proteins induce proteasome-mediated degradation of pRB, p107 and p130 (BOYER *et al.* 1996; GONZALEZ *et al.* 2001; JONES and MUNGER 1997), and this appears to be essential for overcoming pRb induced cell cycle arrest (GIARRE *et al.* 2001). A potential mechanism by which E7 stimulates pRb degradation, was suggested by the discovery that E7 interacts with the S4 subunit of the 26S proteasome (BEREZUTSKAYA and BAGCHI 1997), which is required for proteasome-mediated degradation of ubiquitinated proteins.

The pocket domains of pRB, p107 and p130, are also required for binding to a range of cellular proteins containing an LXCXE motif, among which are cyclins D1 and D2, and histone deacetylases (HDAC) 1 and 2. During the G1 phase of the cell cycle pRB actively represses genes required for

progression to S phase by recruiting HDACs at their promoters (BREHM *et al.* 1998; MAGNAGHI-JAULIN *et al.* 1998). HPV-16E7 has been shown to be able to disrupt these complexes (BREHM *et al.* 1998), by direct binding to the Mi2β, a component of the NURD complex (nucleosome remodelling histone deacetylase complex) through its Zn finger domain (BREHM *et al.* 1999). Other studies have indicated that both p107 and p130 are also able to recruit HDACs, and since Ad E1a has been shown to disrupt these interactions (FERREIRA *et al.* 1998), it seems probable that E7 may also do so.

The essential role of the E7 interaction with the pocket proteins is highlighted by studies showing that it is indispensable for HPV replication and the full viral life cycle (FLORES *et al.* 2000; THOMAS *et al.* 1999).

Cyclins/CDK complexes and cyclins/CDK inhibitors

The stimulatory effect of E7 upon cell proliferation does not only depend on its association with pRb, since E7 targets the function of a plethora of cell cycle regulators including cyclin E (MCINTYRE *et al.* 1996) and the cyclin/CDK inhibitor p21$^{WAF/Cip1}$ (FUNK *et al.* 1997; JONES *et al.* 1997). E7 has also been shown to sequentially activate the cyclin E and cyclin A promoters (ZERFASS *et al.* 1995), and to up-regulate the levels of Cdc25A phosphatase (KATICH *et al.* 2001), which activates the cyclin E/cdk2 and cyclin A/cdk2 complexes. Moreover, E7 has been found to become part of two cyclin-containing complexes: the S-phase-specific E2F/cyclin A/p107/cdk2 complex (ARROYO *et al.* 1993), and the G1-specific E2F/cyclin E/p107/cdk2 complex (MCINTYRE *et al.* 1996).

The ability of E7 to abrogate the inhibitory activities of cyclin-dependent kinase inhibitors such as p21$^{WAF1/Cip1}$ (FUNK *et al.* 1997; JONES *et al.* 1997)

and p21^{KIP1} (ZERFASS-THOME *et al.* 1996) is important for the viral requirement of uncoupling the processes of cellular differentiation and proliferation. Several studies have shown that p21$^{WAF1/Cip1}$ plays an important role in coupling cell cycle arrest and differentiation in keratinocytes (ALANI *et al.* 1998; DI CUNTO *et al.* 1998; MISSERO *et al.* 1996), for example the steady-state levels of p21$^{WAF1/Cip1}$ increase during cellular differentiation independently of p53, resulting in inactivation of CDK2 activity and growth arrest. Recently, it has been shown that inactivation of both the Rb protein and p21 by E7 is necessary to prevent cell cycle arrest (HELT *et al.* 2002). It has also been demonstrated that E7 inhibits p21$^{WAF1/Cip1}$ function in the context of Raf signaling by altering Raf-Akt antagonism and preventing the proper subcellular localization of p21$^{WAF1/Cip1}$ (WESTBROOK *et al.* 2002).

The binding of p21 and p27 by E7 also interferes with the ability of p53 to induce G1 growth arrest following DNA damage (DEMERS *et al.* 1994a; HICKMAN *et al.* 1994; SLEBOS *et al.* 1994). Interestingly, E7-expressing cells contain increased steady-state levels of both p53 (DEMERS *et al.* 1994b) and p21 (THOMAS *et al.* 1999), which is not a consequence of transcriptional induction, but rather of protein stabilization of p53 and/or p21 (JONES and MUNGER 1997; JONES *et al.* 1999; NOYA *et al.* 2001).

Elements of the cellular transcriptional machinery

A striking feature of the HPV E7 targets is that a large majority are involved directly in transcriptional regulation. HPV E7 interferes with the activity of a variety of cellular transcription factors, being able to activate or repress transcription independently of pRB binding (ZWERSCHKE *et al.* 1996). This activity is explained by the finding that E7 can interact with the core component of the TFIID transcription factor complex, the TATA-box binding protein (TBP) (MASSIMI *et al.* 1996), and with the TBP-associated factor TAF-110 (MAZZARELLI *et al.* 1995). TFIID plays a central role in transducing the activation signal from upstream regulatory proteins to the basal transcriptional machinery by directly interacting with transcriptional activators (VERRIJZER and TJIAN 1996). HPV-16E7 has also been found to activate Pol III transcription and TFIIIB, which may be required for its immortalizing activity, since it is evident that a broad range of E7 transformed cells display abnormally elevated levels of Pol III transcripts (WHITE 1998; WHITE *et al.* 1996).

E7 has been demonstrated to interact with TBP through sequences in the E7 cysteine loop, but this association is partially dependent upon phosphorylation of the E7 protein by CKII, since this phosphorylation increases the affinity with which E7 binds TBP (MASSIMI *et al.* 1996). Interestingly mutants of E7 shown to be defective for CKII phosphorylation, also have reduced transforming activity (BARBOSA *et al.* 1990). E7 is phosphorylated by CKII on serines 31 and 32, and mutation of the CKII site to two acidic amino acids significantly increases the affinity of E7 for TBP, indicating that the incorporation of two negative charges at this region of E7 is important in regulating the interaction with TBP (MASSIMI *et al.* 1996). Mutations within the cysteine loop also result in significant reduction in

transformation activity (MASSIMI *et al.* 1997). These observations suggest that the E7:TBP interaction might alter the transcription of genes involved in transformation other than those that are regulated by E2F. It has been demonstrated that E7 is able to block the ability of p53 to stimulate transcription due to the interaction between E7 and TBP (MASSIMI *et al.* 1997). Similar results are also obtained with the Ad E1a protein, indicating a conservation of function between these two viral oncoproteins (SANG *et al.* 1997; SONG *et al.* 1995).

HPV-16E7 has also been found to interact with the fork head domain transcriptional factor M phase phosphoprotein 2 (MPP2) and to stimulate MPP2-specific transcriptional activity (LUSCHER-FIRZLAFF *et al.* 1999). This interaction is functionally relevant for the transforming activity of E7, since MPP2 enhances E7:EJ-ras co-transformation in REFs and neither non-transforming mutants of HPV-16E7 nor low-risk HPV-6E7, are able to stimulate MPP2-specific transcriptional activation.

In addition to associating with components of the basal transcription machinery, E7 interacts with members of the AP-1 family of transcription factors: c-Jun, JunB, JunD and c-Fos (ANTINORE *et al.* 1996). Interaction with c-Jun was shown to occur through the CD3 domain of E7 and this association has been demonstrated to lead to the up-regulation of c-Jun responsive promoters. Dominant negative c-Jun has been further shown to abolish the co-operation between E7 and EJ-ras in primary rodent transformation assays (ANTINORE *et al.* 1996) and to suppress the anchorage independent growth of HPV transformed keratinocytes (LI *et al.* 1998b). More recent data suggest that E7, by binding to both c-Jun and pRb inhibits the ability of pRb to activate a subset of AP-1 driven genes (NEAD *et al.* 1998). Additional transcription factors such as ATF and Oct1

28

may also be activated by E7, since E7 has been shown to complement Ad E1a mutants defective in transactivation of adenovirus early genes (WONG and ZIFF 1996), although no further studies have been done to investigate these aspects.

More recently, HPV-16E7 has been also reported to repress the transcription of fibronectin, a key component of the extracellular matrix. This repression, detected in several HPV-positive nontumourigenic cell lines, was abolished when Cys-X-X-Cys repeats were disrupted (REY *et al.* 2000).

Furthermore, E7 has also been reported to regulate the transcription of cellular genes encoding calcium binding protein S100P and ADP/ATP carrier proteins (HELLUNG SCHONNING *et al.* 2000). S100P belongs to the family of proteins associated with cell differentiation and malignancy (SCHAFER and HEIZMANN 1996) and expression of S100P mRNA was shown to be down-regulated by E7. The function of ADP/ATP carrier protein is unknown, but some reports demonstrate that it interacts with Bax and mediates permeabilization of mitochondria as a first step in the apoptotic pathway (MARZO *et al.* 1998). 16E7 was shown to up-regulate its expression. As S100 protein has been shown to promote apoptosis (HU and VAN ELDIK 1996), it has been suggested that differential regulation of S100 and the ADP/ATP carrier protein may be important for HPV induced cell cycle progression.

16E7 has also been reported to inhibit transcriptional activation by Skip (Ski interacting protein), which is a general transcription co-activator (PRATHAPAM *et al.* 2001). HPV-16E7 associates with Skip via sequences in E7's carboxy terminal region, and the evolutionarily conserved proline rich sequences (PRS) of the SNW domain of Skip. E7 functionally targets Skip *in*

vivo and inhibits its transcriptional activation activity. Two transformation-defective mutants of E7 were identified that failed both to bind Skip and to inhibit its transcriptional activity suggesting that inhibition of Skip function may contribute to cell transformation by HPV-16E7.

E7 oncoproteins have recently also been shown to be able to alter cellular gene expression by their interaction with chromatin remodeling proteins. Interaction of 16E7 with P/CAF (AVVAKUMOV *et al.* 2003), which functions as a transcriptional co-activator, leads to the disruption of protein complexes between P/CAF and transcription factors, thereby impairing transcriptional activation. HPV16-E7 interacts with the acetyltransferase domain of P/CAF, and reduces its acetyltransferase activity *in vitro*. Mutation of a highly conserved leucine residue within the cystein loop of HPV-16E7 disrupts binding to P/CAF and impairs transformation and transcriptional activation, suggesting that interaction between E7 and P/CAF may also contribute to virally induced malignant transformation.

1.5. Cell transformation by HPV oncoproteins

Based on the functions of the respective cellular targets of E6 and E7, it is not surprising that both viral proteins have dramatic effects upon cellular homeostasis, which may ultimately result in malignant transformation.

The first indirect evidence that E6 and E7 were viral oncoproteins came from studies on cervical tumours and derived cell lines, where both proteins were found to be retained and expressed many years after the initial transforming events (ANDROPHY *et al.* 1987; BANKS *et al.* 1987; SCHWARZ *et al.* 1985). Subsequently both E6 and E7 were found to possess intrinsic transforming activity in a variety of different assay systems (Table 3).

Table 3.

Cell type	Name	Assay	Viral genes
Established rodent	NIH3T3 C127 3Y1	Focus formation Growth in soft agar	E5, E6, E7
Primary rat	REF	Focus formation	E6, E7
	BRK	Co-operation with activated oncogene	E7
Primary mouse	BMK	Co-operation with activated oncogene	E6, E7
Primary human	Mammary	Immortalisation	E6
	Cervical and oral kerationctyes	Immortalisation	E6, E7 (E5, E2)
Tumours in transgenic mice	**E7** promotes benign tumour formation **E6** promotes malignant progression of benign tumours		

In all these assay systems the ability of the viral oncoproteins to bring about transformation/immortalization correlates with their association with malignancy. Thus high-risk E6 and E7 possess transforming activities, whilst low-risk proteins usually do not. The first assays used to define E6 and E7 as oncoproteins were based on the transformation of established rodent cells (TSUNOKAWA *et al.* 1986; YASUMOTO *et al.* 1986). This was followed by the demonstration that both E6 and E7 can co-operate with EJ-ras oncogenes in the transformation of the primary rodent cells. (PIM *et al.* 1994; STOREY and BANKS 1993). Much more relevant however, was the demonstration that E6 and E7 can co-operate to bring about the immortalization of primary human keratinocytes (HFKs), the natural host cells of the virus *in vivo* (HAWLEY-NELSON *et al.* 1989; MUNGER *et al.* 1989a). The demonstration that E6 and E7 could indefinitely extend the lifespan of these cells had a profound impact upon our view of the role of these proteins in the development of malignancy. These cell lines, however, are not tumorigenic in nude mice and require additional events such as the

presence of an activated *ras* -oncogene (HURLIN *et al.* 1991), extended passaging in tissue culture (DIPAOLO *et al.* 1989) or treatment with glucocorticoids (DURST *et al.* 1989), to become fully transformed. These observations nicely resemble the process of HPV induced tumourigenicity *in vivo*, where there are long periods between the initial immortalisation events and the ultimate progression to cervical cancer, thereby highlighting the multistep nature of the disease progression. In contrast to oncogenic HPV types, the E6 and E7 proteins from low-risk HPV types are unable to immortalize keratinocytes and are completely inactive in this assay (PECORARO *et al.* 1989; WOODWORTH *et al.* 1989).

More recently, efficient models of HPV-induced carcinogenesis have been obtained through generation of transgenic mice. Expression of E6 and E7 together can induce various types of tumours, depending on the particular tissue in which they are targeted (ARBEIT *et al.* 1993; COMERFORD *et al.* 1995; GRIEP *et al.* 1993). When expression was directed to the basal cells of the squamous epithelium, under control of the human keratin 14 promoter (K14), the transgenic mice developed progressive squamous epithelial neoplasia (ARBEIT *et al.* 1994). When transgenic female mice expressing HPV-16 oncogenes downstream of the K14 promoter were subjected to prolonged estrogen treatment, they developed squamous carcinomas of the cervix (ARBEIT *et al.* 1996), thus providing a model for the multistage process of HPV-induced carcinogenesis *in vivo*, and underlying the importance of estrogen hormones as cofactors in the induction of cervical neoplasia. Individual expression of each oncogene induced epithelial hyperplasia and skin tumours (HERBER *et al.* 1996; SONG and LAMBERT 1999), however E7 was found to primarily cause benign, highly differentiated tumours, whereas those promoted by E6 were mostly
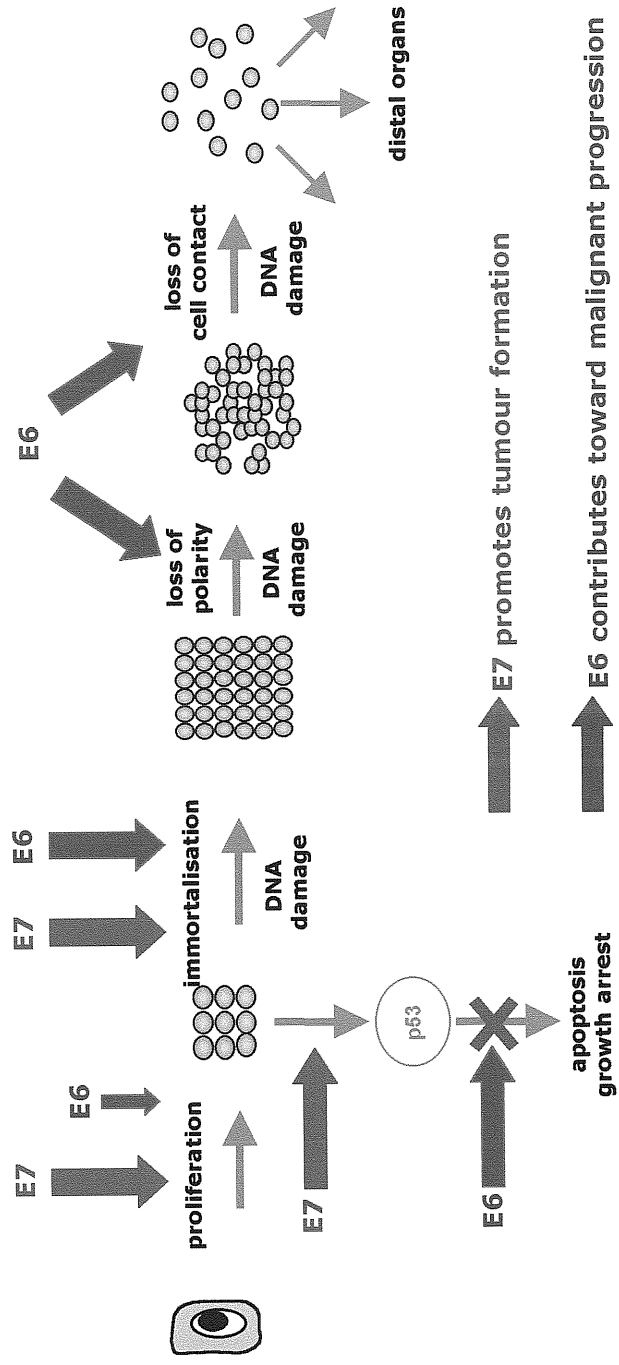
Figure 6. Contribution of E6 and E7 to different stages of tumour progression.

malignant. This suggests that the two oncoproteins play different roles in the process of carcinogenesis, and also supports the notion that they act cooperatively to induce transformation (Figure 6).

1.6. p300/CBP transcriptional co-activators

Since the major aim of this thesis is the analysis of the interaction between HPV proteins and p300/CBP transcriptional co-activators it is appropriate at this stage to consider the activities of these proteins, as well as to highlight their importance for the functioning of different viral transforming proteins.

The p300/CBP proteins belong to a family of transcriptional co-activators and co-repressors participating in multiple cellular processes. p300 is a 2414 aa protein that was initially identified as a nuclear binding target of Ad E1a (ECKNER *et al.* 1994; STEIN *et al.* 1990), whereas CBP is 2441 aa long and was originally recognized as a transcriptional co-activator that bound to phosphorylated cAMP response element- binding protein (CREB) (CHRIVIA *et al.* 1993; HARLOW *et al.* 1986) as well as to Ad E1a (ARANY *et al.* 1995; LUNDBLAD *et al.* 1995). The p300/CBP genes are evolutionarily highly conserved and their genes share considerable sequence homology over their entire length exhibiting 91% sequence identity (ARANY *et al.* 1994). The proteins are 63% identical at the amino-acid level, greater similarity is observed in specific regions (Figure 7A). The highly conserved regions encompass the KIX domain (CREB binding site), the three cysteine-histidine rich domains (CH1-3) and the bromodomain. Each CH region is composed of at least one zinc-finger motif. The histone acetyltransferase (HAT) domain is located in the central region of the protein, partially spanning the CH2 and CH3 domains (Figure 7B).

A.

B.
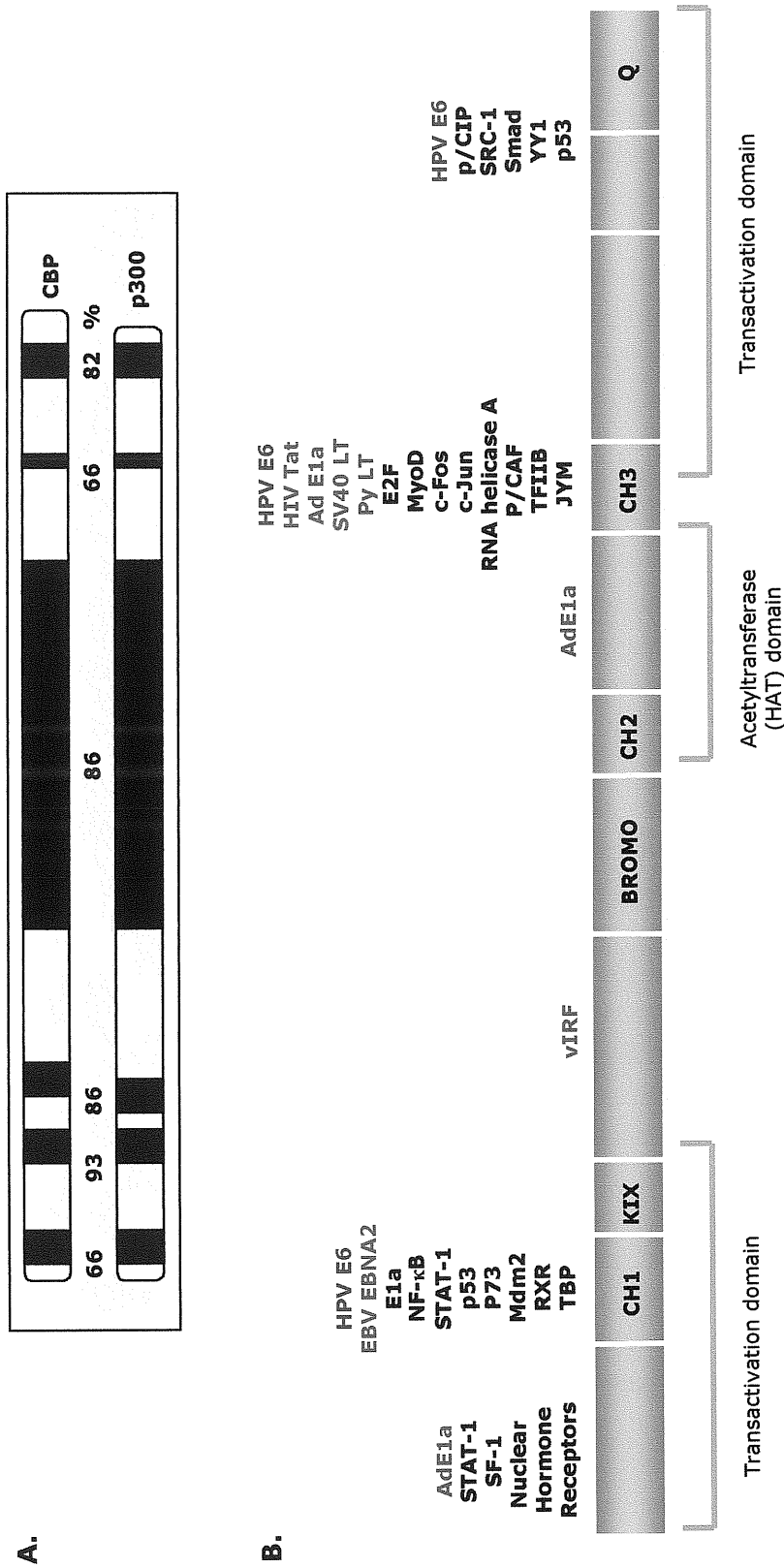
Figure 7. Organisation of the p300/CBP proteins.

Panel A. The regions of highest homology (black boxes), with the percentage of amino acid identity between both proteins. **Panel B.** The functional domains of p300/CBP: (CH1-3: cysteine-histidine rich motifs/zinc finger domains,Q: glutamine-rich domain, HAT: histone acetyltransferase domain, Bromo: bromodomain, KIX: kinase induced domain). The N- and C- terminal domains of p300/CBP can act as transactivation domains,HAT domain is located in the central region of the protein. Some of the cellular target proteins are shown (in black). In blue: various viral proteins shown to interact with p300, in red: previosly desribed HPV proteins targeting p300.

Although p300 and CBP share extensive homology, genetic and molecular analysis suggests that they have not only overlapping, but also many unique functions. The complex phenotypes associated with a loss of p300/CBP function in human and in mice reflect the diverse roles of both proteins in multiple developmental processes.

The known functions of p300 and CBP can be roughly divided into six groups:

- they create a physical bridge between various transcription factors and the basal transcriptional machinery

- they are potent histone acetyltransferases, which link transcription with chromatin remodelling

- they mediate both negative and positive cross-talk between signalling pathways

- they participate in basic cellular functions including DNA repair, cell growth, differentiation and apoptosis

- they play pivotal roles in embryonic development

- they may function as tumour suppressor proteins

1.6.1. p300/CBP in human disease

Two lines of evidence suggest that p300/CBP may function as tumour suppressor proteins. Firstly, mutations in p300 have been found in many types of tumour (GAYTHER *et al.* 2000) and mutation of human CBP causes Rubinstein-Taybi syndrome (RTS), which leads to an increased risk/predisposition of cancer in RTS patients (GILES *et al.* 1998). Secondly, bi-allelic inactivating somatic mutations of the *p300* gene have been observed in gastric and colon cancers (MURAOKA *et al.* 1996). Furthermore, the loss of heterozygosity (LOH) of markers at chromosome band 22q13,

which corresponds with the *p300* locus has been observed in 80% of glioblastomas (MUHAMMAD *et al.* 1997). Likewise LOH around the *CBP* locus has been observed in hepatocellular carcinoma (SAKAI *et al.* 1992).

Mice with monoallelic inactivation of the CBP gene develop multilineage defects in hematopoietic differentiation and, with advancing age, an increased incidence of hematologic malignancies. The latter are characterized, at least in some cases, by loss of heterozygosity at the CBP locus. No such pathology was observed in wild-type or p300 heterozygous null mice of the same age and genetic background. Thus, a full complement of CBP, but not p300, is required for normal hematopoietic differentiation (KUNG *et al.* 2000).

1.6.2. Transcriptional control by p300/CBP

One of the paradoxes in p300/CBP function is that these proteins are capable of contributing to diametrically opposed processes acting both as transcriptional activators and repressors. These diverse properties of p300/CBP appear to be exercised through multiple mechanisms (Figure 8).

They can act as a protein bridge connecting different transcription factors to the basic transcriptional apparatus. The initiation of transcription by RNA polymerase II requires sequence-specific promoter/enhancer-binding transcription factors (TFs) as well as the basal transcriptional machinery. Unless the transcription factors can directly interact with the basal transcriptional apparatus, other proteins must act as bridges that connect them to the basal machinery. p300/CBP is known to interact both with a wide variety of TFs and with components of the basal transcriptional machinery, including TBP, TFIIB, TFIIE, TFIIF and RNA polymerase II, reviewed in (GOODMAN and SMOLIK 2000), therefore p300/CBP provides such
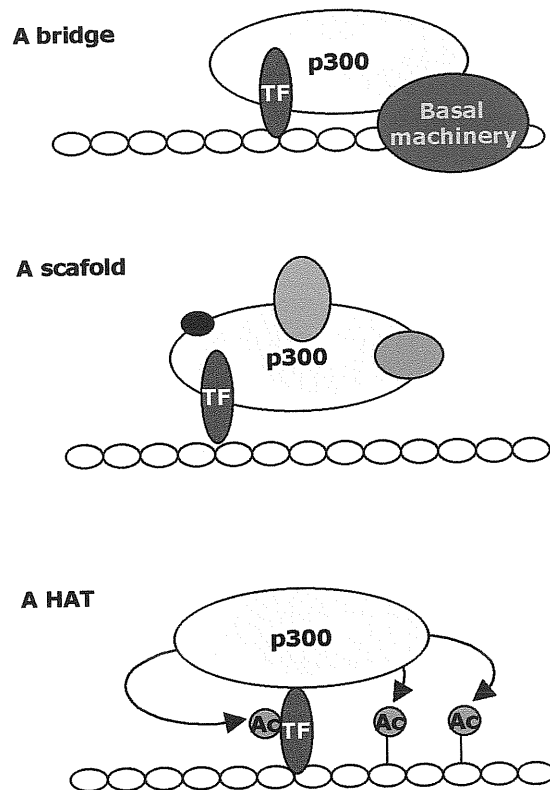
Figure 8. Mechanisms of transcriptional activation by p300/CBP. In a bridging model, p300/CBP proteins connect sequence-specific transcription factors (TF) to the transcriptional apparatus. In the scaffold model p300/CBP act as a protein scaffold for the assembly of multicomponent complexes that confer transcriptional activation. In the HAT model, intrinsic or associated HAT activity of p300/CBP, allows to target chromatin and/or transcription factors and facilitate transcriptional response .

a bridge. Some of the TF interacting with p300/CBP are listed in Table 4 and shown in Figure 7B.

p300/CBP can also provide a scaffold for the assembly of diverse multicomponent transcriptional co-activator complexes. By this activity they increase the relative concentration of these factors in the local transcriptional environment and thereby facilitate protein-protein and protein-DNA interactions. Given a limited repertoire of activators, co-activators and co-factors available for responses of diverse regulatory cues, cells probably use cooperativity and transcriptional synergy, so that a combination of a few ubiquitous, signal- and tissue- specific activators can create a potentially very large number of regulatory complexes (CAREY 1998).

p300/CBP also posses histone acetyltransferase (HAT) activity (BANNISTER and KOUZARIDES 1996; OGRYZKO *et al.* 1996) and this allows p300/CBP to influence chromatin structure by modulating nucleosomal histones. Acetylation of lysine resides within the histone tails may facilitate the TFs access to DNA in chromatin, may weaken internucleosomal interactions and may de-stabilise higher-ordered chromatin structure. It has been shown that p300/CBP is also able to acetylate key transcription factors such as p53 (GU and ROEDER 1997), pRb (CHAN *et al.* 2001), E2F-1, -2 and –3 (MARTINEZ-BALBAS *et al.* 2000; MARZIO *et al.* 2000), GATA-1 (BOYES *et al.* 1998), Myb (TOMITA *et al.* 2000), p73 (COSTANZO *et al.* 2002) and HMGI(Y) (MUNSHI *et al.* 2001). In some of these cases such as with p53, GATA-1 and HNF-4, acetylatation enhances their DNA-binding activity (BOYES *et al.* 1998; GU and ROEDER 1997; SOUTOGLOU *et al.* 2000) resulting in stimulation of target gene expression. Acetylation may also decrease DNA binding activity, such as in the case of HMGI(Y) (MUNSHI *et al.* 1998) as well as

inhibit protein-protein interactions (WALTZER and BIENZ 1998), as in the case of dTCF (T-cell factor) binding to Armadillo. Finally, acetylation has also been demonstrated to influence protein localization, as for example with Ad E1a (MADISON *et al.* 2002).

Recently it has also been demonstrated that purified p300 exhibits another intrinsic enzymatic activity – that of an ubiquitin ligase (GROSSMAN *et al.* 2003), which was shown to be required for p53 polyubiquitination and subsequent proteasome degradation. This activity of p300 was blocked by E1a, explaining one of the mechanisms by which E1a mediates stabilization of p53. Interestingly, the complete ubiquitination of p53 has been shown to require the intrinsic ubiquitin ligase activities of both Mdm2, which catalyses p53 monoubiquitination and p300 catalyzing p53 polyubiquitation and hence subsequent degradation at 26S proteasome.

Table 4. Summary of some p300/CBP targets.

Target/TF	Binding domain		Comment	Reference
	p300	**CBP**		
CREB	1-663	590-669	TF	(CHRIVIA *et al.* 1993) (LUNDBLAD *et al.* 1995)
c-Fos		1621-1877	TF	(BANNISTER and KOUZARIDES 1995)
c-Jun, v-Jun	1596 1752-2370		TF	(BANNISTER *et al.* 1995) (LEE *et al.* 1996a)
YunB	744-1571		TF	(LEE *et al.* 1996a)
YY1	1572-2370		TF	(LEE *et al.* 1995)
c-Myb		461-661	TF	(DAI *et al.* 1996)
Nuclear receptors RXR, RAR, TR, ER, PR, GR	1-117	1-101		(CHAKRAVARTI *et al.* 1996) (KAMEI *et al.* 1996)
Stat-2	CH1		TF	(BHATTACHARYA *et al.* 1996)
MyoD	1514-1922		TF	(YUAN *et al.* 1996)
E2F-1		1621-1877	TF	(TROUCHE and KOUZARIDES 1996)
TBP	1-743 1737-2414		TF	(DALLAS *et al.* 1997)
TFIIB	1737-2414	1680-1812	TF	(LEE *et al.* 1996b)
P/CAF	1763-1966	1801-1880	HAT	(YANG *et al.* 1996)
p53	CH1 C-terminus		TF	(AVANTAGGIATI *et al.* 1997)
p73	CH1		TF	(ZENG *et al.* 2000)

1.6.3. Association of p300/CBP with viral oncoproteins

Many viral proteins have been found to modulate p300/CBP function (Table 5), thereby emphasizing the central importance of p300/CBP in maintaining cellular homeostasis and demonstrating a high degree of conservation of function among different viruses. These comprise DNA and RNA tumour viruses, and include the following viral proteins: Ad E1a, SV40 LT, HPV E2, HPV E6, Polyoma LT, EBV EBNA2 and EBNA-3C/ProT-α, HTLV Tax and p30(II) and HIV Tat (Bernat *et al.* 2002; Eckner *et al.* 1994; Eckner *et al.* 1996; Hottiger and Nabel 1998; Kwok *et al.* 1996; Lee *et al.* 2000a; Nemethova and Wintersberger 1999; Subramanian *et al.* 2002; Wang *et al.* 2000; Zhang *et al.* 2001).

Some of the viral proteins, such as HTLV-1 Tax, EBV EBNA-2 and HPV E2 require p300/CBP for their transcriptional transactivation activities. Others, such as Ad E1a and SV40 LT, appear to block the normal function of p300. The viral oncoproteins that use p300/CBP as co-activator appear to do so by interacting with the CREB binding domain. These viral proteins must be capable of binding, either directly or indirectly, to appropriate DNA sequences in the promoters of their target genes. Viral proteins which block the normal function of p300/CBP typically do not bind DNA and tend to interact with one or more of the zinc finger domains – regions that have been shown to mediate interactions with positive effectors of p300/CBP function.

Table 5. Viral proteins described to interact with p300/CBP.

Virus Protein	Function upon p300/CBP	Reference
Adenovirus <u>E1a</u>	Inhibition of co-activation function Inhibition of p300 HAT activity	(STEIN *et al.* 1990) (ECKNER *et al.* 1994) (HARLOW *et al.* 1986) (CHRIVIA *et al.* 1993)
SV40 <u>LargeT (LT)</u>	Inhibition of co-activation function	(ECKNER *et al.* 1996)
HPV <u>E6</u>	Inhibition of co-activation function	(ZIMMERMANN *et al.* 1999) (PATEL *et al.* 1999)
HPV <u>E2</u>	Activation of E2-dependent transcription	(LEE *et al.* 2000a)
KSHV/HHV-8 <u>vIRF</u>	Inhibition of p300 HAT activity	(LI *et al.* 2000)
Polyoma <u>LargeT (LT)</u>	Inhibition of co-activation function	(NEMETHOVA and WINTERSBERGER 1999) (CHO *et al.* 2001)
EBV <u>EBNA-2</u>	Activation of LMP1 promoter	(WANG *et al.* 2000)
EBV <u>EBNA-3C</u> <u>ProT-α</u>	Regulation of transcription and HAT activity	(SUBRAMANIAN *et al.* 2002)
HTLV-1 <u>Tax</u>	Activation of Tax-dependent transcription	(KWOK *et al.* 1996) (GIEBLER *et al.* 1997) (KASHANCHI *et al.* 1998)
HTLV-1 <u>p30(II)</u>	Activation of p30(II)-dependent transcription	(ZHANG *et al.* 2001)

1.6.3.1. Adenovirus E1a (Ad E1a)

E1a is the first protein to be expressed during adenoviral infection and is essential for viral replication and transformation. Comparison of the amino acid sequences of Ad E1a from different viral serotypes has allowed for the definition of three regions of homology – conserved regions (CR) 1, 2, 3. The amino terminus (2-25aa) along with conserved region 1 (CR1, 35-76aa) of E1a play important roles in p300/CBP binding. This interaction is believed to mediate cell immortalization (ARANY *et al.* 1995; LUNDBLAD *et al.* 1995; STEIN *et al.* 1990) and is thought to be sufficient to promote entry into S phase (WANG *et al.* 1993). The CH3 region of p300 encompasses the domain required for the interaction with E1a (ARANY *et al.* 1995), although

additional studies have reported multiple sites of interaction (KUROKAWA *et al.* 1998; LIPINSKI *et al.* 1999).

Binding to p300 was found to be essential for the oncogenic and growth-stimulatory activities of E1a and for its ability to repress several tissue-specific transcriptional control elements (JELSMA *et al.* 1989; PEEPER and ZANTEMA 1993). The regions of E1a required for transcriptional repression (aa 1 to 30 and 48 to 60) overlap those required for binding p300 *in vivo* (Wang *et al.,* 1993). Overexpression of p300/CBP or p300/CBP-associated factor p/CAF suppresses the cell cycle progressive effects of E1a and subsequently blocks E1a-mediated transformation (SMITS *et al.* 1996; YANG *et al.* 1996).

There appear to be several mechanisms by which Ad E1a blocks p300/CBP function. E1a binds to the same region of p300 that interacts with components of TFIIB and the RNA polymarese II holoenzyme complex (FELZIEN *et al.* 1999; KIM *et al.* 1998; KWOK *et al.* 1994; NAKAJIMA *et al.* 1997). Of particular importance is the observation that E1a binding to p300 is responsible for the repression of p300 transcriptional activity (ARANY *et al.* 1995; SMITS *et al.* 1996). Thus complex formation between p300 and E1a prevents p300 interaction with certain transcriptional factors (JONES 1995), such as c-Fos c-Jun and p53, thereby counteracting their respective transcriptional activities (AVANTAGGIATI *et al.* 1997; BANNISTER and KOUZARIDES 1995; LEE *et al.* 1996a; LILL *et al.* 1997; SOMASUNDARAM and EL-DEIRY 1997).

In addition, E1a binding to p300/CBP was shown to disrupt p300/CBP:P/CAF and p300/CBP:P/CIP co-activation complexes (KUROKAWA *et al.* 1998; YANG *et al.* 1996). Direct binding of E1a to P/CAF results also in a reduction of P/CAF HAT activity (CHAKRAVARTI *et al.* 1999; REID *et al.*

1998). Similarly, direct binding of E1a to the HAT domain or third zinc finger domain of p300/CBP has also been demonstrated to inhibit the HAT activity of p300/CBP (CHAKRAVARTI *et al.* 1999; HAMAMORI *et al.* 1999). However, a note of caution is necessary, since depending on the system used, it has also been suggested that E1a might cause an increase in CBP HAT activity, similar to that seen following phosphorylation by CDK2 (AIT-SI-ALI *et al.* 1998).

E1a-mediated transcriptional repression can be relieved by overexpression of either p300, TBP, or-TFIIB (SONG *et al.* 1995), but cannot be released by overexpression of the pocket proteins. This suggests that p300/CBP and TBP but not the pocket proteins, pRb, p107, and p130 are functional targets of E1A in transcriptional regulation and that transactivation requires the function of the p300/TBP/TFIIB complex delineating a pathway by which E1A may exert its transforming activity (SANG *et al.* 1997). This repression activity, mediated through p300/CBP, can then affect a number of important biological processes such as differentiation, cell cycle regulation and apoptosis.

A model for E1a-mediated inhibition of p300/CBP functions is presented in Figure 9.

1.6.3.2. SV40 LT

The early region of SV40 encodes three proteins: the large T, the small t and the tiny t (17KT) antigens. Large T (LT) is the primary transforming agent of SV40, and its ability to transform cells depends on three domains comprising residues 1-82, 105-114 and 351-626. Several studies have demonstrated that the region spanning residues 1-82 is involved in the interaction with p300/CBP. Early work showed that LT has a biological activity complementary to the p300-associated transforming function of the adenovirus E1a gene products and that LT proteins containing mutations in the 1-82 region could not mediate transformation (YACIUK *et al.* 1991). Interestingly, transformation-defective forms of LT that bind to p300 were found to be able to complement a non-binding p300 and transformation deficient E1a mutant (QUARTIN *et al.* 1994). This led to the suggestion that transformation by T-antigen might require interactions with p300, and this was indeed confirmed by biochemical data and mutational analysis (ECKNER *et al.* 1996; QUARTIN *et al.* 1994). SV40 LT interferes with normal p300 and CBP function on at least two different levels. The presence of LT alters the phosphorylation states of both proteins and inhibits their transcriptional activities on certain promoters (ECKNER *et al.* 1996). It has also been shown that LT antigen interacts exclusively with the unphosphorylated form of p300 (AVANTAGGIATI *et al.* 1996). Because p300 is differentially phosphorylated during the cell cycle, this property could have implications for LT antigen-mediated transformation. LT antigen was found to block activation of a CRE reporter gene through the CPEB/PKA pathway only in cells that contained unphosphorylated p300 (ECKNER *et al.* 1996). The inability of SV40-transformed rat embryo fibroblasts to express a CRE reporter gene in response to forskolin, an activator of adenyl cyclase, is

consistent with the idea that LT-antigen blocks p300 function (AVANTAGGIATI *et al.* 1996). Both studies found that LT-antigen binds primarily to the third zinc finger domain (CH3) of p300/CBP, a region that mediates interaction with many effectors of co-activation function (previously mentioned in the text).

1.6.3.3. HPV E6 and E2

Two HPV proteins have already been demonstrated to interact with p300/CBP: E6 and E2.

The first HPV protein shown to associate with p300/CBP was E6. HPV-16E6 was found to bind to three regions (CH1, CH3 and the C-terminus) of both p300 and CBP and the interaction appeared to be direct. Interestingly, some mutations in HPV-16E6 abrogated CH3-E6 interactions, but did not alter the ability of E6 to associate with the CH1 domain, suggesting that these modified proteins could be used to delineate the functional significance of the CH1 and CH3 domains of p300/CBP (PATEL *et al.* 1999). The low-risk 6E6 was also found to interact weakly with p300 within the CH1 domain, but no binding has been observed with CH3 or the C-terminus of p300.

One of the consequences of 16E6 interaction with p300/CBP was inhibition of the intrinsic transcriptional activity of CBP/p300 and subsequent decreased ability of p300 to activate both p53- and NF-κB-responsive promoter elements. Additionally, it was shown that the HPV-16E6 protein targets CBP in an interaction involving the C-terminal zinc finger of E6 and CBP residues 1808 to 1826, which was identified as a transcriptional adapter motif (TRAM), and which is targeted also by Ad E1a (O'CONNOR *et al.* 1999). Therefore, HPV E6 proteins were suggested to possess two

distinct mechanisms by which they could abrogate p53 function: the repression of p53 transcriptional activity by targeting the p53 co-activator p300/CBP, and the removal of cellular p53 protein through the proteosome degradation pathway (ZIMMERMANN *et al.* 1999).

Recently, it has also been found that in the presence of HPV-16E6, the activity of the IL-8 promoter is repressed (HUANG and MCCANCE 2002). Moreover, from the mutational analysis of the IL-8 promoter, it has been found that E6 down-regulates the IL-8 promoter activity through the NF-κB/p65 binding site. This inhibition appears to result from the ability of HPV-16E6 to compete with NF-κB/p65 and SRC-1 for binding to the N terminus and C terminus of CBP respectively (HUANG and MCCANCE 2002).

The second HPV protein found to interact with the CBP transcriptional co-activator, was the E2 protein. Biochemical, genetic, and functional analysis provided evidence that HPV-18E2 binds directly to the CBP KIX domain which was shown to be required for E2-dependent transcriptional transactivation. Mutations in an amphipathic helix within HPV-E2 abolish its transcriptional activation properties together with its ability to bind to CBP. Interestingly, the HAT activity of CBP was shown to play a role in CBP activation of E2-dependent transcription (LEE *et al.* 2000a). Interaction between BPV-E2 and p300 was also observed, although it has been demonstrated that AMF-1 (Gps2), which binds both E2 (BREIDING *et al.* 1997) and p300 (PENG *et al.* 2000), facilitates the recruitment of p300 and its histone acetylase activity into complexes with E2 and stimulates levels of E2-dependent transcription (PENG *et al.* 2000). In a set of separate studies it was also found that HPV-8E2 binds strongly to the HAT domain of p300 resulting in strong activation of HPV8 gene expression (MULLER *et al.* 2002). In the same study it was also demonstrated that E2 proteins from

different PV types, including 8-, 18- and BPV-E6, show different pattern of interaction with p300, suggesting that E2 proteins differ in their binding to p300 and CBP.

Therefore it would appear that HPV proteins are able to form two functionally different complexes with p300/CBP: first the co-activator complex with E2:p300/CBP and a second inhibitory complex with E6:p300/CBP.

The scheme of HPV proteins interacting with p300 is presented in Figure 10.
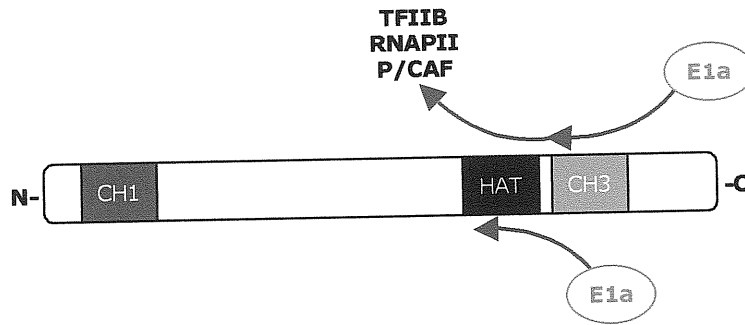
Figure 9. Model for the E1a inhibition of p300/CBP.
Interaction of E1a amino terminus with the third zinc finger domain of p300/CBP is proposed to displace TFIIB, RNA polymarase II holoenzyme and P/CAF. Interactions involving other associated factors may be disrupted as well. The carboxy terminus of E1a is proposed to inhibit p300/CBP HAT function directly.
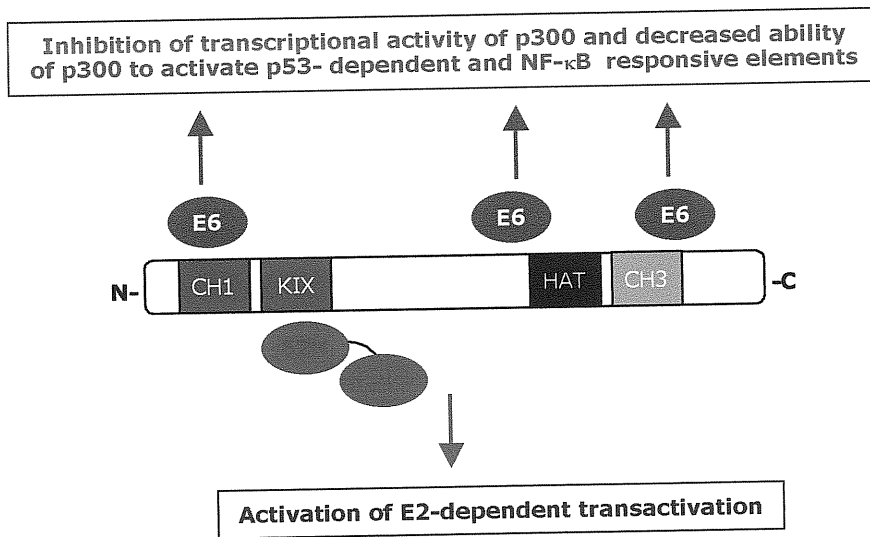
Figure 10. Interaction of HPV proteins with p300/CBP.

1.7. Objectives of the study

From the above introduction it is clear that the co-operation between viral and cellular oncogene products dramatically increases the possibility of conversion of a pre-malignant lesion to an invasive cancer.

The current studies on p300/CBP firmly place these proteins in the spotlight of transcription research. The increasing variety of transcription factors identified that utilize p300/CBP suggest that multiple cellular activities may be coordinated by this family of proteins. Many viral oncoproteins have been found to modulate p300/CBP function, further emphasizing the central importance of p300/CBP in maintaining cellular homeostasis and underlying conservation of function between different viruses. Here, the analysis of the interaction between p300/CBP and two HPV oncoproteins, E6 and E7, will be presented.

As highlighted before, p300/CBP play a central role in the life cycles of a large number of the viruses. In many cases the interaction with p300/CBP is also crucial for the ability of these viruses to bring about cellular transformation. In HPV two viral proteins are known to associate with p300/CBP, but at least in the case of E6, the biological relevance is unclear. The other issue is the uncertain role played by E7 if any, with respect to its interaction with p300/CBP.

Therefore in this thesis I will try to address these concerns.

2. RESULTS

2.1. HPV E6 interactions with p300

Recent studies have demonstrated that the HPV-16 E6 and Bovine Papillomavirus type 1 (BPV-1) E6 can interact with the p300/CBP co-activators (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999). As a result of this interaction, the transcriptional activity of p300 is greatly reduced, suggesting that E6 can interfere with the normal functioning of p300 (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999). This was a particularly important observation, since a number of other DNA tumour virus oncoproteins, including SV40 LT and Adenovirus E1a, have also been shown to require interaction with p300 in order to efficiently transform cells (ECKNER *et al.* 1996; SMITH and ZIFF 1988; STEIN *et al.* 1990). Thus, the demonstration that E6 could likewise inhibit p300 activity, suggested that this interaction may be important for the ability of HPV E6 to contribute towards cell transformation. At present, however, there is little biological evidence to support this hypothesis. In addition, there have appeared conflicting results as to whether the interaction between E6 and p300 is restricted only to the oncogenic HPV types (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999).

2.1.1. E6 interaction with p300 is not only limited to the high-risk HPV E6 proteins

In order to determine whether the interaction between E6 and p300 is high-risk specific or not, we proceeded to investigate the ability of high-and low-risk HPV E6 proteins to interact with p300 in a series of *in vitro* GST pull-down assays. Since it was not possible to obtain a full-length GST-p300 fusion protein, we used different regions of p300 fused to GST and these are shown in Figure 11A. This set of GST fusion proteins covers all the major functional domains of the p300 protein. These proteins were induced and purified as described previously (MASSIMI *et al.* 1996) and then incubated with *in vitro* translated, radiolabelled low-risk HPV-11E6 and high-risk HPV-18E6. HPV-16E6 and a previously described mutant of 16E6 (Δ123-7) (PATEL *et al.* 1999), defective in p300 binding, were also included as a positive and negative control, respectively. The results from a representative binding assay are shown in Figure 11B. As can be seen, both HPV-11 and HPV-18 E6 proteins show significant levels of interaction with p300. Interestingly, the fragments of p300 bound by both E6 proteins are the same, and overlap with the CH1, CH2 and CH3 domains of p300. No significant binding is seen with the fragments of p300 spanning residues 672-1193 and 1893-2414. It can also be seen, that the pattern of the binding obtained with 16E6 is similar and in addition, the p300-binding deficient mutant - Δ123-7.16E6 shows only a minimal binding to p300, demonstrating the specificity of the interaction. These results are largely in agreement with earlier observations where the CH1 and CH3 domains were found to be bound by HPV-16E6, although binding to CH2 was not assessed in those studies (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999). To further verify the

A.

| | CH1 | KIX | | BROMO | CH2 | | CH3 | | | Q |

HAT (1195-1810)

1 (1-672)
2 (672-1193)
(1069-1459) 3
(1459-1892) 4
(1893-2414) 5

B.

GST-p300 fragments

	1	2	3	4	5	GST-alone	0.25 Input	
								11E6 (ivt)
% of protein bound	16	12	19	19	8.2	3		
								18E6 (ivt)
% of protein bound	17	8	17	17.4	9	3.4		
								16E6 (ivt)
								Δ123-7.16E6 (ivt)

C.
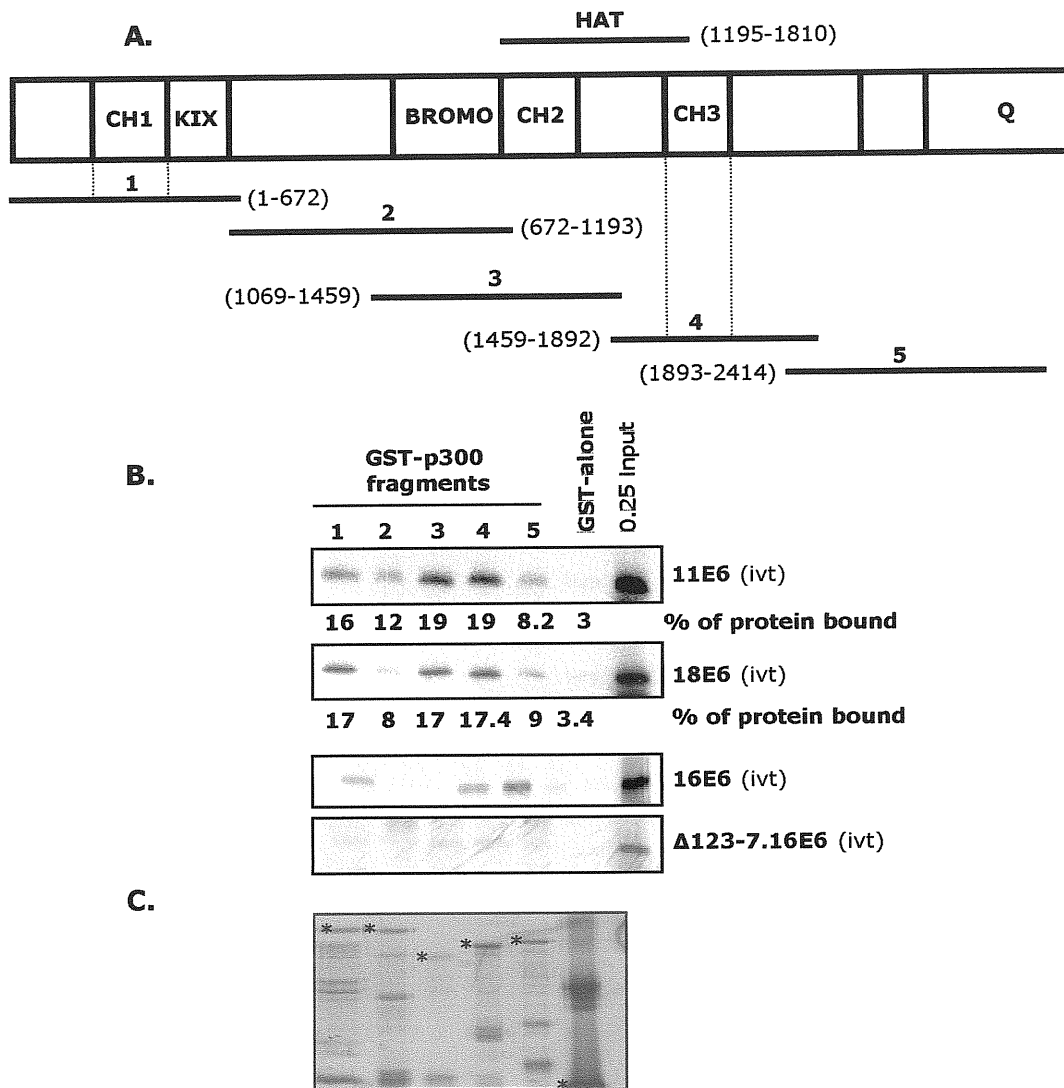
Figure 11. HPV E6 proteins interact with p300 *in vitro*.

Panel A. Schematic representation of the GST-p300 fusion clones used for *in vitro* binding assays. The most conserved domains of p300 are indicated (CH1-3: cysteine-histidine rich motifs/zinc finger domains, Q: glutamine-rich domain, HAT: functional histone acetyltransferase domain, Bromo: bromodomain, KIX: kinase induced domain).

Panel B. Interaction between the HPV-11E6, HPV-18E6, HPV-16E6 oncoproteins and the p300-binding defective mutant Δ123-7.16E6 with GST-p300 fusion proteins. Major sites of interaction encompass p300 fragments 1, 3 and 4 which encompass the CH1, CH2 and CH3 domains. The numbers below each panel show the percentage of input protein bound to the beads.

Panel C. Coomassie stain of purified GST-fusion proteins indicates the protein input; * marks the position of the full length GST-fusion protein fragment.

specificity of these interaction assays, we then compared the relative ability of the HPV-11 and HPV-18E6 proteins to bind p300 in comparison with their abilities to interact with p53 and the amino terminal region of the Discs Large (Dlg) protein (NTdlg), as positive and negative controls respectively (GARDIOL *et al.* 1999; WERNESS *et al.* 1990). As can be seen from the Figure 12A HPV-18E6 binds strongly to p300 and this is similar to the level of interaction seen between p53 and p300. HPV-11E6 also shows some degree of interaction with p300 when compared with the GST-alone and GST-NTdlg negative controls, although the levels of interaction between HPV-11E6 and p300 are considerably weaker than that observed with HPV-18E6.

It should be noted that the strength of interaction between 11E6 and p300 varies depending on the assay. The result obtained using full length GST fusion 11E6 and full-length *in vitro* translated p300 (Figure 12A) suggests that the interaction between low- and high-risk E6 and p300 differ in strength of interaction, while pull-down assays with GST p300 fusion proteins (Figure 11B) would imply that both 11 and 18 E6 bind to p300 with similar efficiency. This difference may arise from the fact that subdivision of p300 into five fragments may have led to the exposition of protein sequences on the surface not normally accessible on the native protein, which may have affected the binding of 11E6 to p300. Another possibility is that the overall conformation of the full-length p300 may be important for the binding to E6 protein.

Taken together these results demonstrate that both HPV-18 and HPV-11E6 also interact with p300, with stronger levels of association being observed with the high-risk E6 protein compared with the low-risk E6.

A.

GST-11 E6 GST-18 E6 GST-NTdlg GST-p53 GST-alone 0.25 Input

p300 (ivt)

12 14.2 8 19 6.2   **% of protein bound**
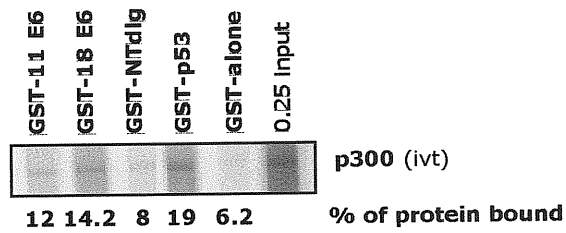
B.
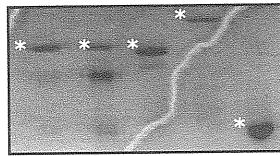
Figure 12. HPV E6 proteins interact with p300 *in vitro*.

Panel A. Interaction between *in vitro* translated p300 and HPV-11E6, HPV-18E6, p53 and amino terminal Dlg (amino acids 1-222) GST fusion proteins. The numbers below show the percentage of input protein bound to the resin.
Panel B. Commassie stain for protein input of the purified GST-fusion proteins used in the binding assay; * marks the position of the full length GST-fusion protein.

2.1.2. High-risk E6 is able to complement the Ad E1a transformation deficient mutant

Having confirmed that the interaction between E6 and p300 is common to both high-and low-risk HPV E6 proteins, we next focused our attention on investigating the potential biological consequences of this association. To do this, we used an amino terminal deletion mutant of Adenovirus E1a (ΔN 2-36). This mutant had previously been shown to be defective both in its ability to bind p300 and to cooperate with EJ-ras in the transformation of primary baby rat kidney cells – BRKs (STEIN *et al.* 1990; WHYTE *et al.* 1988). We reasoned that if the E6-p300 interaction was important for the ability of E6 to contribute towards cell transformation then E6 should be able to complement the p300 binding- and transformation-deficient mutant of Ad E1a in a BRK transformation assay. To examine this possibility, primary BRK cells were obtained from 9-day old Wistar rats, and co-transfected with the Ad E1a mutant together with different combinations of the E6 expression vectors, in the presence of EJ-ras as a cooperating, activated oncogene. After 2 weeks under G418 selection, the cells were fixed, stained and the colonies were counted. The results obtained from two typical assays are shown in Figure 13A together with the results from a series of independent assays in Figure 13B. It is apparent from Figure 13 that the ΔN 2-36 E1a mutant, when compared with wt E1a, shows greatly reduced ability to cooperate with EJ-ras, and this is consistent with previous reports (STEIN *et al.* 1990; WHYTE *et al.* 1988). Similarly, HPV-16E6 and HPV-18E6 show little co-transforming activity in the primary BRK cell transformation assays (Table 6, Figure 13B), also in agreement with previous studies (STOREY *et al.* 1988). Nevertheless, it is clear

A.

| +ΔNE1a | +ΔNE1a
+16E6 | +ΔNE1a
+Δ123-7.16E6 | +ΔNE1a | +ΔNE1a
+11E6 | +ΔNE1a
+18E6 |

B.

G418 Colony Number

Ras	+	+	+	+	+	+	+	+	+	+	+
E1a	-	+	-	-	-	-	-	-	-	-	-
ΔN	-	-	+	+	+	+	+	+	-	-	-

| | | 11E6 | 16E6 | 18E6 | Δ123-7.
16E6 | ΔA.
18E6 | 11E6 | 16E6 | 18E6 |
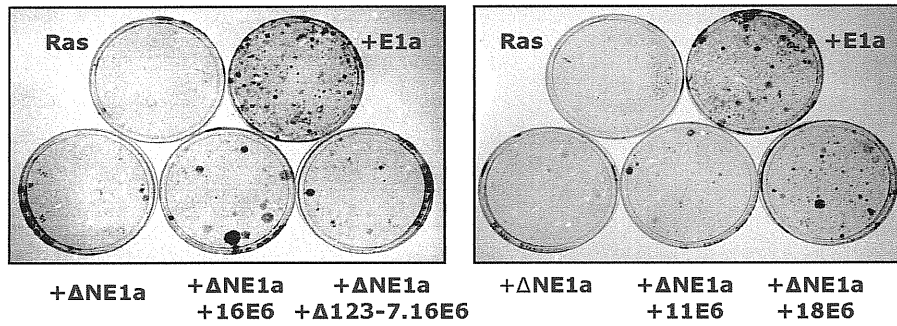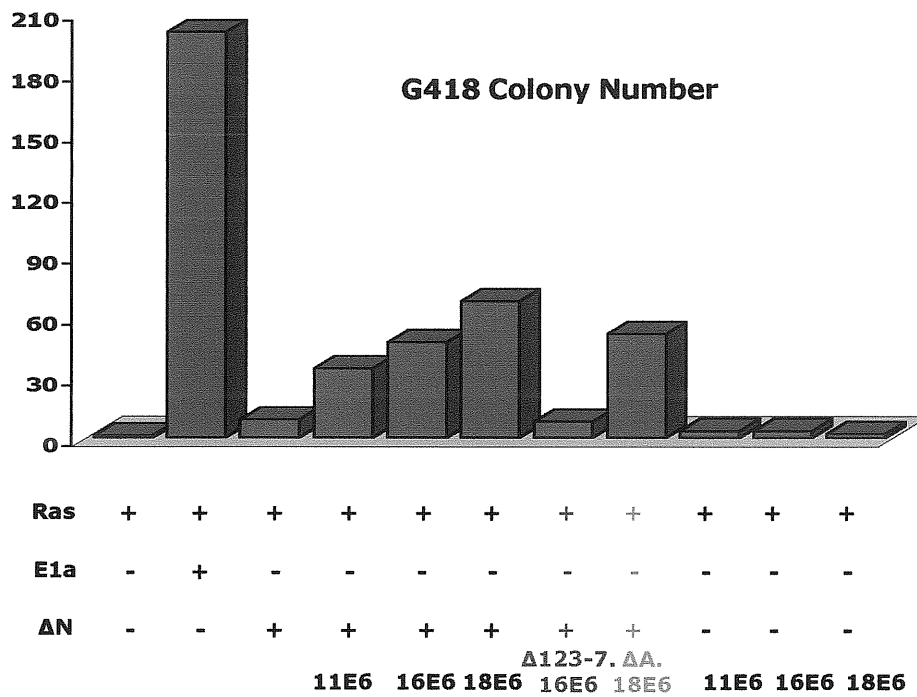
Figure 13.

Panel A. Focus formation assay in BRK cells. Representative plates from two separate transformation assays co-transfected with the indicated expression plasmids are shown.
Panel B. Graph representing the mean number of G418 resistant colonies from 3 to 6 independent BRK transformation assays. The co-transfected vectors are indicated. The mutants used are Ad E1a ΔN (Δ2-36), HPV-16E6.Δ123-7 and HPV-18E6.ΔA (Δ47-49).

that HPV-16E6 is able to complement the defect in ΔN 2-36 Ad E1a protein

when both proteins are co-expressed. In addition, the mutant of HPV-16E6

(Δ123-127), defective in its ability to interact with p300 (PATEL *et al.* 1999) is

likewise defective in its ability to complement the transformation deficient E1a

mutant, suggesting that the capacity of E6 to bind p300 is required for

efficient complementation of the E1a mutant. We also investigated the ability

of the HPV-18E6 and HPV-11E6 proteins to rescue the E1a mutant. The results

obtained are shown in Figure 13 and Table 6. It is clear that HPV-18E6

efficiently complements the ΔN 2-36 mutant of Ad E1a. In contrast the ability

of HPV-11E6 to complement this mutant is significantly reduced, with fewer,

smaller, and slower growing colonies being obtained.

To exclude any indirect effects due to inactivation of p53 in these assays, we

also included a mutant of HPV-18E6 (ΔA, 47-49aa), which we had previously

shown to be defective in its ability to target p53 for degradation (PIM *et al.*

1994). As can be seen from the results presented in Figure 14A, this mutant

binds to p300 as efficiently as the wild type HPV-18E6 protein and easily

complements the Ad E1a mutant (Table 6, Figure 13B). This suggests that the

ability of E6 to complement a p300 binding- and transformation- defective

mutant of E1a is unrelated to its capacity to target p53 for degradation.

2.1.3. The interaction between p300 and E6 is conserved among different E6s but not all E6s complement the Ad E1a transformation deficient mutant

Having found that both HPV-11E6 and HPV-18E6 could interact with p300, we wanted to investigate whether this interaction is common for E6 proteins from different HPV types. In particular we wished to determine if this interaction is found in cutaneous HPV derived E6 proteins, since to date, very few targets of mucosal HPV E6 proteins have been found to be shared with cutaneous E6 proteins.

In order to investigate this further we performed *in vitro* pull down assays, using high- and low-risk mucosal E6 proteins (18 and 6, respectively), high- and low-risk cutaneous (1 and 8) and also high-risk cutaneous animal PV: BPV-1 and CRPV E6s. These were *in vitro* translated and radiolabelled and then incubated with the GST p300 fusion proteins (fragment 1 and the HAT domain fragment spanning CH2 and CH3: aa 1185-1810). GST-alone was used as a negative control. The results from a representative binding assay are shown in Figure 14A. It can be seen that all the E6 proteins analysed show significant levels of interaction with the two fragments of p300, indicating that it is a highly conserved feature of the PV E6 proteins.

Having found that both mucosal and cutaneous derived HPV E6 proteins can interact with p300, we then examined the ability of these E6s to complement the E1a mutant defective for p300 binding and transformation in the BRK focus formation assays. The results obtained are summarized in Table 6 and presented as a graph in the Figure 15. As can be seen, when compared with the ability of the high-risk mucosal E6 to complement the Ad E1a ΔN mutant
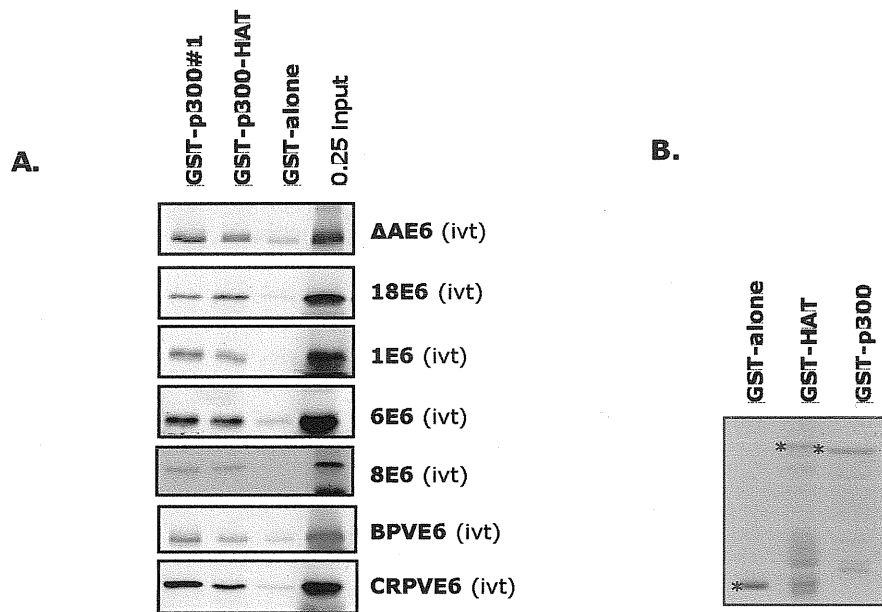
Figure 14. Interaction between different E6s and p300.

Panel A. High- and low-risk mucosal E6 proteins (18 and 6, respectively), high- and low-risk cutaneous (1 and 8) and also high-risk cutaneous animal PV: BPV and CRPV E6s interact with GST-p300 fragment 1 and GST-HAT (spanning over CH2 and CH3 domain). ΔA (Δ47-49).18E6 mutant defective in p53 degradation, shows also significant level of interaction with p300 fragments.
Panel B. Coomassie stain of the GST-fusion proteins represents protein input;
* marks the position of the full length GST-fusion protein.
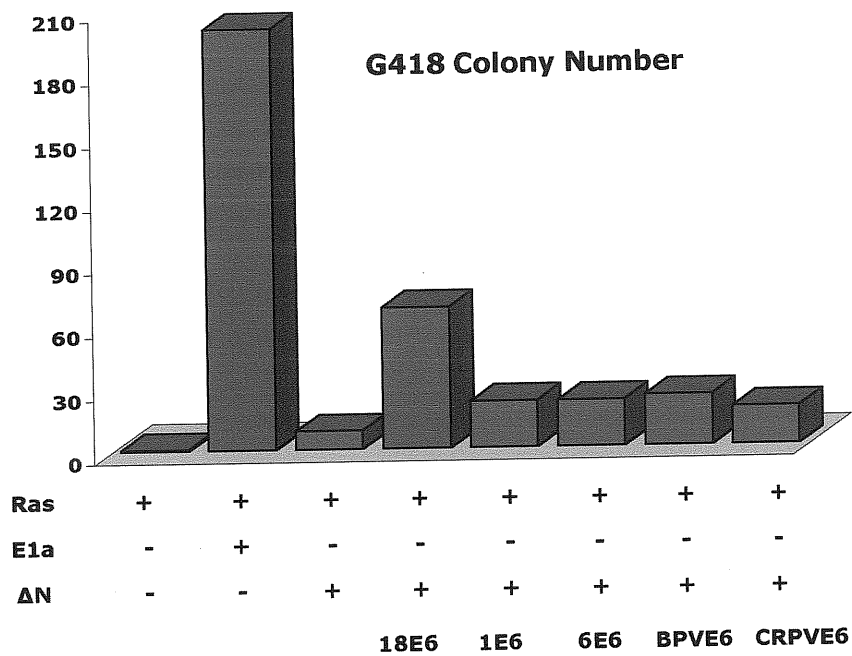
Figure 15.
Graph representing the mean number of G418 resistant colonies from 3 to 6 independent BRK transformation assays. The co-transfected expression vectors are indicated.

other E6s show only a much weaker capacity to rescue the Ad E1a transformation deficient mutant in BRK focus formation assays. However, in all cases the complementation observed is greater than this seen with the mutant of HPV-16E6 defective in p300 (Δ123-7), suggesting that the interaction between PV E6 proteins and p300 is relevant in a biological context as defined by BRK cell transformation assay.

2.2. HPV E7 interaction with p300

A noticeable feature of the Ad E1a and the HPV E7 proteins are the remarkable parallels in both their structure and biological activities. This is reflected both in their common cellular targets (CHELLAPPAN *et al.* 1992) as well in their ability to induce cell cycle progression and induce cell transformation (PHELPS *et al.* 1988; STOREY *et al.* 1988). One of the key cellular targets described for E1a are the p300/CBP transcriptional co-activators. Until now, however, such an interaction had not been demonstrated for the HPV E7 protein and a series of experiments were therefore initiated in order to investigate this.

2.2.1. HPV-16E7 interacts with p300 *in vitro*

In order to investigate whether E7 could interact with p300, several overlapping fragments of p300 were expressed as GST fusion proteins (Figure 16A) and purified (Figure 16C). These recombinant proteins were then incubated with *in vitro* translated, radiolabelled HPV-16E7. The complexes were then washed, separated by SDS-PAGE and analyzed by autoradiography. As can be seen in Figure 16B, the HPV-16E7 protein binds strongly to GST-p300 fragment 1, which encompasses the CH1 domain of p300. Weaker interaction was observed with the CH2 and CH3 domains, while no significant binding was seen with fragments of p300 spanning residues 672-1193 and 1893-2414. In order to investigate whether the ability of E7 to bind to p300 is specific to the high-risk types, we repeated the binding assays using low-risk HPV-11E7. The results obtained are also shown in the Figure 16B and demonstrate a similar pattern of interaction, with strong binding to the CH1 domain of p300 and weaker interaction with the CH2 and CH3 domains.

A.

KIX (568-646)

TAZ (336-430)

	CH1	KIX			BROMO	CH2		CH3			Q

1 (1-672)

2 (672-1193)

(1069-1459) **3**

(1459-1892) **4**

(1893-2414) **5**

B.

GST-p300 fragments

					GST-alone	0.25 Input	
1	2	3	4	5			

16E7 (ivt)

19	1.5	4.3	5.4	2.5	1.3	% of input bound

11E7 (ivt)

20	2.4	2.8	2.1	1.8	1.1	% of input bound

C.
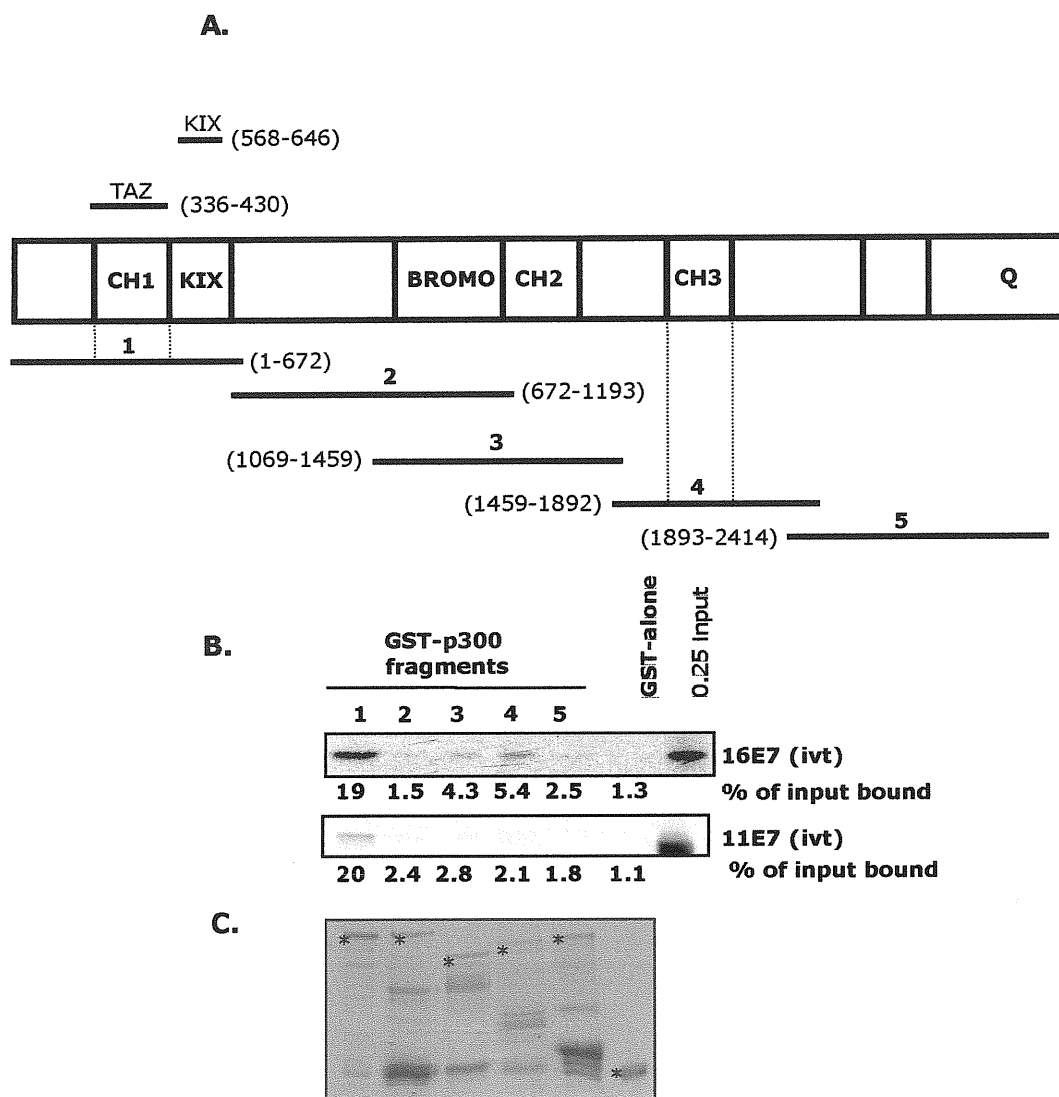
Figure 16. HPV E7 interacts with p300 *in vitro.*
Panel A. Schematic representation of the GST-p300 fusion clones used for *in vitro* binding assays. The most conserved domains of p300 are indicated (CH1-3: cysteine-histidine rich motifs, TAZ: zinc finger domain composed of CH1 region, Q: glutamine-rich domain, HAT: functional histone acetyltransferase domain, Bromo: bromodomain, KIX: kinase induced domain).
Panel B. Interaction between the HPV-16E7 and HPV-11E7 oncoproteins with p300. The major site of interaction on p300 lies within fragment 1, which encompasses the CH1 domain; weak interaction is seen with domains encompassing CH2 and CH3. The percentage of *in vitro* translated E7 protein bound to the different fusion proteins is also indicated.
Panel C. Corresponding GST-fusion protein inputs. The position of the full length GST-fusion protein fragment is marked by *.

Therefore this implies that the interaction between HPV E7 and p300 is common to low- and high-risk mucosal HPV types.

To map more accurately the site of HPV E7 interaction on p300, the assay was repeated using the TAZ (residues 336-430) and the KIX (residues 568-646) domains of p300 fused to GST. These proteins were expressed and purified as described before. The results obtained from a typical binding assay can be seen in Figure 17A. No interaction was found between HPV-16E7 and the KIX domain, whilst strong binding to the TAZ (CH1 region) domain was observed. In addition, the interaction observed between E7 and TAZ was comparable with that seen for the entire fragment 1 (1-672), thereby demonstrating that the major E7 binding site on p300 lies within residues 336-430, encompassing the CH1 domain.

In order to further verify the specificity of the interaction between E7 and p300, the GST pull-down assay was performed using full-length GST-11E7 and GST-16E7 and binding with *in vitro* translated, radiolabelled full-length p300. GST-TBP was used as positive control. As can be seen from Figure 17B, the high-risk 16E7 interacts with p300 with an affinity comparable to the interaction between p300 and TBP. Interestingly, the low-risk HPV-11E7 protein is also able to bind to p300, although the interaction is weaker than that of the high-risk 16E7.

A.   **B.**

16E7 (ivt)

Input 0.25 | GST-alone | GST-KIX | GST-TAZ | GST-p300 #1

GST-11E7 | GST-16E7 | GST-TBP | GST-alone | 0.25 Input

p300 (ivt)

3 5 18 19

2.5 4.3 5 1   % of input bound

C.   **D.**
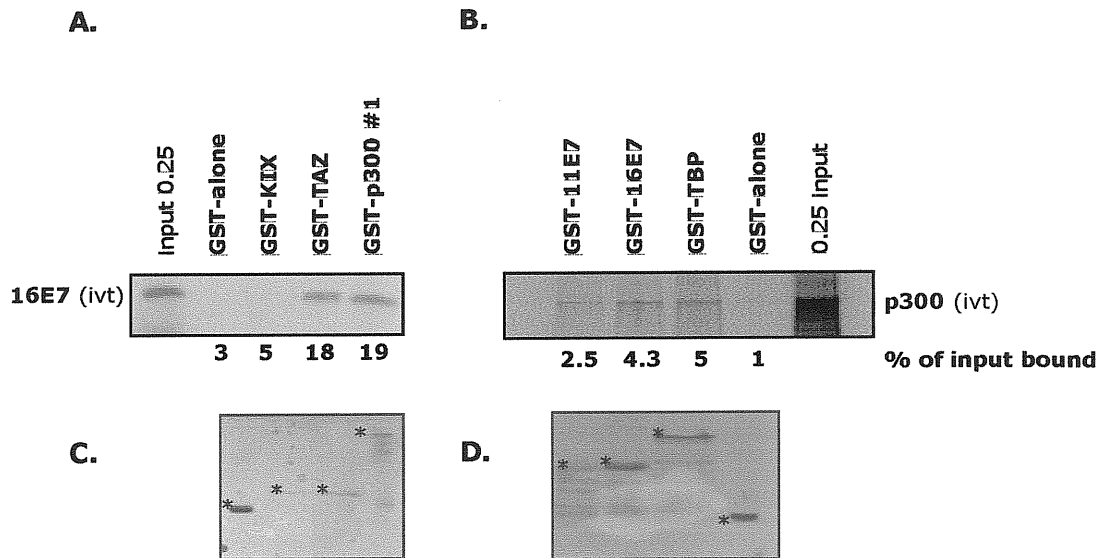
Figure 17.

Panel A. Fine mapping of the 16E7 interaction site on p300. *In vitro* translated E7 protein was incubated with the different GST fusion proteins and binding assessed following extensive washing, PAGE and autoradiography. The major binding site lies within the TAZ domain, which encompasses the CH1 domain and spans residues 336-430. The percentage of *in vitro* translated 16E7 bound to the protein is also indicated.

Panel B. Interaction between the low-risk 11E7 and high-risk 16E7 (produced as GST fusion proteins) and full length *in vitro* translated, radiolabelled p300. GST-TBP was used as positive control. The percentage of *in vitro* translated p300 bound to the protein is also shown.

Panel C, D. The bottom panels show the inputs of the GST-fusion proteins used in the two binding assays. The percentage of *in vitro* translated 16E7 and p300 bound to the protein is also indicated.

2.2.2. Mapping the binding site of 16E7 involved in the interaction with p300

To determine which residues of HPV-16E7 are required for complex formation with p300, we repeated the binding assays with a number of previously described (BANKS *et al.* 1990; EDMONDS and VOUSDEN 1989; MASSIMI *et al.* 1997) mutants of HPV-16E7 (Figure 18). These mutants lie within known functional domains of the E7 protein including the N-terminus (p2), CD2 (p24 and pCKII) and CD3 (Δ1-Δ4). Since the principal binding site of E7 on p300 lies within the CH1 domain, we used GST-p300 fragment 1, and for comparison, GST-p300 fragment 2 and GST-alone as negative controls. The different E7 mutants were *in vitro* translated and radiolabelled and the binding assays were performed with the purified GST-fusion proteins as already described. A series of typical results are shown in Figure 19A. As can be seen, all the deletion mutants (Δ1:52-55aa, Δ2:65-67aa, Δ3:75-77aa, Δ4:79-83aa) within the C-terminal half of HPV-16E7 retained the ability to bind to p300 at levels comparable with the wild type E7 protein. In contrast, mutants within the amino terminal domain of E7: p24 (C24G: deficient in pRb binding), pCKII (S31R, S32P: deficient in CKII phosphorylation), as well as p2 (H2P: within N-terminus) showed greatly reduced binding to p300. In order to try to completely abolish the binding between E7 and p300, we generated the double mutant p24-CKII, but as can be seen from the binding assays, this mutant retained levels of the binding similar to those observed for the p24 and pCKII mutants by themselves. These results demonstrate that the entire region of 16E7 encompassing the N-terminal half of E7 (aa 1-32) is required for the interaction with p300. Interestingly, all the N-terminal mutants used in these
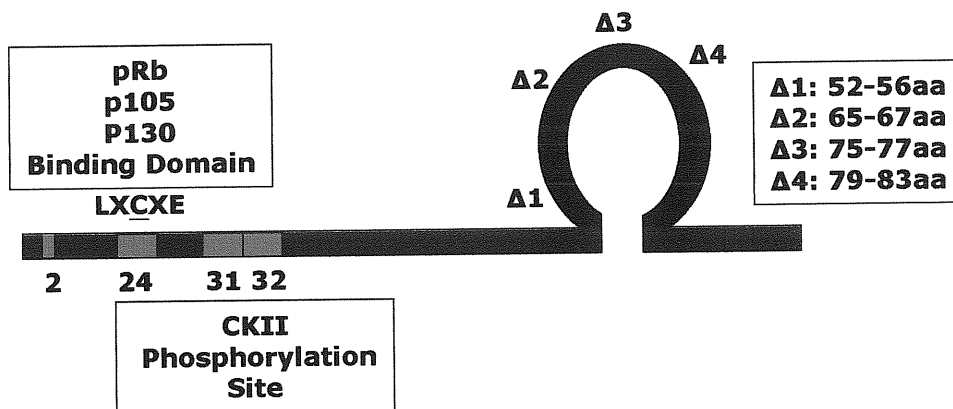
Figure 18.
Schematic representation of the HPV-16E7 protein together with the location of mutants used for the *in vitro* pull-down assays; p24 (C24G) - mutant deficient in pRb binding; pCKII (S31R, S32P) - mutant deficient in CKII phosphorylation; C-terminal deletion mutants - Δ1:52-55aa, Δ2:65-67aa, Δ3:75-77aa, Δ4:79-83aa, p24-CKII (double mutant C24G, S31R, S32P) and p2 (H2P).
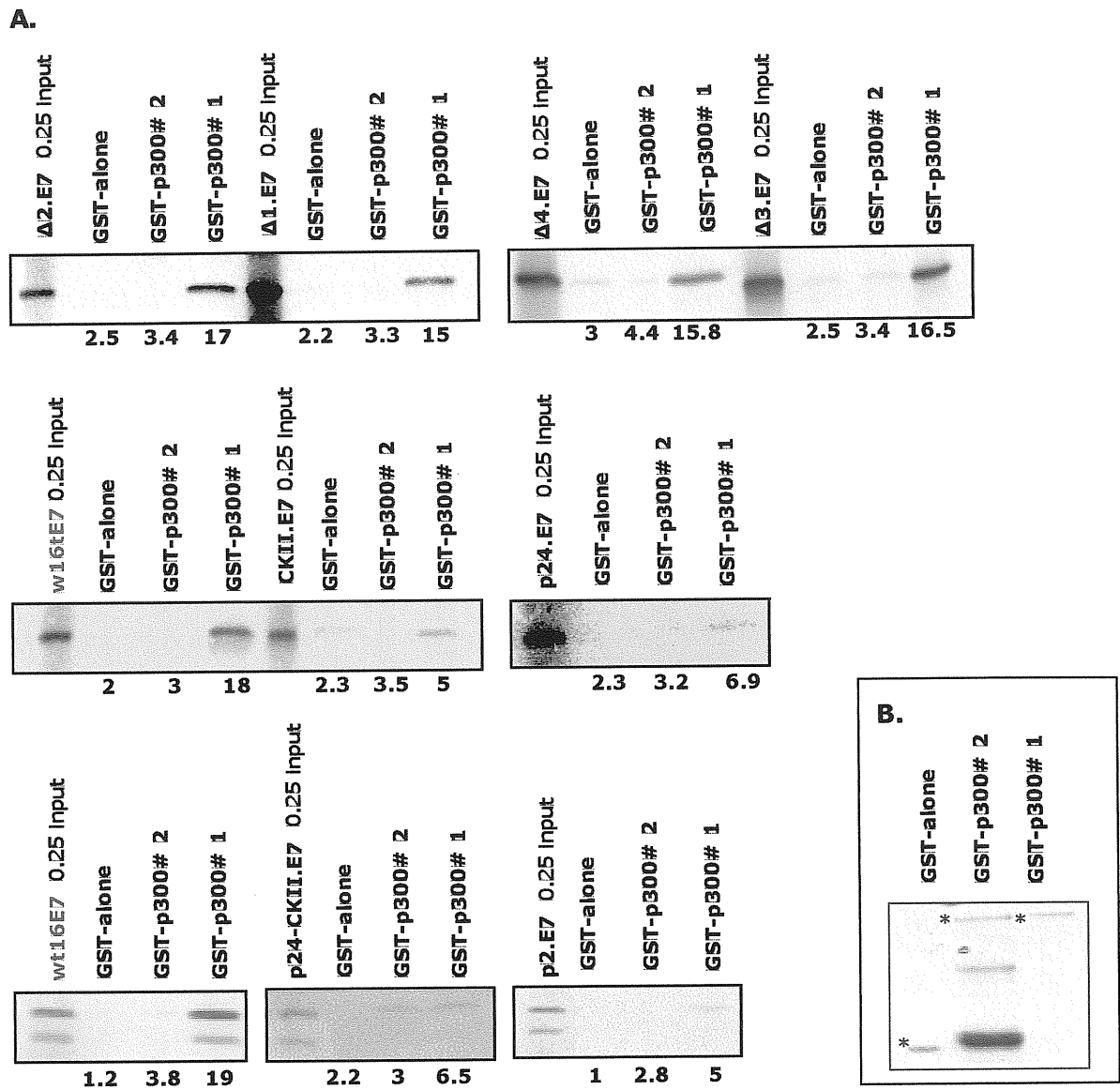
Figure 19.
Panel A. Interaction of the HPV-16E7 mutants (indicated in Figure 18) and the wild type 16E7 with GST-p300 fragments #1 and #2. GST-alone was used as a negative control. The numbers below each panel show the percentage of input protein bound on the beads.
Panel B. Coomassie stain for the GST-fusion protein inputs used in the binding assays; * marks the position of the full length GST-fusion protein fragment.

assays: p2, p24 and pCKII, have been shown to be defective in cell transformation assays (BARBOSA *et al.* 1990; EDMONDS and VOUSDEN 1989) demonstrating that the ability of E7 to bind p300 correlates with its transforming activity.

2.2.3. The interaction between 16E7 and p300 is direct

Since E7 has previously been shown to interact with a variety of cellular proteins, including TBP and P/CAF (AVVAKUMOV *et al.* 2003, Mazzarelli, 1995 #546; HUANG and MCCANCE 2002; MASSIMI *et al.* 1996) all of which can also interact with p300 (ABRAHAM *et al.* 1993; YANG *et al.* 1996), it was obviously possible that the interaction between E7 and p300 was indirect. To investigate this possibility we repeated the pull-down assays using purified His-tagged HPV-16E7 and purified GST-p300 fusion fragments. The bound E7 protein was then analyzed by western blotting with an anti-HPV-16E7 antibody. As can be seen from the Figure 20, 16E7 shows a very strong interaction with GST-TBP, but no interaction is detected with GST-alone. Interestingly, 16E7 binds to the p300 fragment 1, with an efficiency similar to that seen with TBP. In addition, weaker interaction is also observed between 16E7 and p300 fragments 3 and 4, whilst there is no binding of E7 to p300 fragments 2 and 5. This pattern of interaction is very similar to that observed using *in vitro* translated E7 and further demonstrates the specificity of the interaction. In addition these results show clearly that the interaction between 16E7 and p300 is direct and that the main binding site of 16E7 on p300 lies within its CH1 domain. There are also weaker E7 binding sites within fragments 3 and 4, which overlap with the CH2

A.

GST-p300
fragments

GST-alone | GST-TBP | 0.5 input

| 1 | 2 | 3 | 4 | 5 |

16E7

32 0.4 10.3 7.3 0.3 0.8 29   **% of protein bound**

WB α-16E7

B.

◄—His₆-16E7
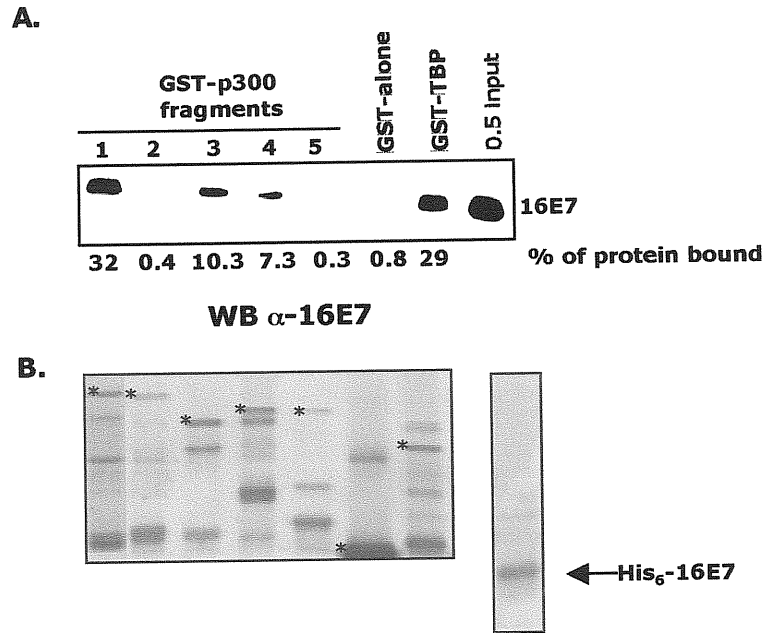
Figure 20. The interaction between HPV-16E7 and p300 is direct.
Panel A. Bacterially derived 6-His tagged 16E7 was incubated with the GST-p300 fusion protein fragments. The bound complexes were removed from glutathione beads, separated on 15% SDS-polyacrylamide gel and the presence of bound 16E7 was detected with anti-16E7 antibody (Santa Cruz, ED17). GST-TBP and GST-alone were used as positive and negative controls, respectively. The percentage of protein bound is indicated as determined by densitometric scanning (UltroScan XL, Pharmacia LKB).
Panel B. Coomassie stain of the GST-fusion proteins and the purified his-tagged 16E7 protein used for experiment.

and CH3 domains, and most importantly these fragments encompass the HAT domain of p300.

This pattern of multiple sites of interaction on p300 suggest that E7 may be able to moderate p300 activities by more that one mechanism.

2.2.4. E7 interacts with p300 *in vivo*

The above studies demonstrate a strong direct interaction between HPV-16E7 and p300 *in vitro*. To confirm that this was a physiologically relevant interaction, a series of co-immunoprecipitation assays were performed from cells expressing HPV-16E7 and p300. In the first series of experiments we used an HA-tagged HPV-16E7, which was transiently expressed in U2-OS cells. 24 hours after transfection cells were harvested, and cellular extracts were used for co-immunoprecipitation experiments with an anti-p300 antibody (NM11 Pharmingen), which were followed by WB (with anti-16E7 antibody, ED17 Santa Cruz) analysis in order to detect 16E7 within the co-immunoprecipitates. In all the experiments where WB was followed by E7 detection using anti-16E7 antibody, the antibodies were previously crosslinked to protein A or protein G –sepharose resin in order to avoid the interference of immunoglobulin light chains which would be expected to migrate just above the E7 protein on SDS-PAGE. As can be seen from Figure 21A a significant amount of E7 protein co-precipitates with the immunoprecipitated p300 protein. The co-immunoprecipitation assay was then repeated on cells expressing endogenous E7 protein. For this we used transformed BRK cells expressing EJ-ras and 16E7 and cervical tumour derived CaSki cells, containing HPV-16 DNA sequences. The co-immunoprecipitation was done

using either anti-E7 (ED17 Santa Cruz) or anti-p300 antibodies (NM11 Pharmingen and Santa Cruz C-20), and the immune complexes were subsequently analyzed by western blotting. Using the E7-transformed BRK cells as a source of E7 protein, it is clear that the anti-p300 antibody co-immunoprecipitates a protein of approximately 18-20kDa that co-migrates with the *in vitro* translated E7 protein. As can be seen, the species of E7 co-immunopreciptating with p300 migrates just below that immunoprecipitated from the cells with the anti-E7 antibody (Figure 21B). Likewise, similar results were obtained when the experiment was performed in CaSki cells (Figure 21C). Two different anti-p300 antibodies, NM11 (Pharmingen) and C20 (Santa Cruz) co-immunoprecipitated HPV-16E7 from the CaSki cell extracts, although the C20 antibody appeared to be much less efficient than the NM11 antibody (Figure 21C). In order to verify that E7 did indeed co-immunoprecipitate with p300, the reverse experiment was then performed. Extracts of CaSki cells were immunoprecipitated with an anti-E7 polyclonal antibody (MASSIMI *et al.* 1996) and the presence of p300 in co-immunoprecipitates was detected by WB analysis with an anti-p300 antibody (NM11 Pharmingen). The results obtained are shown in Figure 20D and as can be seen the anti-E7 polyclonal antibody was able to co-immunoprecipitate a protein of approximately 280-300kDa which was recognized by the anti-p300 antibody in western blot analysis (Figure 21D). These results suggest that the HPV-16E7 protein can complex with p300 *in vivo*, either when over-expressed in a transient transfection or when the endogenous proteins are present.

As a final verification that HPV-16E7 can interact with p300 *in vivo*, a series of mammalian two-hybrid assays were also performed (done by Nikita
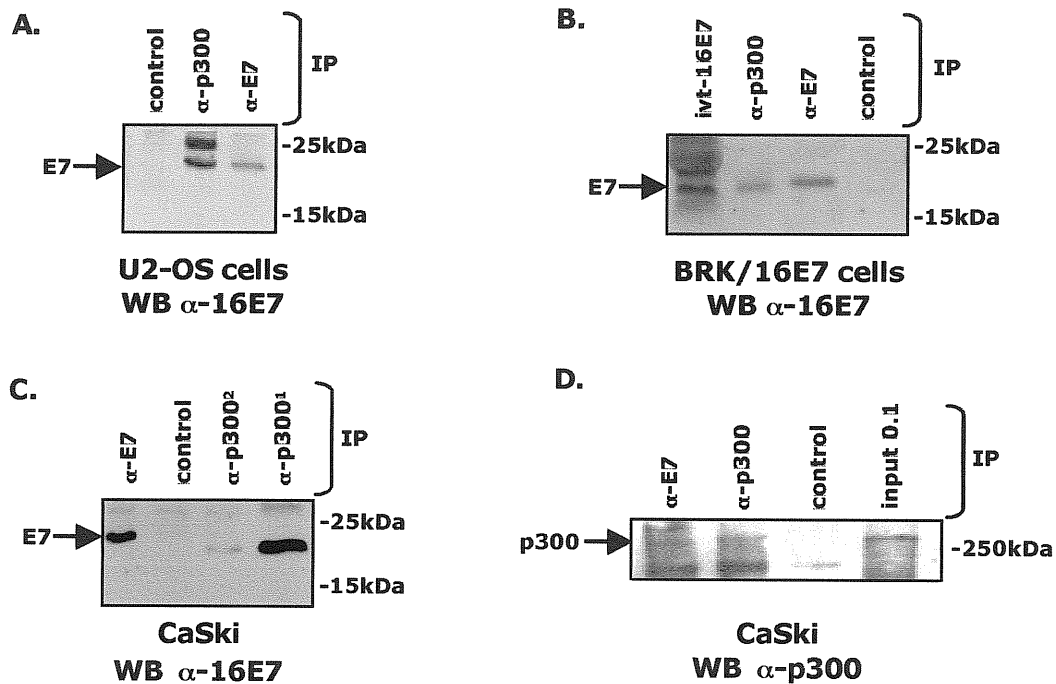
Figure 21. HPV-16E7 interacts with p300 *in vivo*.

Panel A. An anti-p300 antibody (NM11) co-immunoprecipitates HPV-16E7 from whole U2-OS cell extracts transfected with HA-tagged HPV-16E7. Anti-16E7 (Santa Cruz ED17) and unrelated antibody were used as positive and negative controls, respectively. Immunoprecipitates were separated on 15% SDS-polyacrylamide gel and the presence of 16E7 was detected by western blotting with anti-16E7 antibody.

Panel B. An anti-p300 antibody co-immunoprecipitates endogenous HPV-16E7 from a BRK cell line transformed with E7 and EJ-ras. Whole cell extracts from the BRK cell line expressing 16E7 were immunoprecipitated with anti-p300 antibody (NM11 Pharmingen), anti-16E7 (Santa Cruz ED17) or an unrelated antibody (control). Immunoprecipitates were separated on 15% SDS-polyacrylamide gel followed by western blotting with anti-16E7 antibody. *In vitro* translated (ivt) 16E7 was included as a control.

Panel C. Two different anti-p300 antibodies (#1: NM11 Pharmingen and #2: C-20 Santa Cruz) co-immunoprecipitate endogenous E7 from CaSki cells (HPV-16 positive). Anti-16E7 antibody (Santa Cruz ED17) and unrelated antibody were used for immunoprecipitation as positive and negative controls, respectively.

Panel D. Anti-16E7 polyclonal antibody was able to co-immunoprecipitate a protein of approximately 280-300 KDa from CaSki cells expressing endogenous E7 and p300, which was recognized in Western Blot by anti-p300 antibody (NM11 Pharmingen). Unrelated antibody was used for immunoprecipitation as a negative control, input of cellular extract is also shown.

Avvakumov, Departments of Microbiology and Immunology, Oncology and Physiology and Pharmacology, The University of Western Ontario, London Regional Cancer Centre, London, Ontario, Canada). HPV-16E7 was fused to the GAL4 DNA binding domain and co-transfected into CV-1 cells with constructs expressing the CH3 domains of p300 or CBP fused to the VP16 activation domain. After 48 hours the cells were harvested and luciferase activity measured. As can be seen from the results in Figure 22A, E7 interacts specifically with the CH3 domain of p300 and also with the equivalent region of CBP. No interaction was observed with the region of CBP spanning residues 1892-2441. In addition, HPV-16E7 was also found to bind to a fragment spanning residues 300-695, which encompasses the KIX domain of CBP (Figure 22B). This result is particularly interesting since this is the equivalent domain which was found to be bound by E7 on p300 in the *in vitro* binding assays. Taken together these results are in agreement with the mapping studies performed *in vitro* and demonstrate that E7 and p300/CBP are able to form a complex *in vivo*.

2.2.5. E7 and p300 co-localize

Although p300 is a nuclear protein, HPV E7 has been described to reside in several different cellular compartments. Thus, our next goal was to determine whether E7 and p300 localize within the same cellular compartments. To do this U2-OS cells were either transfected with HA-tagged HPV-16E7 alone or with HA-tagged E7 and pCMVβ.p300. 12 hours after transfection cells were fixed with PFA and permeabilized with 1% Triton. Endogenous and overexpressed p300 was visualized using an anti-p300 monoclonal antibody

A.

Relative luciferase activity

1.0	3.7	26.4	2.2	30.3	27.6

CV-1

| E7 | - | + | + | + | + | + |

| prey | - | - | CBP$_{1679-1891}$ | | p300$_{1573-1825}$ | |
| | | | CBP$_{1892-2441}$ | | p300$_{1639-1855}$ | |

B.

Relative luciferase activity

1.0	3.0	1.8	14.3	12.5	14.4

CV-1

| E7 | - | + | + | + | + | + |

| prey | - | - | CBP$_{1-300}$ | | CBP$_{200-695}$ | |
| | | | CBP$_{100-695}$ | | CBP$_{300-695}$ | |
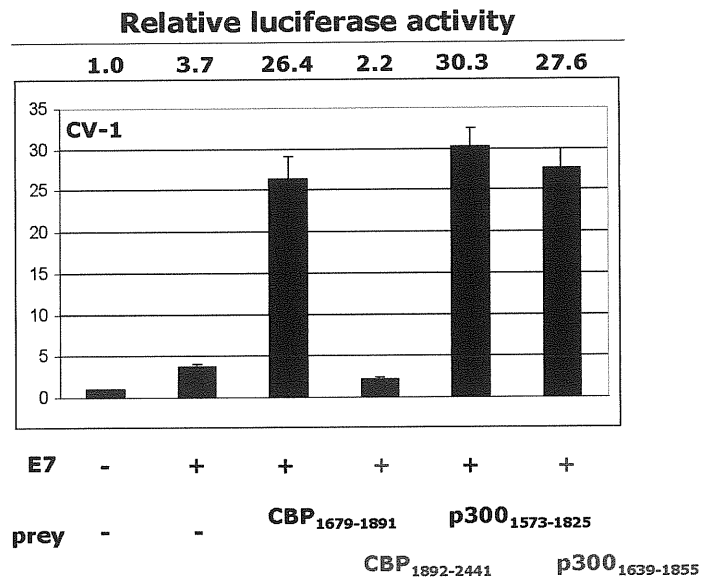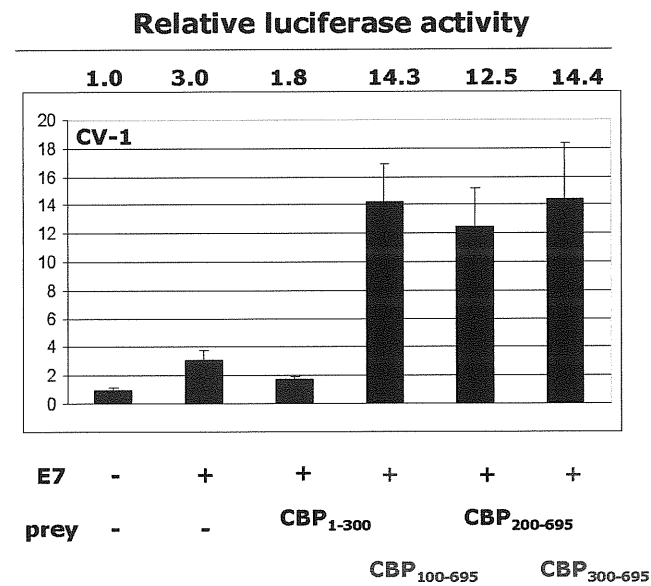
Figure 22. Mammalian two hybrid tests.

HPV-16E7 interacts with the CH3 domains of p300 and CBP (**Panel A**) and with the CH1 of CBP (**Panel B**) in mammalian two hybrid test. CV-1 cells were co-transfected with the (gal)6-luc reporter plasmid,together with vectors expressing HPV-16E7 fused to the Gal4 DBD and vectors expressing the indicated portions of p300 or CBP fused to the VP16 transcriptional activation domain. 48h post-transfection cells were harvested and assayed for luciferase activity. The results show the mean of three independent experiments and standard deviations are also shown.

(NM11 Pharmingen) while HA-tagged E7 was visualized using a rabbit anti-HA antibody (Santa Cruz Y-11). These were followed by secondary antibodies (rhodamine conjugated goat anti-rabbit and fluorescein conjugated goat anti-mouse) and proteins visualized using confocal microcopy. The results obtained are presented in Figure 23. As can be seen, endogenous p300 in U2-OS cells is present in a meshwork pattern throughout the whole nucleoplasm, overlaid with several brighter foci of variable size with dot-like structures (Figure 23A). This pattern of expression of p300 is in agreement with the previous studies (VON MIKECZ *et al.* 2000) and was even more evident when p300 was overexpressed (Figure 23B) from the pCMVβ.p300 expression plasmid. In agreement with previous studies (GUCCIONE *et al.* 2002; SMITH-MCCUNE *et al.* 1999) the HPV-16E7 protein shows also a predominantly nuclear pattern of expression with nucleolar exclusion (Figure 23A and 23B). The merged images show multiple regions and dot-like structures of E7 and p300 co-localization in the case of endogenous p300 (Figure 23A), and a similar pattern of co-localization is also seen when both proteins are overexpressed (Figure 23B). Since we had shown that low-risk 11E7 could bind p300 *in vitro*, but had no evidence of this association *in vivo*, we repeated the experiments using HA-tagged HPV-11E7. As can be seen from Figure 22C, over-expression of both HA-tagged HPV-11E7 and p300, showed that low-risk 11E7 also co-localizes with p300 (Figure 23C). HA-11E7 shows distinct regions of concentration within the nucleus, with the presence of clear dot-like structures. Very similar patterns of expression have been observed for proteins that localize to PML oncogenic domains (PODs) (STERNSDORF *et al.* 1997), including p300/CBP. In the presence of HPV-11E7, p300 appears to be accumulated within dot-like
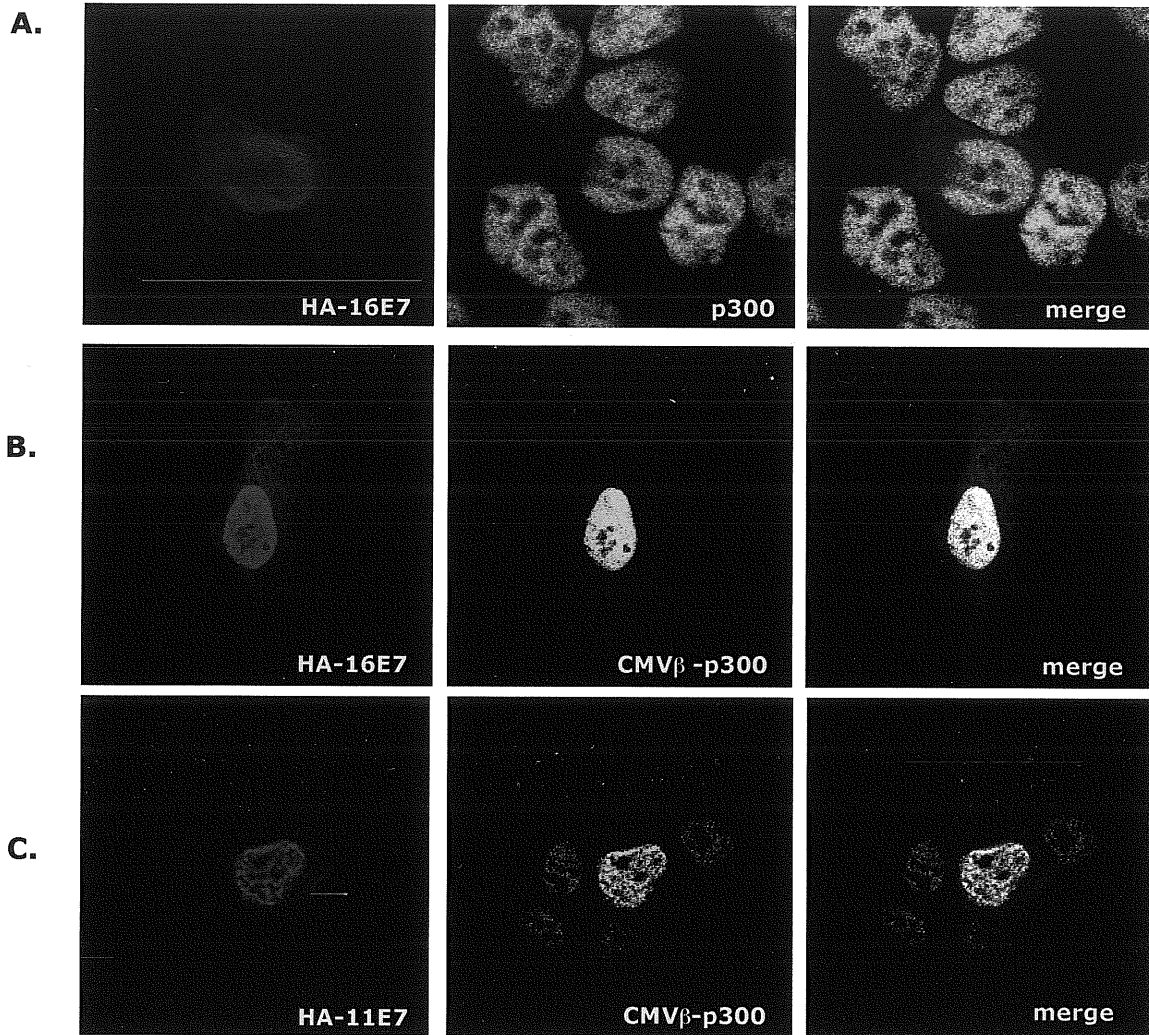
Figure 23. HPV-E7 and p300 co-localize in the nucleus of U2-Os cells.

Panel A. HA-tagged 16E7 (red) transiently transfected into U2-OS cells co-localizes with the endogenous p300 (green). Cells were fixed in 3% PFA, permeabilized with Triton X-100 and stained with anti-HA antibody (Santa Cruz Y-11) and anti-p300 antibody (NM11 Pharmingen). **Panel B.** U2-OS cells were transiently transfected with HA-tagged 16E7 and CMVβ.p300. Cells were fixed in 3% PFA, permeabilized with Triton X-100 and stained with anti-HA (Santa Cruz Y-11) antibody and anti-p300 antibody (NM11 Pharmingen). **Panel C.** U2-OS cells were transiently transfected with HA-tagged 11E7 and CMVβ.p300. Cells were fixed in 3% PFA, permeabilized with Triton X-100 and stained with anti-HA (Santa Cruz Y-11) antibody and anti-p300 antibody (NM11 Pharmingen).

nuclear structures, suggesting that HPV-11E7 may indeed be able to recruit p300 to these nuclear domains.

In summary, these results demonstrate that E7 and p300 exist in the same cellular compartments and further support the *in vitro* and *in vivo* interaction studies. However there is a remarkable difference between 11E7 and 16E7 localization with respect to p300, since the cellular compartments where 11E7 and p300 co-reside, are not the same as for 16E7 and p300.

2.2.6. HPV-16E7 represses the transcriptional co-activation function of p300 upon E2

Since p300/CBP have been shown to be potent transcriptional co-activators, it is not surprising that this is the function that is targeted by many viral proteins, albeit by different strategies. These strategies include sequestration of p300/CBP from transcriptionaly active complexes, displacement of positive transcription factors by competitive binding and blocking p300 HAT activity.

Recent reports (LEE et al. 2000a; MARCELLO et al. 2000) have shown a strong synergistic transcriptional activity between the HPV E2 protein and p300/CBP. It has also been demonstrated that p300 and P/CAF form a co-activator complex with E2 to facilitate transcription (LEE et al. 2002). Additionally, it has also been demonstrated that the binding of E2 to p300 represents a rate-limiting step in the transcriptional transactivation for activation by E2 (MULLER et al. 2002). Since the p300-E2 synergy is highly relevant in the context of HPV infection, we sought to investigate what effect HPV-16E7 might have upon this activity of p300.

A.

Fold Conversion (CAT activity) 6xE2BSCAT

| 1.0 | 23.8 | 62.2 | 20.5 | 3.3 | 7.8 | 47.2 | 23.3 |

16E2	-	+	+	+	-	-	+	+
p300	-	-	+	-	+	-	+	+
16E7	-	-	-	+	-	+		

B. β-gal

C. 16E2
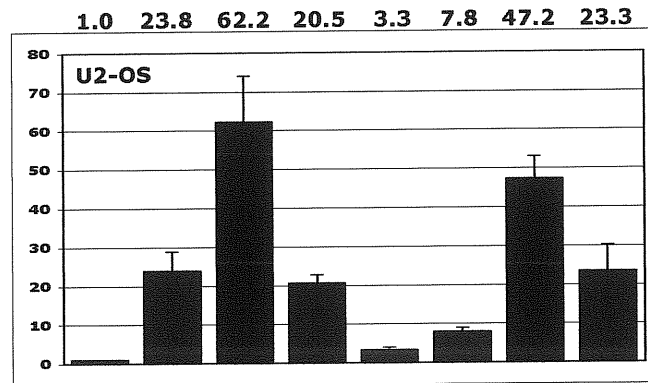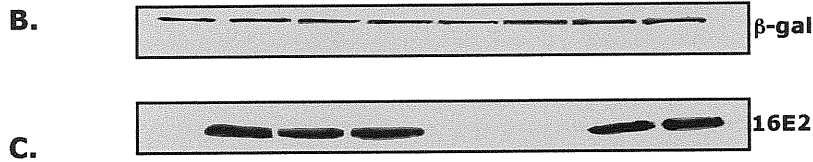
Figure 24. HPV-16E7 inhibits the p300 transcription co-activation function.

Panel A. The transcriptional co-activation function of p300 was measured in the context of the HPV-16E2 protein in transiently transfected U2-OS cells. To measure the transcriptional activity of E2, 1µg of 6xE2CAT, 0.5µg of pSV.β-gal (to standardize E2 activation values for transfection efficiency) along with 1µg of E2 (pJ4Ω), 5µg of CMV-β p300 and 1 or 5µg of 16E7 (pJ4Ω) were co-transfected. The total amount of DNA was equalized with empty plasmids. The mean values with standard deviations from three independent experiments are presented. An anti-β-gal WB was done as a control for transfection efficiency (**Panel B**). The same blots were also probed with an anti-E2 polyclonal antibody against HPV-16E2 in order to verify that changes in transcriptional activity were not due to the changes in the level of E2 expression (**Panel C**).

The transcription co-activation CAT reporter assays were performed as follows. U2-OS cells were transiently transfected with a CAT reporter construct containing six E2 binding sites, together with plasmids expressing E2, p300 and E7. After 48 hours cells were harvested and CAT assays were performed.

As can be seen in Figure 24, p300 strongly stimulates E2-dependent transcriptional activity, and this is in agreement with previous studies (LEE *et al.* 2000a; MARCELLO *et al.* 2000). In contrast HPV-16E7 alone showed only minimal effect upon E2 transcriptional activity, which is not surprising, since E2 associates with a large number of transcription factors and it is not clear what proportion of E2's transcriptional activity alone is dependent upon interaction with the endogenously expressed p300. However, the addition of 16E7 to the p300/E2 co-activator complex results in a dramatic reduction in the ability of p300 to co-activate E2-dependent transcription.

In order to verify that the levels of E2 were unaffected by the addition of E7, control western blots of the cellular lysates, were performed using a polyclonal anti-16E2 antibody (BOUVARD *et al.* 1994b), and as can be seen in Figure 24C, the levels of E2 remain constant in all transfectants. Likewise a western blot for β-gal (Figure 24B) shows the equal level of transfection efficiency between all transfectants.

Having shown that E7 could specifically antagonise the transcriptional co-activation function of p300, we next wished to investigate whether this was a consequence of E7 binding to p300. To do this we used the 16E7 p24 (C24G) mutant, which we had shown to be deficient in p300 binding. HPV-11E7 and Ad E1a were also included in the assay for comparison. The results are shown in Figure 25. As can be seen Ad E1a is a very potent inhibitor of p300 co-

Fold Conversion (CAT activity) 6xE2BSCAT

| 1.0 | 22.4 | 67.2 | 48.7 | 25.8 | 52.1 | 8.2 |

| E2	-	+	+	+	+	+	+
p300	-	-	+	+	+	+	+
	-	-	-	11E7	16E7	p24	E1a

β-gal
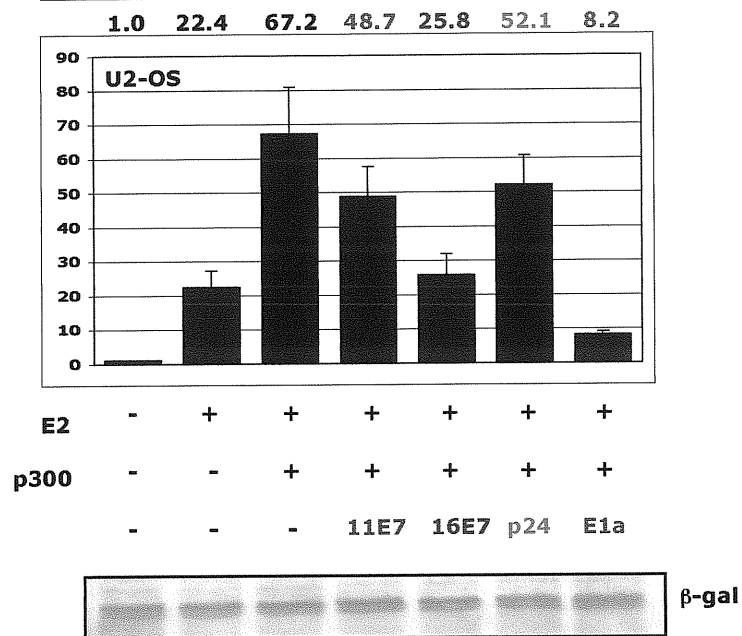
Figure 25.
Inhibition of p300 co-activation function by E7 and E1a.
The effect of low-risk HPV-11E7, Ad E1a and the p24 mutant (C24G) on p300-mediated E2-dependent transcription. 5μg of the indicated plasmids were co-transfected.
Anti-β-gal WB was done to control for transfection efficiency.

activation function and this is in agreement with previous studies (ARANY *et al.* 1995; SMITS *et al.* 1996). Likewise, the increase in E2-driven transcription mediated by p300 is strongly reduced in the presence of 16E7. The 16E7 p24 mutant showed a greatly reduced ability to inhibit the p300-mediated transcriptional co-activation function in this assay, suggesting that this activity of 16E7 is related to its ability to bind p300.

However, the results with 11E7 would suggest that it has a minimal effect upon p300-transcription co-activation function and this may be related to its apparent weaker degree of interaction.

2.2.7. 16E7 inhibits p300-mediated transcriptional activation of the p73 responsive pBax promoter

The above results demonstrate clearly that HPV-16E7 is able to repress the p300 transcriptional co-activation function in the context of E2-driven transcriptional activation. However, we were interested in determining whether this was a more general activity of E7. We decided to assess this using the p73 responsive promoter. This was done for two reasons. First, it has been demonstrated that the p73 N-terminal domain interacts with the CH1 domain of p300, which results in transcriptional co-activation of p73 responsive promoters and subsequent induction of apoptosis (ZENG *et al.* 2000). Moreover, since E7 also interacts with the same region of p300 as p73 does, p73 might be one of the targets of the E7:p300 interaction. Secondly, since HPV-16E7 was shown to be able to repress p53-mediated transcriptional activity (MASSIMI and BANKS 1997), we were interested in determining whether E7 may also exert a similar activity on the p53 family member, p73. For these

studies, we chose the p73α isoform, since this is the one which is predominantly expressed in differentiating keratinocytes (DE LAURENZI *et al.* 2000), the natural target cells of HPV *in vivo*. Since many of the p73-responsive promoters are also responsive to p53, we performed the promoter activity assays in the p53 null, SaOS-2 cells. In order to assay for p73 transcriptional activity we used the promoter of the proapoptotic Bax protein in the pBax Luc vector. Cells were transfected as indicated in materials and methods. 12 hours after transfection luciferase assays were performed using the dual-luciferese system to control also for transfection efficiency. The results obtained are shown in Figure 26 and show that transcription from the Bax promoter is p73-dependent. In agreement with previous studies (ZENG *et al.* 2000), it can be seen that p300 acts as a potent co-activator of p73-mediated transcription, inducing a four-fold increase in the transcription from the pBax promoter. 16E7 appeared to inhibit the p300 transcriptional co-activation function on the p73α-responsive promoter in a dose-dependent manner. In addition, p300 was also unable to transactivate the p73-responsive promoter in the presence E1a protein, further underlining the conservation of function between these two viral proteins. Similarly to the inhibition of p300-mediated transactivation of the E2-dependent promoter, both the low-risk 11E7 and p300-binding defective 16E7 mutant (p24-CKII) exerted only a reduced ability to abolish p300 activity upon the p73α-responsive pBax promoter, demonstrating that the repression of p300 transcriptional activity is specific and results from the interaction between 16E7 and p300.

Fold conversion (Luciferase activity) pBaxLuc

| 1 | 90 | 385 | 66 | 58 | 24 | 320 | 370 | 138 |

SaOS-2

(y-axis: 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500)

| p73α | - | + | + | + | + | + | + | + | + |
| p300 | - | - | + | - | + | + | + | + | + |

| | - | - | - | 16E7 | 16E7 (wedge) | | 11E7 | p24-CKII | E1a |
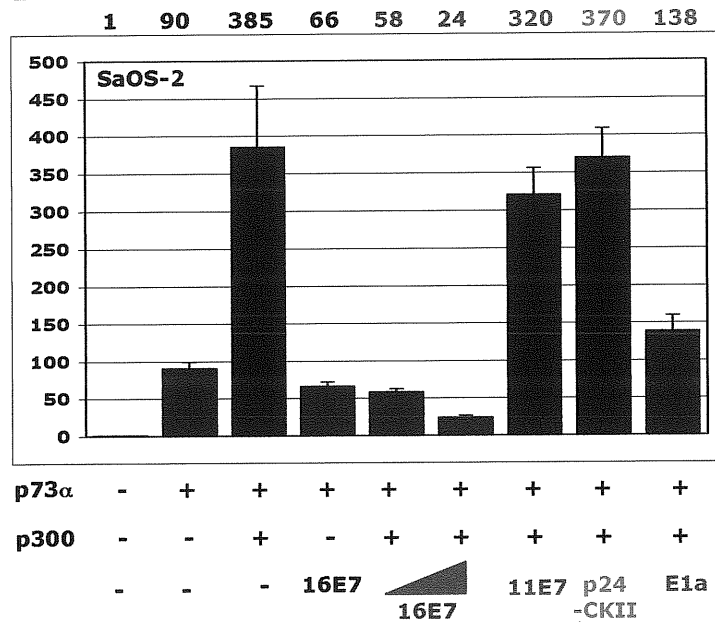
Figure 26. Effect of E7 on p300-mediated transactivation of p73α responsive Bax promoter.

SaOS-2 cells were transfected with the indicated plasmids by Lipofection with 1μg of reporter pBaxLuc, 0.1μg of pcDNA₃.p73, 0.2μg CMV-β.p300 and 0.2, 0.4μg of 16E7 constructs and 0.4 μg of E1a, p24-CKII and 11E7 (all as pcDNA₃ vectors). The total amount of DNA was equalized with empty plasmids. Simultaneous expression of pRL plasmid allowed for the normalization of transfection efficiency. Dual-luciferese assay was performed as described in Materials and Methods. The mean values from three independent experiments are presented, together with standard deviations.

Taken together these results demonstrate that 16E7 is a potent inhibitor of p300 co-activation function in the context of at least two different promoter reporter assays.

2.2.8. HPV-16E7 competes with E2, but not with p73 for the CH1 domain of p300

Previous studies have shown that p73α and HPV-18E2 both bind to the N-terminal region of p300 spanning the first 500 residues (MULLER et al. 2002; ZENG et al. 2000). Since this is the same region bound by the HPV E7 protein we were interested in investigating whether the interaction of E7 with p300 could affect E2 and p73α binding to p300 and therefore provide an explanation for the inhibitory effect of E7 upon the p300 transcriptional co-activation function. This is a particularly interesting question since the levels of p300 have been demonstrated to be limiting within the cell (KAMEI et al. 1996; YAO et al. 1998), and such a competition could therefore seriously affect p300 function. In order to investigate this, a series of *in vitro* competition binding assays were performed. The first series of experiments were carried out in order to investigate the potential ability of E7 to influence the E2:p300 interaction. A GST-p300 fusion protein covering the CH1 and KIX domains (fragment 1) was incubated with *in vitro* translated, radiolabelled HPV-16E2 and increasing amounts of *in vitro* translated, radiolabelled 16E7. After extensive washes, the complexes were separated by SDS-PAGE and the pattern of bound proteins was determined by autoradiography. The results obtained are shown in the figure 27A. In agreement with previous studies, 16E2 shows strong binding to p300 (MULLER et al. 2002). Interestingly,

increasing amounts of 16E7 specifically reduce the amount of E2 bound to the N-terminal domain of p300. This demonstrates that the binding of these two proteins to p300 is mutually exclusive, suggesting that this is one mechanism by which 16E7 antagonizes the p300 ability to co-activate 16E2-dependent transcription and transactivation.

In order to further investigate whether E7 might be able to interfere with other proteins binding to the CH1 domain of p300, we proceeded to investigate if 16E7 might perturb the binding of TBP to this region. The assay was performed as described above. Purified GST-p300 fragment 1 was incubated with *in vitro* translated, radiolabelled TBP and increasing amounts of 16E7. As can be seen from the figure 25C, TBP shows a strong binding to the p300 N-terminal fragment overlapping the CH1 domain and this binding is reduced in the presence of 16E7 protein. This result suggests that 16E7 may also compete with TBP for the binding site on p300 and that the binding of these two proteins to p300 may be a mutually exclusive event.

Finally, the same assays were performed with p73α. Purified GST-p300 fragment 1 was incubated with *in vitro* translated, radiolabelled p73α and increasing amounts of 16E7. The results obtained are shown in Figure 27B. In agreement with previous reports, p73α shows a strong interaction with the p300 N-terminal fragment covering the CH1 domain. However, in contrast to results obtained for 16E2 and TBP, increasing amounts of 16E7 appeared to have no effect upon the p73α binding to the p300 CH1 domain.

Therefore, these results demonstrate that the mechanism by which 16E7 inhibits the p300 transcriptional co-activation function with respect to p73α and E2 are most likely different.
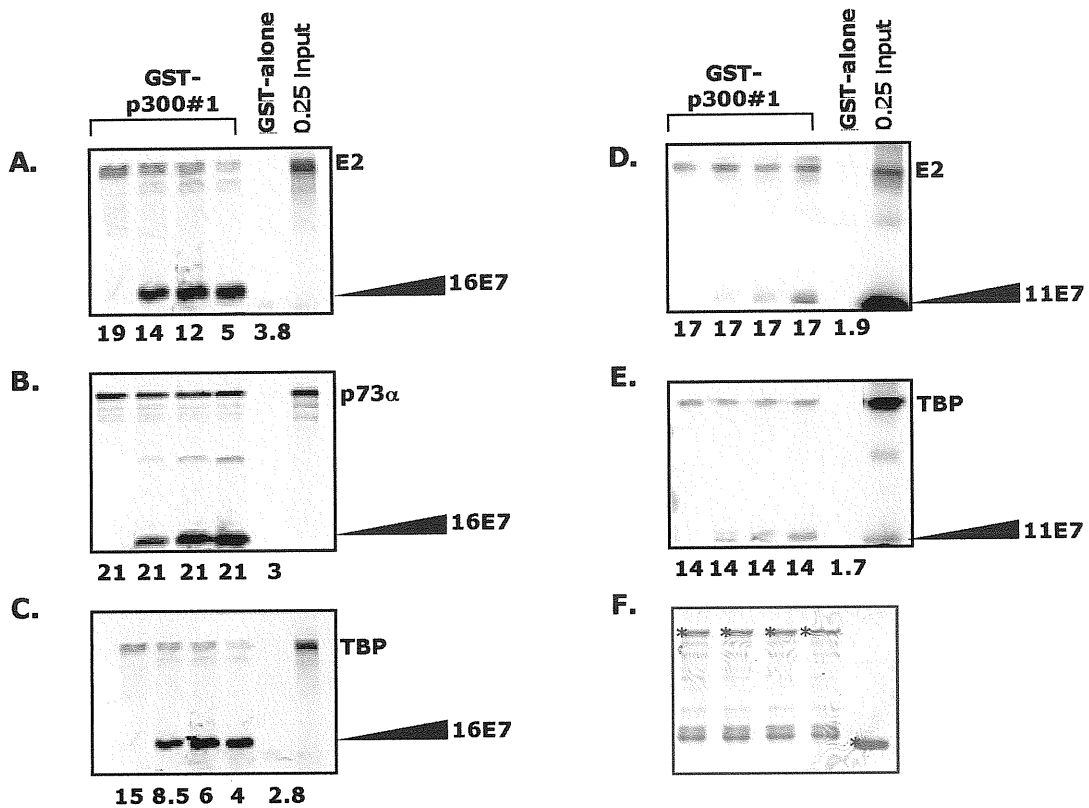
Figure 27. *In vitro* competition assays between GST-p300 fragment 1, E2, p73α, TBP and increasing amounts of 11- and 16E7 protein.

GST-p300 fragment 1 spanning the N-terminus of p300, was incubated with ivt E2 (**Panel A**), p73 (**Panel B**) and TBP (**Panel C**) and increasing amounts of ivt 16E7. The same GST-p300 fragment was incubated with ivt E2 (**Panel D**), TBP (**Panel E**) and increasing amounts of 11E7. GST-alone was used as a negative control. After extensive washing, complexes were separated by SDS-PAGE and autoradiographed. The numbers below each panel show the percentage of input protein bound on the beads. **Panel F**. Coomassie stain of the GST-fusion proteins used in the assays;

* marks the position of the full length GST-fusion protein.

Additionally, from the competition binding assays done in the presence of increasing amounts 11E7 (Figure 27D,E), it is clear that 11E7 is not able interfere with the binding of E2 and TBP to the CH1 domain of p300, explaining its minimal effect seen upon p300 transcriptional co-activation function.

2.2.9. 16E7 blocks p300 HAT activity *in vitro*

As described previously, E1a has been demonstrated to inhibit p300 transactivation function in at least three ways: by sequestration of p300/CBP from transcriptionaly active complexes, by displacement of positive transcription factors due to competitive binding and by blocking p300/CBP HAT activity. The above studies suggest that 16E7 can block p300 activity through a steric hindrance mechanism with respect to E2 co-activation, yet other mechanisms would appear to be involved in repression p73α co-activation. Therefore we proceeded to investigate whether E7 is able, similarly to E1a, to alter p300 HAT activity. Although many partners of p300 are acetylated, acetylation of hisones is one of the best characterized processes and thus histones were chosen as a substrate for the acetylation reaction. In order to carry out this experiment we used a purified fragment of p300 encompassing the HAT domain (residues 1195-1810), bound to the GST resin and possessing HAT activity. The acetylation reaction was performed in the presence of increasing amounts of his-tagged purified 16E7 and carried out as described in materials and methods. The reactions were also done in the presence of the histone deacetylase inhibitor - sodium butyrate as well as Acetyl-CoA, as a donor of an acetyl group. Complexes were separated by SDS–PAGE and the

presence of acetylated products was detected by WB with the anti-acetylated lysine antibody (Cell Signalling) and the results obtained are presented in Figure 28. As can be seen, no acetylation products are obtained in the absence of Ac-CoA, however autoacetylation products of p300 can be easily noted, thereby verifying the specificity of reaction. Increasing amounts of purified 16E7 were able to inhibit the ability of p300 to acetylate histones – the amount of acetylated histones decreases with the increasing amounts of HPV-16E7. Therefore this experiment suggests that 16E7 is a potent inhibitor of p300 HAT activity and this offers an alternative mechanism by which E7 may moderate the transcriptional co-activation function of p300.

A.

B.
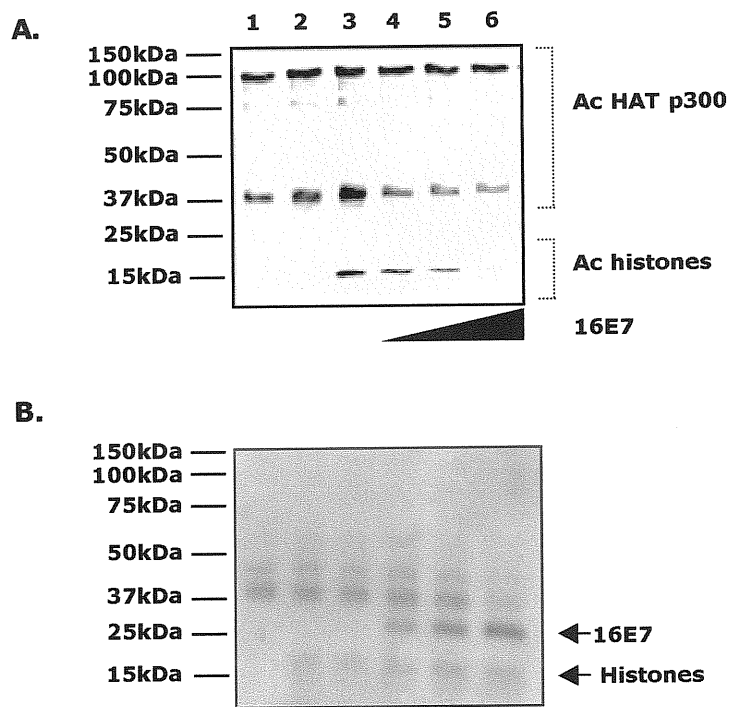
Figure 28. Inhibition of p300 HAT activity by 16E7.

Panel A. GST-HAT p300 was incubated with histone substrates and Ac-CoA in the presence of purified 16E7 (Lanes 4-6). Lane 1: GST-HAT with Ac-CoA (no substrate), Lane 2: GST-HAT with substrate, without Ac-CoA. Lane 3: GST-HAT with Ac-CoA and histone substrate. The presence of acetylated products was detected by WB with anti-acetylated lysine antibody. **Panel B.** Ponceau S staining of the membrane is also shown.

3. DISCUSSION

The major transforming potential of HPV has been attributed to the action of the virally encoded E6 and E7 proteins. These gene products are sufficient to initiate cell transformation by virtue of their ability to interact with, and manipulate the functions of several growth-regulatory proteins that control cell cycle progression and programmed cell death. Overcoming the action of tumour suppressor gene products has emerged as being a prerequisite for achieving the tumourigenic phenotype in many cancers. The major transforming activities of E6 and E7 include the targeted degradation of p53 by E6 and the inactivation of the cellular retinoblastoma protein pRB by E7. Even though inactivation of p53 and pRb by these viral oncoproteins is necessary for transformation, it is clear that other activities are also required, suggesting that other cellular targets are involved in the transformation pathways induced by E6 and E7. The findings presented in this study suggest that one of the additional common functions of the HPV oncoproteins is to overcome the action of the p300/CBP transcription co-activators, which interestingly have also been shown to exhibit tumour-suppressor activities (GAYTHER *et al.* 2000; KUNG *et al.* 2000; MURAOKA *et al.* 1996).

The increasing variety of transcription factors identified that utilize p300/CBP as a co-activator, suggest that multiple cellular activities are co-ordinated by this family of proteins. In addition, many viral oncoproteins have been found to modulate the p300/CBP function, further emphasizing the central importance of p300/CBP in maintaining cellular homeostasis as well as underlining the conservation of function between many different virus types.

What does HPV gain through the binding to p300? I will try to answer this question in the light of the experiments presented in this thesis.

3.1. E6 interactions with p300

In this study, the interaction between E6 and p300 has been further analyzed. It has been found that the E6 proteins derived from different PV types, both human and animal in origin, were capable of interacting with p300, thus indicating that this activity is conserved across many PV types. The fact that evolutionary divergent PV types exhibit conserved interaction with p300 suggests that it plays a fundamental role in the PV life cycle.

The HPV-18 and HPV-11E6 binding sites on p300 overlap with the CH1, CH2 and CH3 domains of the protein, although at present, it is not known whether these three regions of p300 are bound directly by E6, or whether any of these interactions are mediated via an unknown, intermediate protein, present within the reticulocyte lysates. However previous studies would indicate that HPV-16E6 binding to CH1 and CH3 was indeed direct (PATEL *et al.* 1999). In addition, multiple sites for interaction on p300/CBP have been described for the Ad E1a protein. Kurokawa *et al.*, (1998) show that Ad2 E1a interacts with at least three different domains of p300/CBP with a comparable affinity: (1) aa 1-450, (2) aa 1459-1891, spanning CH3, and (3) aa 2058-2163, containing a Gln-rich region. In contrast, Lipinski *et al.*, (1999) show that Ad12 E1a and Ad2 E1a interact strongly only with 2 regions of p300: a fragment spanning the CH3 domain (aa 1519-1850) and a fragment encompassing the Gln-rich region (aa 1999-2200). The discrepancy between these two findings may have

arisen from the different conditions used for the assays, however they seem to be in agreement about multiple binding sites of E1a existing on p300.

The finding that many transcription factors and viral proteins interact with the same domains of p300 and CBP suggest that these regions exhibit considerable flexibility in accommodating diverse protein ligands. The CH1 and CH3 domains of p300 largely contribute to the binding of p300/CBP with transcription factors, elements of the basal transcription machinery as well as with some of the viral proteins. Proteins binding to CH1 include p73, Mdm2, TBP, Stat2 and Pit-1 to mention but a few, while CH3 has been demonstrated to be a domain necessary for interaction with P/CAF, TFIIB, RNA helicase II, E2F, C/EBP, JunB, c-Fos, MyoD, GATA-1 and Ad E1a, SV40 LT, Py LT and HIV Tat (all reviewed in (GOODMAN and SMOLIK 2000)).

A novel transcriptional adapter motif (TRAM) has been identified within the CH3 domain of p300/CBP that has been demonstrated to represent a hot spot for transcription factor binding as well as for Ad E1a and HPV-16E6 interaction (O'CONNOR *et al.* 1999; ZIMMERMANN *et al.* 1999). Since the CH3 region and TRAM of p300 is also the interaction point for E2F (TROUCHE *et al.* 1996), TFIIB (KWOK *et al.* 1994), P/CAF (YANG *et al.* 1996) and components of the RNA polymerase II holoenzyme such as RNA helicase A (NAKAJIMA *et al.* 1997), it has been suggested that E1a may function in part by preventing these factors from associating with the co-activator or by displacing them from the binding domain by competitive binding. Whether this represents a common mechanism of action of both E1a and E6 needs further examination. However since both proteins interact within the same regions of p300, it does imply that these viral transforming proteins may have evolved common strategies

for interfering with the normal function of p300. Association of the E6 viral oncoprotein with the same domains in a manner that prevents the binding of essential p300 effectors may therefore give the virus the possibility to control multiple signalling pathways responsible for cell-growth control, differentiation, cell adhesion and apoptosis.

It has also been demonstrated in this study that the low-risk HPV E6s (1, 6, 11) as well as high-risk animal BPV and CRPV E6s show a significant level of interaction with p300, and this is in agreement with previous studies showing interactions between the low-risk 6E6 (PATEL *et al.* 1999) and BPV E6 proteins (ZIMMERMANN *et al.* 2000) with p300. However, not all studies are in agreement with respect to the domains on p300 that are bound by E6. Patel *et al.,* (1999) demonstrated that 6E6 interacts with the CH1 domain of p300, somewhat more weakly than 16E6. In contrast Zimmerman *et al.* (1999) failed to detect any interaction between the HPV-6 and -11 E6s with a fragment of CBP equivalent to the CH3 domain of p300, although other regions of CBP were not tested and this may account for this discrepancy. Obviously one of the other reasons for this discrepancy may be the fact that the high- and low-risk HPV E6 proteins display different affinities for p300 and CBP. This could certainly offer an explanation for the different results presented here and by other studies. In this thesis we found that all the HPV E6 proteins tested (1, 6, 8, 11, 16, 18, BPV and CRPV) interact with the CH1 and the HAT fragment of p300, spanning the CH2 and CH3 domain, although some differences were observed in the efficiency of these interactions with respect to particular domains of p300, and the possible implications of this observation with respect to p300 function should be further analysed.

Differences in results obtained between groups may also be a result of the diverse p300 or CBP fragments used for the interaction assays as well as different conditions in which experiments were performed. Our results demonstrate however, that E6 proteins bind to the regions covering three CH domains of p300.

The studies of Patel *et al.,* (1999) and Zimmerman *et al.,* (1999), showed that the high-risk HPV E6s could block the intrinsic transcriptional activity of p300/CBP and decrease the ability of p300/CBP to activate NF-κB- and p53-responsive promoter elements. p53 has been shown to interact with several domains of p300 (AVANTAGGIATI *et al.* 1997; GU *et al.* 1997; LILL *et al.* 1997) and this stimulates the transcriptional activity of p53 on p53-regulated promoters and enhances the response to ionizing radiation. However, the interaction of E6 with p300 was found to be p53-independent since mutants of E6 defective in p53 binding and degradation, nonetheless retain the ability to bind p300/CBP and to inhibit p300-mediated p53 co-activation. 16E6 was shown to be able to interfere with complex formation between p53 and the p300/CBP TRAM region resulting in p53 transcriptional inhibition (ZIMMERMANN *et al.* 1999). The same mechanism has also been demonstrated for Ad E1a, which is also a potent inhibitor of p53 transactivation function (SOMASUNDARAM and EL-DEIRY 1997; STEEGENGA *et al.* 1996), and moreover, the p300/CBP-interacting region of E1a, required for transformation, was also required for inhibition of p53-dependent gene expression. The mechanism of p53 transcriptional inhibition by E1a includes the displacement of p53 from the TRAM domain of p300/CBP (O'CONNOR *et al.* 1999), suggesting that

competition for the p300/CBP binding represents a potential mechanism by which these viral proteins may manipulate cell fate.

From the above it would appear that the HPV E6 proteins possess two distinct mechanisms by which they can abrogate p53 function: first, the removal of cellular p53 protein through the E6-AP-mediated proteasome degradation and second, the repression of p53 transcriptional activity by targeting the p53 co-activator p300/CBP. Targeting p53 for degradation is the major route by which E6 overcomes its anti-proliferative effects (CROOK *et al.* 1994; CROOK *et al.* 1991; LECHNER *et al.* 1992) and is required to prevent p53-induced apoptosis (THOMAS *et al.* 1996b) and this activity is partly independent of its ability to act as transcriptional activator (HAUPT *et al.* 2003; SHEIKH and FORNACE 2000). The studies mentioned above, indicate clearly that E6 proteins may also use other pathways to abrogate p53's function - an example being the down-regulation of p53 transcriptional activity through binding to p300/CBP (ZIMMERMANN *et al.* 1999). Induction of G1 cell cycle arrest is largely based on transcriptional activation of the inhibitor of the cyclin-dependent kinases, p21 (EL-DEIRY *et al.* 1993), and also on inhibition of DNA replication, by both p21-dependent and independent mechanisms. The ability of p53 to mediate important functions such as the response to DNA-damage and the subsequent G1-checkpoint activation was shown to be p300/CBP dependent (LILL *et al.* 1997). Since transactivation of specific target genes is the principal mechanism by which p53 induces growth arrest, binding of E6 to p300/CBP allows it to overcome p53's growth suppressive effect independently from targeting it for degradation (Figure 29). In addition, since during viral infection and in HPV-induced cervical lesions not all the p53 is degraded (COOPER *et al.* 1993; HOLM

et al. 1993; MANTOVANI and BANKS 1999; TERVAHAUTA *et al.* 1994), repression of p300 transcriptional activity by HPV E6 proteins provides an alternative means of interfering with p53 function. Interestingly, it has also been also suggested that p53 can induce apoptosis through mechanisms dependent on transcriptional activation (CHEN *et al.* 1996c; HAUPT *et al.* 1995) and p300 has been shown to influence p53-induced transcription-dependent early apoptosis (LILL *et al.* 1997). Thus by blocking p300/CBP transcriptional co-activation function E6 would be able to interfere with growth arrest and apoptosis pathways induced by p53 in a manner which does not require degradation of the p53 protein (Figure 29).

Recent studies have also shown that p300/CBP contributes to the mechanisms that regulate p53 degradation. It has been demonstrated that generation of the polyubiquitinated forms of p53 that are targeted for proteasome degradation requires the intrinsic ubiquitin ligase activities of hdm2 and p300. Consistent with the role for p300 as E4 for p53, expression of E1a, but not a p300-binding deficient mutant, caused a dramatic decrease in polyubiquinated, but not-mono/oligoubiquinated species of p53 (GROSSMAN *et al.* 2003). This effect may account for the reports of E1a-mediated stabilization of p53 (LOWE and RULEY 1993; NAKAJIMA *et al.* 1998). However, it does not seem likely that E6 would target the p300-ubiquitin ligase activity for p53 whilst simultaneously targeting it for degradation. Indeed, it has been reported that in HPV-positive cancer cells degradation of p53 depends entirely on E6, while the hdm2-dependent pathway is inhibited (HENGSTERMANN *et al.* 2001). This indicates that E6 can target p53 for degradation under conditions when this would be normally blocked, such as after DNA damage, thereby
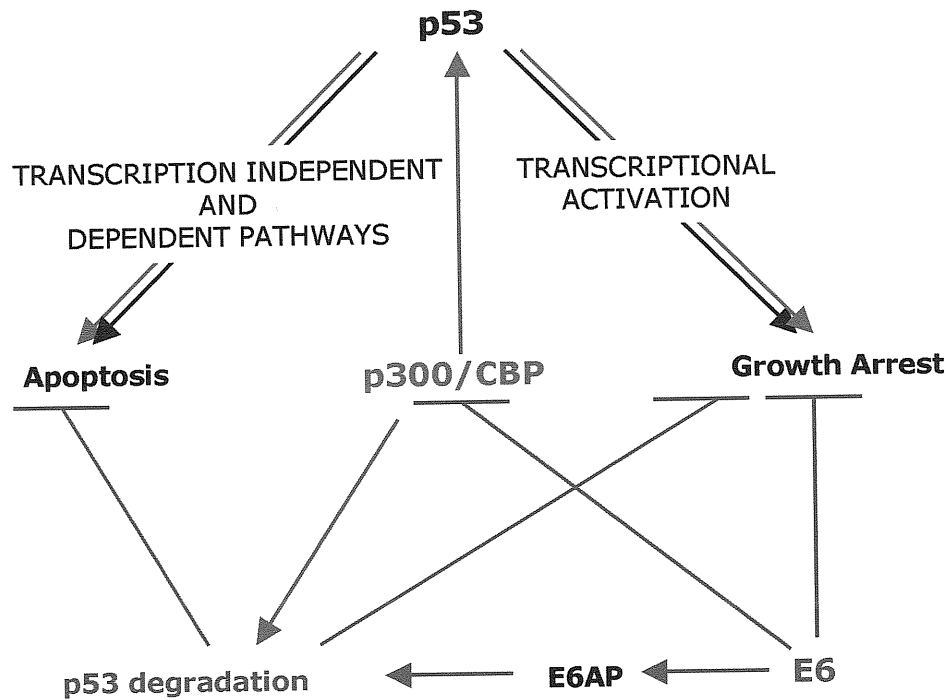
Figure 29. The interactions between p53, p300 and HPV E6. p53 interferes with the replicative cycle of HPVs either by inducing growth arrest or apoptosis. In both processes p300/CBP is involved. In the case of mucosal HPVs, E6 directly stimulate p53 degradation. E6 may also abolish p53 function through interaction with p300/CBP.

allowing the accumulation of genomic mutations in the infected keratinocytes, and thus contributing towards malignant progression. E6 reactivates degradation of p53 by recruiting the prototype HECT-domain ubiquitin ligase, E6-AP (SCHEFFNER *et al.* 1993), which normally is not involved in the ubiquitination of p53.

In the light of our results demonstrating strong interaction between HPV E7 protein and p300 (see below) as well as documented stabilization of p53 by 16E7 (EICHTEN *et al.* 2002; JONES and MUNGER 1997; JONES *et al.* 1997b), it is possible to speculate that E7 may exhibit an effect upon the p300-ubiquitin ligase activity similar to that of Ad E1a. Thus, E7 may block the p300-ubiquitin ligase activity, whilst E6 could target p53 for degradation through the E6-AP mediated pathway, however this model requires validation.

HPV E6 interference with NF-κB function due to targeting of p300/CBP is particularly intriguing, since deregulation of the NF-κB pathway can result in hyperproliferation of the stratum spinosum (HU *et al.* 1999; TAKEDA *et al.* 1999). NF-κB is activated upon infection by a number of viruses including HPV (NEES *et al.* 2001) and promotes transcription of a number of genes involved in the local immune response such as class I MHC, interleukins and GM-CSF, some of which are synthesised directly by the keratinocytes (BALDWIN 1996; TOMIC-CANIC *et al.* 1998). Therefore E6 inhibition of the NF-κB pathway through its association with p300 may result in down-regulation of the local immune response and thereby help the virus to escape immune recognition in the epithelium. This in turn will allow persistence of the viral infection leading to hyperproliferation of the stratum spinosum and granulosum providing obvious advantages for viral replication. Additionally, the suppression of the

local immune surveillance may also have important consequence for the development of cervical cancer.

The demonstration that the high-risk HPV E6s could, in common with other viral proteins, inhibit p300 activity, suggested that this interaction might be important for the ability of HPV E6 to contribute towards cell transformation. However, there was little biological evidence to support this hypothesis. Hence, we next focused our attention on investigating the potential biological consequences of this association. To do this, we used an amino terminal deletion mutant of Ad E1a ΔN (Δ2-36). It has been demonstrated that any mutations in E1a that destroy binding with pRb and/or p300 inactivates the ability of E1a to co-operate with an activated ras oncogene in the transformation of primary BRK cells (JELSMA *et al.* 1989; WHYTE *et al.* 1988). The Δ2-36 mutant of Ad E1a had previously been shown to be defective both in its ability to bind p300 and its ability to cooperate with EJ-ras in the transformation of primary BRK cells (STEIN *et al.* 1990; WHYTE *et al.* 1988). The use of primary rodent cells is a relevant assay for assessing the transforming potential of the viral proteins *in vivo*, and in addition BRK cells represent a good model for the HPV studies, as the cells are principally epithelial in origin. Since HPV E6 by itself shows little transforming potential in the BRK assay, even in the presence of a cooperating, activated oncogene, it was possible to set up this system to investigate whether E6 could complement the (Δ2-36) Ad E1a mutant in the transformation of primary BRK cells by virtue of its ability to interact with p300. We reasoned that if the E6-p300 interaction was important for the ability of E6 to contribute towards cell

transformation then E6 should be able to complement the mutant of Ad E1a in this assay system.

In the BRK transformation assays presented here, co-transfection of high-risk HPV E6s was able to efficiently rescue the defect of the ΔN E1a mutant. These results suggest that the interaction between high-risk E6s and p300 is biologically relevant and may also contribute towards E6 induced cellular transformation. These results appear also to be specific and dependent upon the E6:p300 interaction since a mutant of 16E6 (Δ123-7), which was shown to be unable to bind p300 (PATEL *et al.* 1999) failed to complement the E1a mutant, lacking the p300-binding region. In addition, this function of E6 appears to be independent of its ability to target p53 for degradation, since a mutant of 18E6 (Δ47-49) unable to target p53 for ubiquitin-mediated degradation (PIM *et al.* 1994), nonetheless retained the capacity to complement the Ad E1a mutant in the transformation of the BRK cells.

Although the high-risk HPV-E6 proteins are capable of complementing the E1a mutant defective in p300 binding, they nonetheless never restore the transforming activity to that seen with the wild-type E1a protein. There are several possible explanations for this apparent discrepancy. One possibility is that E1a may have other activities/targets other than p300-binding within its amino terminal domain that are not shared with E6. An obvious candidate for this could be the p400 protein shown to interact with an N-terminal fragment of Ad E1a (BARBEAU *et al.* 1994; HOWE and BAYLEY 1992). The p400 complex consists of the p400 protein, a novel SWI2/SNF2 family member and TRAPP/PAF400, a *c-myc*- and P/CAF-associated nuclear protein with a PI-3 kinase-like domain (MCMAHON *et al.* 1998; VASSILEV *et al.* 1998). This complex

is essential for E1a induced transformation (FUCHS *et al.* 2001) and E1a

binding results in functional perturbation of this complex, as has been shown

to be necessary for E1a transforming activity.

An alternative possibility is that Ad E1a and HPV E6 may exhibit different

affinities for p300 and have divergent effects upon its function, although

further studies on the effects of E6 on p300 function are required in order to

clarify this point. Finally, a more trivial explanation could be related to the

relative differences in levels of expression between Ad E1a and HPV E6

proteins. E1a is a highly abundant protein, whilst HPV E6 is notoriously difficult

to detect owing to the low levels of the protein found in the cell (ANDROPHY *et*

al. 1987; BANKS *et al.* 1987) suggesting that the levels of E6 may simply not

be high enough to fully complement the E1a mutant in the BRK transformation

assays.

Usually, comparison between the high- and low-risk E6 proteins indicates

functional distinctions based on differential affinities for cellular targets and

represents a cardinal factor in their ability to transform cells. The results

presented here indicate that the interaction between HPV E6 and p300 is

conserved across many PV types, suggesting that it is a common requirement

for viral replication. However, although low-risk HPV E6 proteins would appear

to bind p300 with affinities similar to that of the high-risk HPV E6 proteins, it

is clear that they show much weaker capacity to rescue the Ad E1a

transformation deficient mutant lacking the p300-binding domain, in the BRK

transformation assays. There are several possible explanations for this obvious

discrepancy. We can speculate that transcriptional inhibition of p300 is a

prerequisite for cellular transformation mediated by high-risk mucosal HPV

E6s, hence the observation that only high-risk E6s were able to repress p300 transcription activity, while low-risk 6E6s appeared also to have only marginal effects (PATEL *et al.* 1999).

There are also some differences in the precise patterns of localization between high- and low-risk associated viral oncoproteins and differences at such a basic level of function could profoundly influence their abilities to contribute to cell transformation. The low-risk E6 proteins may have a tendency to accumulate in dot-like structures within the nucleus, corresponding to PML oncogenic domains (PODs, PML bodies), while high-risk E6 proteins have a slightly different pattern of cellular localization, being distributed in many compartments of the cell (GUCCIONE *et al.* 2002). p300/CBP has been shown to co-localize within a subset of PML bodies together with RNA polymerase II (VON MIKECZ *et al.* 2000) in the transcriptionally active domains in the nucleus. This finding supports the hypothesis that low-risk E6 proteins need the interaction with p300/CBP for efficient replication and probably uses p300/CBP, which accumulate in POD structures. It is a particularly important observation since several studies have demonstrated that targeting viral proteins to PML oncogenic domains is an important step in viral replication (EVERETT and MAUL 1994; MULLER and DEJEAN 1999).

3.2. E7 interaction with p300

The Ad E1a and the HPV E7 oncoproteins exhibit remarkable parallels in their biological activities such as their ability to transform and immortalize cells and to induce cell cycle progression. In many cases both proteins apparently act through similar molecular mechanisms. However, it has long been thought that E7 and E1a although similar, differ in certain biological and biochemical functions. Thus E1a being able to interact with p300/CBP, whilst E7 did not, represented one example of such a difference.

In this study we demonstrate that the E7 proteins derived from both low- and high-risk HPV types are capable of interacting with p300. The principal site of interaction on p300 lies within the p300 CH1 domain. Weaker interactions are also observed with the p300 CH2 and CH3 domains, and interestingly, binding to the equivalent CH1 and CH3 domain of CBP was also found using the mammalian two-hybrid analysis, suggesting that CBP may also be a target of E7.

Several transcriptional regulators have been shown to interact with p300 within the CH1 finger domain, including TBP (ABRAHAM *et al.* 1993), p73 (ZENG *et al.* 2000) and Mdm2 (GROSSMAN *et al.* 1998). The complexes of these regulators with p300 could serve as functional targets of the HPV E7 protein in transcriptional regulation through which E7 might exert its transforming activity and this will be discussed further on. It is also demonstrated here, that the interaction between 16E7 and p300 is direct, and that p300-16E7 complexes are relatively stable, since they could be extracted from cells in the presence of 300mM NaCl. The residues of HPV-16E7 involved in binding to p300 lie within the N-terminal domain of 16E7. Interestingly, previous studies

have shown that an amino terminal mutant of 16E7 was unable to complement an E1a mutant defective in p300 binding (DAVIES and VOUSDEN 1992), and that E7 can complement the transcriptional co-activation function of the E1a amino terminus in the transactivation of viral early promoters (WONG and ZIFF 1996). These results might be explained, at least partially, by the interaction between p300 and E7. It is also interesting to note that, similarly to E1a and SV40 LT (EDMONDS and VOUSDEN 1989; VOUSDEN and JAT 1989) the regions of E7 required for binding p300 overlap with the pRb-binding domain. This also raises the possibility that E7s interactions with p300 and pRb are mutually exclusive events and this requires further investigation.

It is also worth noting that the endogenous form of E7 co-immunoprecipitating with p300 from cells expressing both proteins had a molecular weight slightly smaller than the major E7 species found in these cells and instead co-migrated with E7 obtained from *in vitro* translations. This suggests that the form of E7 preferentially binding to p300 may be an unmodified form of the protein. Since the CKII phosphorylation site is also required for the interaction, this raises the possibility that phosphorylation may be a means of regulating the E7:p300 association. CKII regulation of E7 appears to vary during the cell cycle, therefore this may provide a means of specifically targeting E7 to p300, at a given point within the cell cycle (MASSIMI *et al.* 2001).

The problems with detecting the interaction between p300 and E7 by other laboratories may have arisen for several reasons. Failure to detect E7:p300 complexes may reflect insufficient sensitivity or inability of antibodies to recognize the complex. In the experiments described by Davies and Vousden (1992), different cell lines were used for detection of E7:p300 and E1a:p300

interactions. The failure to detect p300 in E7 immunoprecipitates may be due to the fact that the levels of E1a expression in 293 cells (used as a positive control) are much higher than the levels of expression of 16E7 in CaSki or SiHa cells. Choice of which antibody to use, may also represent a critical factor in these type of experiments, as demonstrated in the work of Eckner *at al.,* (1996), where only one, out of 10 antibodies raised against p300, was able to co-immunoprecipitate SV40 LT from cellular extracts. Likewise in the studies presented here, one of the anti-p300 antibodies (NM11) worked much better in the co-immunoprecipitation experiments. As the p300 is extensively modified at different sites by phosphorylation, sumolation and acetylation, it may be that specifically modified forms of p300 are better/weaker targets of E7. As mentioned before, it is also possible that the complex formation between E7 and p300 is restricted to a specific phase of the cell cycle and these aspects require further investigation. Nonetheless, there was some biological evidence that E7 can encode an activity analogous to p300 binding by E1a. Rescue of a conditionally immortalized rat embryo fibroblast cell line requires only the p300-binding function of E1a (RILEY *et al.* 1990), and E7 was shown to complement the growth defect in this cell line (VOUSDEN and JAT 1989) implying that E7 can either bind p300 or provide another function important for this activity. Additionally, functional analysis of HPV-16E7 by complementation with adenovirus E1A mutants (DAVIES and VOUSDEN 1992) revealed that an N-terminal E7 mutant was unable to complement an E1A mutant defective in p300 binding, indicating that the two mutants were defective for functionally equivalent activities.

Taken together, the results of numerous biochemical and biological approaches undertaken in this study confirm that the HPV E7 protein mirrors another function of E1a - that of p300 binding.

Several studies have highlighted the potential role of p300/CBP proteins in the processes of transcriptional co-activation and it is now apparent that a diverse and constantly increasing array of transcription factors are able to form a stable physical complex with p300/CBP and respond to the co-activating properties of p300/CBP. The HPV E2 protein has been shown to use p300/CBP (LEE *et al.* 2000a; MARCELLO *et al.* 2000) in order to enhance its own ability to activate viral gene expression. Thus, E2 may use p300/CBP as a classical co-activator or/and anchor to reach promoters in the absence of E2 binding sites. One consequence of the E7:p300 interaction presented in this thesis, is suppression of p300 transcriptional co-activation function in the context of the HPV-16E2 transactivator. 16E7 can suppress E2-dependent transcription and block activities of E2 by sequestrating p300 and preventing E2:p300 association which may be an important step in overcoming E2-mediated growth arrest in PV-infected cells. This suggestion is supported by the observation that low-risk E7 only marginally inhibited the p300 co-activation function of E2-dependent transcription. One of the possible explanations for the divergent results obtained with high- and low-risk E7 proteins can be provided by the immunofluorescence analysis (GUCCIONE *et al.* 2002), and this thesis) showing differential localization of both E7 proteins within the cell. Both proteins co-localize with p300, however, HPV-16E7 protein exhibits a predominantly diffuse nuclear pattern of expression with nucleolar exclusion, while HPV-11E7 has regions of concentration within the nucleus, as well as the

presence of clear dot-like structures, which also co-stain for p300. Very similar patterns of expression have been observed for proteins that localize to PML oncogenic domains (PODs) (STERNSDORF *et al.* 1997), including p300/CBP (VON MIKECZ *et al.* 2000). It has been also demonstrated that 11E7 protein co-localize with PML (GUCCIONE *et al.* 2002) as does 11E6. However, previous studies have suggested that the sites of DNA replication of the low-risk HPV types are also concentrated close to PODs (SWINDLE *et al.* 1999). This suggests that low-risk types may have evolved an efficient mechanism to make use of POD structures and proteins accumulated there, in this case p300/CBP. Bearing in mind, that p300/CBP are POD resident proteins, we can speculate that low-risk E6 and E7 proteins use p300/CBP for their efficient replication.

The results from the *in vitro* competition binding assays imply a possible mechanism by which 16E7 may inhibit p300-mediated transactivation of E2. It has been demonstrated that 16E7 is able to displace 16E2 from its p300 binding site. This was a particularly interesting observation since analysis of $p300^{+/-}$ and $Cbp^{+/-}$ mice suggest that the amount of p300/CBP is limiting within cells (KAMEI *et al.* 1996; YAO *et al.* 1998). These findings argue that different signal transduction pathways compete for a pool of p300/CBP and thereby target their effects toward specific sets of responsive genes. p300 can be directed toward specific genes to the exclusion of others, hence some of the transcription factors use p300/CBP preferentially for regulating a certain category of genes, or alternatively for responding to particular transcriptional stimuli that, if inactivated would be advantageous to the functions of the viral oncoprotein. It is possible to imagine that there would be sufficient p300/CBP to associate only with one group of factors (either those linked to proliferation

or differentiation) at any given time. Thus the role of HPV E7 would be to redirect the cell's regulatory machinery in order to meet the requirements of viral replication, possibly by blocking the transcription of the genes involved in differentiation. As it has also been demonstrated that binding of E2 to p300 may represent a rate-limiting step for activation by E2 (MULLER *et al.* 2002), it is possible that 16E7 blocks the binding of E2 to p300 and this may provide a means of fine-tuning the levels of viral oncoprotein expression during viral infection.

Although p300/CBP are believed to be expressed ubiquitously, tissue-specific changes in protein levels have been described (PARTANEN *et al.* 1999). p300 was shown to be required during terminal keratinocyte differentiation by inducing the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ (MISSERO *et al.* 1995). Analysis of p300 protein levels within differentiating keratinocytes revealed that p300 levels increase during terminal differentiation of skin keratinocytes (MULLER *et al.* 2002). p300 is mainly expressed within the stratum granulosum, in the cells where the expression of the late genes of HPV is restricted. Transcription factors such as the AP-1 family, which are thought to be important during this process, bind to the region of p300 also bound by E7 (JANG and STEINERT 2002; KAMEI *et al.* 1996). Possible disruption of AP-1 and p300 transactivation complexes by 16E7 may therefore have an effect on keratinocyte differentiation. In the sequential step model of transcriptional regulation, co-activators such as p300/CBP, are the key regulators in the assembly and mobilization of the basal transcription machinery. Transcription processes are regulated through the sequential interactions of a large number of modulatory multiprotein complexes, and

assembly of basal transcription factors at the promoter represents the end result of these interactions. Regulation is imparted by additional components such as enhanceosomes and mediator complexes, which, along with co-activators integrate specific extracellular events to intracellular signals. The critical role of p300/CBP in this context is to promote rapid formation of the pre-initiation complex to facilitate multiple rounds of transcription (YIE *et al.* 1999a). Thus, depletion of p300/CBP from this complex decelerates the rate of transcription (YIE *et al.* 1999b), so that the competition between E7 and different transcription factors for the binding site on p300 may therefore represent a mechanism by which E7 can interfere with p300 transcriptional function and subsequently redirect the transcriptional machinery to the promoters of the proliferation specific genes.

Interestingly, the HPV E2 has also been shown to recruit P/CAF into a co-activator complex with p300 (LEE *et al.* 2002). These two co-activators have been shown to synergistically activate E2-dependent transcription. Recent studies have also shown that P/CAF is a target of HPV E7 protein (AVVAKUMOV *et al.* 2003; HUANG and MCCANCE 2002). Therefore an interesting possibility is that HPV E7 may target both of these co-activators in order to inhibit E2-dependent transcription.

The histone acetylase activity of P/CAF is also required for efficient activation of E2 transcriptional activity. P/CAF acetyltransferase also interacts with p300 and was shown to be displaced by Ad E1a (YANG *et al.* 1996). The ability of E1a to induce cells to enter S phase, which requires its N-terminal p300-binding domain (WANG *et al.* 1993), can be compromised by co-expressing P/CAF, suggesting that the interaction between p300/CBP and P/CAF has a

growth suppressing outcome (YANG *et al.* 1996). Given the capacity of E7 to interact both with p300 and P/CAF it is therefore possible that E7 by interfering with the function of both co-activators, gains the opportunity to influence genes controlling cell-cycle arrest and differentiation, in a manner analogous to E1a.

P/CAF has been shown to inhibit cell cycle and promote cellular differentiation through its acetyltransferase properties (PURI *et al.* 1997; YANG *et al.* 1996), and as interaction of 16E7 with the P/CAF acetyltransferase leads to reduction of its acetyltransferase activity this may result in interruption of differentiation pathways. *In vitro* acetylation assays, presented here, revealed that 16E7 is also able to reduce HAT activity of p300 upon a histone substrate. Further investigation as to whether p300/CBP and/or P/CAF HAT inhbition results in p300 transcriptional repression, is however required.

Although stimulation of p300/CBP HAT activity by cyclin E-CDK2 has been shown to promote entry into S phase (AIT-SI-ALI *et al.* 2000), in this report, p21 was shown to inhibit p300- and CBP-bound cyclin E-CDK2 activity, which resulted in repression of HAT activity and subsequent cell cycle arrest. This does not explain how p21 is able to stimulate the activity of transcription factors known to promote cell cycle arrest or differentiation through induction of p21, such as p53 and MyoD (reviewed in (SNOWDEN and PERKINS 1998). However it is possible that 16E7 might be able to indirectly stimulate HAT activity by blocking p21 function. The involvement of E1a in opposing processes, such as the inhibition and stimulation of p300/CBP HAT activity, has already been demonstrated (AIT-SI-ALI *et al.* 1998; CHAKRAVARTI *et al.* 1999; HAMAMORI *et al.* 1999). E1a binds TRAPP/GCN5, p300/CBP and P/CAF

HAT complexes, which leads to deregulated cellular transcription (LANG and HEARING 2003). Differential usage of the HAT domain may suggest that regulation of the HAT activity is cell cycle- and promoter- context dependent.

Apart from interaction with various histone acetyltransferases such as p300/CBP and P/CAF, E7 has also been demonstrated to recruit the Mi2β NURD (nucleosome remodelling and histone deacetylase) complex (BREHM *et al.* 1999), which leads to the E7-mediated transcriptional repression of target genes. E7's ability to bind components of both acetyltransferase and deacetylase complexes may suggest that such interactions are regulated by post-translational modifications of E7. Alternatively, E7 may preferentially bind the p300/CBP or NURD complex in the context of certain phases of the cell-cycle or a particular promoter, to which it initially becomes tethered via an interaction with a promoter-specific factor.

It has been postulated that recruitment of co-activators bearing HAT activity by promoter-bound transcription factors results in histone acetylation of nearby nucleosomes thus enhancing access of the transcriptional and replicational machinery to DNA (GRUNSTEIN 1997; MIZZEN and ALLIS 1998; STRUHL 1998). In this study we show that the other effect of 16E7 upon p300 is inhibition of its HAT activity. Since p300 is a chromatin-regulating factor, inhibition of p300 HAT activity by 16E7, which results in the hypoacetylation of the histones, may lead to the subsequent alteration of chromatin structure and perturbation of gene expression. It is also possible that in addition to disrupting p300 and/or p/CAF complexes, E7 alters the enzymatic activities of the complexes or directly targets them to new substrates including other E7-binding proteins or, alternatively, blocks the acetylation of other proteins. If

E6 or E7, as E1a and SV40 have been shown to do, prevent p53 acetylation by interacting with p300/CBP, then the affinity of p53 for its target promoters may be reduced. This represents an interesting possibility since a number of reports have also demonstrated that the presence of high-risk E6 proteins significantly reduces p53 DNA binding activity (LECHNER and LAIMINS 1994; THOMAS *et al.* 1996a; THOMAS *et al.* 1995).

The results presented in this thesis, show that one consequence of the E7:p300 interaction is suppression of p300 transcriptional co-activation function in the context of the HPV-16E2 transactivator, however p300/CBP transcriptional repression mediated by 16E7 is not only restricted to viral promoters. 16E7 has been demonstrated to interact with p300 mainly within the CH1 domain of p300. This domain has been shown to mediate the interaction of p300 with important transcription factors, and amongst these there are two we wished to focus on, p73 (ZENG *et al.* 2000) and TBP (ABRAHAM *et al.* 1993). The complexes of these regulators with p300 could serve as functional targets of the HPV E7 protein in transcriptional regulation through which E7 might exert its transforming activity.

p73 needs to interact with the p300 for transactivation of responsive target genes such as Bax or p21 and for activation of apoptosis (ZENG *et al.* 2000). In this study, high-risk E7 protein was found to be able to inhibit p300-mediated transcriptional co-activation of the p73- responsive pro-apoptotic Bax promoter. However, the mechanism of this inhibition seems to be different from that observed for E2, since 16E7 did not appear to compete with p73 for the p300 binding site. It has been demonstrated that acetylation of p73 following DNA damage increases its ability to activate the transcription of pro-

apoptotic genes (COSTANZO *et al.* 2002). As demonstrated here, 16E7 is able to reduce the HAT activity of p300, which leads to the speculation that the 16E7 repression of p300-mediated transcriptional activity of p73 may result from the inhibition of p73 acetylation and further studies need to establish whether or not 16E7 may block p73-mediated apoptosis as a result of this inhibition of acetylation. Interestingly, Ad E1a also inhibits the transactivation by p73 and a deletion mutant of E1a incapable of interacting with p300/CBP is defective in this assay (DAS *et al.* 2003). Furthermore, CBP increases the transactivation mediated by p73 suggesting that CBP may function as a co-activator so that E1a inhibits p73-mediated transactivation by sequestering p300 or CBP. However, in the experiments presented in this thesis high-risk HPV-16E7 protein seems to be a more potent inhibitor of p300 transactivation function upon a p73-responsive promoter than E1a and this may suggest that both viral proteins employ different mechanisms in order to inhibit p300 co-activation function with respect to p73.

It has been demonstrated that E1a transcriptional repression involves interaction with p300/CBP and interference with the formation of a TBP-TATA box complex (BOYD *et al.* 2002). The region of E1a involved in the binding to p300 is also critical for E1a transcriptional repression and for its ability to interfere with TBP-TATA box complex formation. Some studies suggest that one consequence of the E7:TBP interaction is abolition of TBP DNA binding activity thereby providing an explanation for the transcriptional inhibitory effects of E7 (MALDONADO *et al.* 2002). It has also been speculated that E7 interaction with TBP mediates the inhibition of p53 transcriptional activity (MASSIMI and BANKS 1997). Whether E7 mediated p300/CBP co-transcriptional

repression accounts for the effects observed should be further examined, the results from the competition binding assays allow us to speculate that since 16E7 can displace TBP from the binding site on p300, then the binding of TBP and E7 to p300 is apparently mutually exclusive. This suggests that E7 may interfere with TBP function through its interaction with p300.

In summary, the results presented in this part of the thesis would suggest that repression of p300 transactivation function by high-risk E7 provides means of regulating both viral and cellular gene transcription both of which may be important steps in the process of viral mediated oncogenesis.
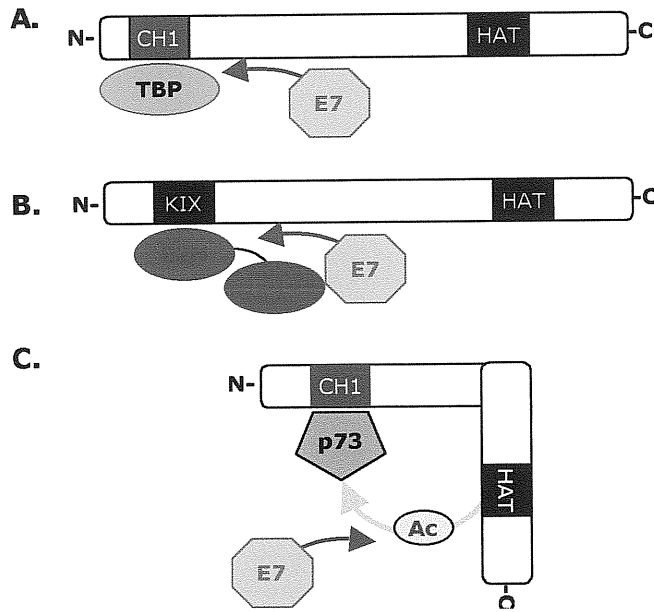
Figure 30. Mechanisms by which HPV-16E7 may inhibit p300 transcriptional co-activation function. **Panel A** and **B.** 16E7 may compete with other proteins for the binding site on p300 as in the case of TBP and E2 binding to the CH1 domain of p300. **Panel C.** 16E7 may inhibit the HAT activity of p300 affecting acetylation of p73 and/or other substrates.

3.3. CONCLUSIONS

In this thesis I have presented evidence that both HPV E6 and E7 interact with the same cellular target – the p300 transcriptional co-activator. Such a phenomenon is not a rare event among viral transforming proteins. An example of this is the adenovirus E4orf6 and E1B55K proteins both interacting directly with the p53 tumour suppressor (QUERIDO *et al.* 1997) and EBV EBNA-2 and EBNA-3C/ ProT-α interacting with p300 (SUBRAMANIAN *et al.* 2002; WANG *et al.* 2000).

Here, I have also demonstrated that the binding to p300 is not limited only to high-risk HPV E6 proteins, but is common for mucosal, cutaneous and animal PV E6 proteins. This high degree of evolutionarily conserved function suggests that the interaction between E6 and p300 is a common requirement for viral replication. The functional significance of this interaction is supported by the observation that high-risk E6s can complement a transformation deficient Ad E1a mutant, which, together with the data demonstrating p300 transcriptional repression by high-risk E6 (PATEL *et al.* 1999; ZIMMERMANN *et al.* 1999) would suggest that transcriptional repression of p300/CBP may be necessary for viral-induced transformation.

Both the high- and the low-risk HPV E7 protein were also found to interact with p300 and, as a consequence of this interaction, the transcriptional activation function of p300 is greatly reduced. HPV-16E7 represses the transcriptional co-activation function of p300 in at least two ways: by blocking protein-protein interactions and by blocking HAT activity of p300. The role of the E7:p300 interaction in cellular transformation is highlighted by the fact that mutants of E7 which fail to bind p300 are reported to be defective in

transformation.

p300/CBP has been shown to activate transcription by at least 2 mechanisms: one involving the bridging of DNA-bound transcriptional factors to components of the basal transcriptional machinery and the other involving histone and non-histone protein acetylation. p300/CBP are potent transcriptional co-activators, involved in regulation of diverse aspects of cellular homeostasis, and it is therefore not surprising that this function of p300/CBP is targeted by the HPV oncoproteins. It seems likely that the HPV oncoproteins can abrogate the function of p300 by targeting both mechanisms of p300-mediated transcriptional activation, and the results presented here favour these assumptions. Competition for the p300-binding site as well as interference with its acetyltransferase activity (Figure 30) seem to be a common mechanism of interference with p300/CBP function and is well conserved among the different viral proteins.

Taken together the results presented in this thesis demonstrate a complex pattern of interaction between HPV oncoproteins and the p300 transcriptional co-activator. At this point a dissection of the respective functions of E6 and E7 upon p300/CBP activity is necessary. As the binding sites of E6 and E7 partially overlap, an interesting approach would be to determine whether the functions of both viral oncoproteins overlap, synergise or whether there are different mechanisms which they employ in order to inhibit different p300 functions. It is possible that differential usage of different p300 domains by viral oncoproteins may be responsible for the diverse effects seen upon p300 function. It seems likely that E6 would express its function upon p300 mainly through interaction with the CH3 domain, and by this interfere with the p53-

related functions, whilst E7 binding mainly to the CH1 domain may affect

other p300 effectors such as p73 and TBP.

The work presented here provides an indication of the possible consequences

of impairment of p300 function. Loss of p300 transcriptional co-activation

function parallels some aspects of the acquisition of a malignant phenotype,

confirming the importance of E6 and E7 in targeting this cellular protein in the

malignant progression of HPV-induced cervical dysplasia.

Most of the work described in this thesis is contained in the following articles:

1. E. Guccione, P. Massimi, A. Bernat, L. Banks. (2002). Comparative analysis of the intracellular location of the high- and low-risk Human Papillomavirus oncoproteins. *Virology;* 293:20-25.

2. A. Bernat, P. Massimi, L. Banks. (2002). Complementation of a p300/CBP defective binding mutant of adenovirus E1a by human papillomavirus E6 proteins. *J Gen Virol;* 83:829-33.

3. A. Bernat, N. Avvakumov, J.S. Mymryk and L. Banks. (2003). Interaction between the HPV E7 oncoprotein and transcriptional co-activator p300. *Oncogene;* 22:7871-7881

4. MATERIALS AND METHODS

4.1. Plasmids

The following expression constructs were used in this study:

E6

A number of previously described PV-E6 containing plasmids were used:

Fo *vivo* expression: pcDNA$_3$: 1E6, 6E6, 11E6, 16E6, 18E6, BPVE6, CRPVE6, pcDNA$_3$.ΔA (47-49aa) 18E6 (PIM *et al.* 1994), pcDNA$_3$-Flag.Δ123-127 16E6 (PATEL *et al.* 1999) was a kind gift of D. McCance.

For *in vitro* expression: pSP$_{64}$: 11E6, 18E6; some of the constructs used for *in vivo* were also used for *in vitro* expression: pcDNA$_3$: 1E6, 6E6, 8E6, BPVE6, CRPVE6, pcDNA$_3$.ΔA (47-49aa) 18E6, pcDNA$_3$-Flag.Δ123-127 16E6.

E7

The following wild type and mutated E7 expression plasmids were used in this study: pETHis$_6$.16E7 (PRATHAPAM *et al.* 2001), pJ4Ω.16E7 (STOREY *et al.* 1988), pcDNA$_3$-HA.16E7, pGEX2T.11E7 and pGEX2T.16E7 (MASSIMI *et al.* 1996). A number of previously described mutants of HPV-16E7 were used: the deletion mutants within CR3 domain of 16E7: Δ1:52-55aa, Δ2:65-67aa, Δ3:75-77aa, Δ4:79-83aa (MASSIMI *et al.* 1996); mutants within the CR2 domain of E7 (EDMONDS and VOUSDEN 1989): p24 (C24G, deficient in pRb binding) and pCKII (S31R, S32P, mutant deficient in CKII phosphorylation) as well as N-terminal mutant p2 (H2P). The pCKII mutant subcloned into pcDNA$_3$-HA (*BamHI/EcoRI*) was used as a substrate for p24 mutagenesis (C24G) using the Gene Tailor^{TM} Site-Directed Mutagenesis System (Invitrogen) to generate the double mutant p24-CKII (C24G, S31R, S32P). This was done using the following pair of primers:

forward: CAGAGACAACTGATCTCTCTAACGGATATGAGCAAT

and reverse: GTAGAGATCAGTTGTCTCTGGTTGCAAATC.

The resulting double mutant was then verified by DNA sequencing.

p300

The following plasmids were used for p300 expression studies:

For *in vivo* expression: pCMVβ.p300 which was a kind gift from J.Mymryk.

For *in vitro* studies: pGEX.p300 plasmids (fragments 1-5, spanning residues: 1-672; 672-1193; 1069-1459; 1459-1892; 1983-2414, respecively) were

kindly provided by S. Hasan, pGEX.HATp300 (1195-1810) was a gift from E. Verdin. pGEX2T.TAZp300 and pGEX2T.KIXp300 were generated by cloning fragments spanning 336-430 aa for TAZ and 568-646aa for KIX into pGEX2T (*BamHI/EcoRI*) PCR products obtained by amplification with following primers:

TAZ:

forward: TAATAAGGATCCCCAGAGAAGCGCAAG

and reverse: TAATAAGAATTCACTCAAAATTGGCTG;

KIX:

forward: TAATAAAGATCTCGGAAACAGTGGCAC

and reverse: TAATAAGAATTCACTTTCTTCTAGTTCTTTCTC

Constructs were then verified by DNA sequencing.

The following additional plasmids were used in this study: for CAT analysis: pJ4Ω.E2 (LEES *et al.* 1990), pTKM$_{32}$.6xE2CAT (THIERRY *et al.* 1990), pCH$_{10}$.β-gal, pEJ6.6 (EJ-ras) (SCHNEIDER *et al.* 1987); for E1a complementation assays: pCE.E1a (SCHNEIDER *et al.* 1987), pCMV.ΔNE1a (2-36) was a gift from T. Dobner; for GST-pull down assays: pGEX2T.p53 and pGEX2T.TBP (MASSIMI *et al.* 1996), pGEX2T.11E6 and pGEX18E6, pGEX2T.NTdlg (GARDIOL *et al.* 1999); for p73α studies: pcDNA$_3$-HA.p73α and pTkRLuc (*Renilla*) (pRL) were kind gifts of F. Mantovani, pBaxLuc (*Firefly*), was a kind gift of M. Gostissa (ZACCHI *et al.* 2002).

4.2. Antibodies

The following antibodies were used in this study:

Primary antibodies: anti-p300 mouse monoclonal (NM11, Pharmingen); anti-p300 mouse monoclonal (Rw128, Upstate Biotechnology); anti-16E7 mouse monoclonal (ED17, Santa Cruz); anti-16E7 rabbit polyclonal antibody (MASSIMI *et al.* 1996); anti-HA rat monoclonal (3F10, Roche); anti-HA mouse monoclonal (Y-11, Santa Cruz); anti-B-galactosidase mouse monoclonal (Promega); anti-acetylated lysine, rabbit polyclonal (9441, Cell Signalling); anti-α-actin, (rabbit polyclonal, Sigma); anti-16E2 (BOUVARD *et al.* 1994b). If not otherwise stated, these were used for WB in 1:1000 or 1:500 dilution. Secondary antibodies were used as follows: anti-mouse-biotin (DAKO); anti-rabbit-biotin (DAKO); followed by avidin-HRP (DAKO) incubation; **OR** anti-

mouse-HRP, (Sigma); anti-rat-HRP (DAKO); anti-rabbit-HRP (Sigma), rhodamine conjugated goat anti-rabbit, Molecular Probes and fluorescein conjugated goat anti-mouse, Molecular Probes. If not stated, these were used in 1:1000 or 1:2000 dilution.

4.3. GST fusion protein expression and purification

For protein production and purification, 50 ml of an overnight culture of *E. coli* strain BL-21 previously transformed with the appropriate expression plasmids were diluted 1:10 in Luria Broth containing ampicillin and grown at 37°C up to an OD of 0.6. Recombinant protein expression was induced for four hours with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (20mM Tris-HCl pH 7.5, 250mM NaCl, 0.5% NP40, 2μg/ml aprotinin, 100μM TPCK and 50μM TLCK, 1mM PMSF **OR** 50mM Tris-HCl pH 8.0, 250mM NaCl, 5% glycerol, 5mM EDTA, 0.5% NP40, 2mM DTT, 2μg/ml aprotinin, 100μM TPCK and 50μM TLCK, 1mM PMSF) and the lysates were then cleared from cell debris by centrifugation at 13000 rpm. The GST-fusion proteins were then incubated for one hour at 4°C with glutathione-conjugated agarose beads (Sigma) and bound proteins were washed extensively several times with lysis buffer. The levels and purity of proteins were determined by SDS-PAGE and subsequent Coomassie Brilliant Blue R (Sigma) staining.

4.4. Purification of His$_6$ 16E7

The BL-21 cells transformed with pETHis$_6$ 16E7 were induced with 1mM IPTG for 3 hours. The cells were harvested in lysis buffer (10mM Tris-HCl pH 8.0, 300mM NaCl, 0.1M phosphate buffer pH 8.0) disrupted by sonication and cell debris were removed by centrifugation. The supernatant was incubated with Ni-NTA (Qiagen Inc.) for 1 hour at 4°C. The complexes were then washed and the bound protein was eluted with increasing amounts of imidazole. The purity of the protein was determined by SDS-PAGE and Commassie Brilliant Blue R (Sigma) staining.

4.5. Cell culture and transfections

The following cell lines were used in this study:

- U2-OS (human osteosarcoma),
- SaOS-2 (human osteogenic fibrosarcoma),
- CaSki (human cervical epidermoid carcinoma, HPV16 positive),
- BRK (baby rat kidney cells) obtained from 9day-old Wistar rats; the BRK cell line expressing HPV-16E7 was obtained by transfecting primary BRK cells from 9-day old Wistar rats with EJ-ras (pEJ6.6) plus HPV-16E7 in pJ4Ω. After 2 weeks under G418 (Genticin, GibcoBRL) selection (100mg/ml stock, final concentration: 2.5µg for 1ml of DMEM) single transformed colonies were picked and expanded.
- CV-1 (African green monkey kidney cells)
- HaCaT (immortalized human skin keratinocytes)

All cells were grown in DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and 100 µM streptomycin at 37^0C and 10% CO_2. Unless otherwise stated, transfections were done using standard calcium phosphate precipitation (WIGLER *et al.* 1979) or in case of luciferease assays by lipofection with Superfect® Transfection Reagent (Qiagene Inc.).

4.6. Mammalian two-hybrid assay

The HPV16-E7 gene was expressed as a fusion to GAL4p DNA binding domain in the vector pM, while the indicated in the Figure 20, portions of p300 or CBP were expressed as fusions to the VP16 activation domain in vector pVP16 (Clontech). The (gal)$_6$-Luc reporter plasmid contains a luciferase gene immediately downstream of six repeates of GAL4p binding region and a minimal TATA box and was generously provided by Dr. J. Torchia. CV-1 cells were transfected using Effectene transfection reagent (Qiagen Inc.) according to the manusfacturer's instructions. Luciferase assays were performed 48h post-transfection.

4.7. BRK focus formation assay (E1a complementation assays)

Primary BRK cells were obtained from the kidneys of 9-day old Wistar rats. The tissues were first washed several times in DMEM without serum, then homogenised and finally trypsinised using 0.25% Trypsin solution in PBS. After at least three steps of trypsinisation, 15 minutes each at 37°C, the reaction was blocked with 50% FCS. The cells were then cenrtifugated for 10 minutes at 12000rpm and the pellet was resuspended in supplemented DMEM. From one rat enough cells were obtained for plating 6-8 tissue culture dishes (Ø100 mm). After two days in culture the BRK cells were co-transfected with the Adenovirus E1a mutant ΔN (2-36), defective in p300-binding and deficient in transformatin assays, together with different combinations of the E6 expression vectors, in the presence of EJ-ras as a cooperating, activated oncogene. After 2 weeks under G418 (Genticin, GibcoBRL) selection (100mg/ml final concentration: 2.5µg for 1ml of DMEM), the cells were fixed with formaldehyde (3,7% solution, Merck), stained with Giemsa-Blue (10% solution, Diagnostica Merck) and colonies were counted.

4.8. Chloramphenicol acetyltransferase (CAT) reporter assay

To measure the co-transcriptional activity of E2 and p300, cells were transfected with 1µg of 6xE2CAT, 0.5 µg of pSV.β-gal (to standardize E2 activation values for transfection efficiency) plus 1µg of pJ4Ω.16E2, 5µg of pCMV-β.p300 and either 1 or 5µg of pJ4Ω.16E7, 5µg of pJ4Ω.11E7, 5µg pJ4Ω.p24, 5µg pCE.E1a as indicated in the text. The total amount of DNA was equalized with empty plasmids. Cells were harvested after 48h, lysed (40mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA) and subjected to three cycles of freezing and thawing, cell debris were removed by centrifugation. CAT assays were then carried out as described previously (PIM *et al.* 1994). Anti- β-gal WB was done for control of transfection efficiency.

4.9. Dual Luciferase Reporter Assay

SaOS-2 cells were transfected by lipofection (Superfect® Transfection Reagent Qiagene Inc.) with 1µg of reporter pBaxLuc, 0.1µg of pcDNA$_3$ p73, 0.2µg pCMV-β.p300 and 0.2 or 0.4µg of the HPV-16E7 expression plasmids (all as pcDNA$_3$ vectors). Total amount of DNA was equalized with empty plasmids. Simultaneous expression of pRL plasmid provided an internal control of baseline response and allowed for the normalization of transfection efficiency. 24 hours post transfection, cells were lysed and luciferese activity was measured using the Dual-luciferese® assay kit as recommended by the manufacturer (Promega).

4.10. *In vitro* transcription-translation and GST pull-down assays

Proteins were expressed *in vitro*, using the TNT reticulocyte lysate system (Promega) in the presence of L-^{35}S-labeled cysteine (Amersham 0.6µCi/1µl of reaction volume) or tran-^{35}S-labeled methionine (ICN) for 1-2h. This was then incubated for 1h at room temperature with equal amounts of GST fusion proteins bound to glutathione-linked agarose beads. The reaction was carried out in a final volume of 50µl, in a binding buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2.5 mM EDTA. Bound proteins were washed extensively in PBS/0.5%NP40. SDS-PAGE and autoradiography were used to analyze the pattern of bound proteins. Assays were quantitated using a Phosphoimager (Instant Imager, Packard). For the *in vitro* competition assay the amount of *in vitro* translated products in each sample were equalized with water-primed reticulocyte lysate.

4.11. Western blotting and co-immunoprecipitation

For co-immunopreciptations cells were rinsed in ice-cold PBS and lysed on ice in buffer B (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0,5% NP40, 5% glycerol, 100 µM TPCK, 50 µM TLCK). Lysates were cleared by centrifugation at 13000 rpm for 10 minutes, incubated (around 1µg/reaction) with the appropriate specific antibodies (specified in the text) for times ranging from 1 hr to overnight at 4°C, and subsequently mixed with either protein A or protein G agarose beads (Amersham Pharmacia Biotech.) for 40 minutes. Where

necessary, antibodies were covalently bound to the protein A or protein G using 5mg/ml dimethylpimelimidate (Pierce) as crosslinker.

For western blotting, cellular extracts were prepared as described above. Protein concentrations were determined using the Bio-Rad Protein Assay System. Equal amounts of cell extract were then separated on SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell, pore size Ø 0.22µm or Ø 0.45µm) Proteins were detected with the appropriate specific antibodies (specified within the text) and developed using either the Amersham ECL System according to the manufacturer's instructions or with SuperSignal® West Femto Maxiumum Sensitivity Substrate (Pierce).

4.12. Immunofluorescence and confocal microscopy

24h after transfections cells were washed in PBS and fixed with 3% PFA for 15 minutes at room temperature. After further washing, the cells were permeabilized with 1% Triton for 5 minutes and then washed extensively with PBS. Endogenous and overexpressed p300 was visualized using an anti-p300 monoclonal antibody (NM11 Pharmingen) diluted 1:100 in PBS. HA-tagged E7 was visualized using a rabbit anti-HA antibody (Santa Cruz Y-11) diluted 1:200 in PBS. After 1h incubation at 37^0C and extensive PBS washing, secondary antibodies (rhodamine conjugated goat anti-rabbit and fluorescein conjugated goat anti-mouse), both diluted 1:1000 were added. The cells were incubated at 37^0C for 20 minutes then washed extensively, first with PBS and then with water, and finally mounted on glass microscope slides using Vectashield mounting medium (Vector Labs Inc.). A Zeiss Axiovert 100M microscope with 100x objective oil-immersion lens was used for confocal laser scanning microscopy. An LSM 510 confocal unit with two lasers giving excitation lines at 543nm and 488nm was attached. Data were collected at 1024x1024-pixel resolution. Scanning conditions were kept constant in each experiment to ensure that the signal overlap between channels was essentially eliminated.

4.13. *In vitro* acetylation assay

Resin-bound GST-HATp300 was washed several times with HAT buffer (50mM Tris-Hcl pH 8.0, 0.1mM EDTA pH 8.0, 10% glycerol, 1mM DTT, 1mM PMSF, 10mM sodium butyrate) and then mixed with 2µl histones (all four histones, 10mg/ml, Sigma), Ac-CoA (10mM final concentration, Boehringer Mannheim) and purified His-tagged HPV-16E7. The reaction was carried out in 30µl-50µl volume in HAT buffer for 1h at 30ºC. The reaction was stopped by adding SDS-PAGE sample buffer. Complexes were separated by SDS-PAGE and the presence of acetylated products was verified by WB with anti-acetylated lysine antibody (1:4000, Cell Signaling) followed by anti-rabbit-HRP (1:2000, Sigma).

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