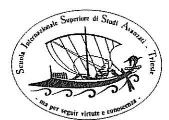

ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

New Cellular Targets of HPV-16 E7

Thesis submitted for the Degree of
Doctor of Philosophy

Candidate:
Dr. Tulasiram Prathapam

Supervisor:
Dr. Lawrence Banks

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TRIESTE
Via Beirut 2-4

TRIESTE

Dedicated to

My beloved parents

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INDEX

Title: New Cellular Targets of HPV-16 E7
Acknowledgements
Summary........1
Introduction........2

1. General Introduction........2
   1.1. History
2. Genome Organization........4
3. Viral life cycle........5
   3. 1. Early genes involved in transcription and replication
4. Association of HPV with cancer........9
   4.1. Epidemiological studies
   4.2. HPV-E6 and E7 are continually expressed and retained in cervical tumours and derived cell lines
   4.3. Transformation assays
      4. 3.1. Established rodent cells
      4. 3.2. Primary rodent cells
      4. 3.3. Primary human cells
         3. 3 a. Keratinocyte immortalisation
         3. 3 b. Mammary epithelial cells
5. Properties of the HPV Oncoproteins........13
   5. 1. HPV E5 Oncoprotein
   5. 2. HPV E6 Oncoprotein
   5. 3. HPV E7 protein
6. HPV E6 and E7 proteins target the products of two tumor suppressor genes, p53 and pRb, respectively........20
   6.1. p53
   6.2. Retinoblastoma gene
   6.3 Role of p53 and pRb in cell cycle regulation
7. Interactions of E7 with the cellular proteins........26
   7. 1. Disruption of cell cycle control through its interaction with the pocket proteins
   7. 2. Interaction of E7 with the cyclin/cdk2 inhibitors
   7. 3. Interaction with cyclins and cyclin/cdk complexes
   7. 4. Interaction with elements of cellular transcriptional machinery
   7. 5. Association with IGFBP-3, involved in growth control and apoptosis
   7. 6. Association of E7 with the carbohydrate metabolism
   7. 7. Interaction of E7 with immune modulating molecules

Results........43

1. Identification of new interacting partners of HPV-16 E7........43
2. Targets........44
3. E7 interacts with Skip through its carboxy terminal domain........47
4. The interaction between HPV-16 E7 and Skip is direct........48
5. Identification of the Skip binding domain on HPV-16 E7........49
6. Identification of the E7 binding motif on Skip........50
7. Skip stimulates the activation of diverse viral and cellular promoters........51
8. Cellular Ski and Skip cooperate in transcriptional transactivation........54
9. HPV E7 abrogates the transactivation function of a Skip-Ski complex........55
10. Identification of the Ski interacting domain on Skip........57
11. Characterization of SNW domain of Skip........58

Discussion......59

1. Identification of novel binding partners of HPV-16 E7...... 59
   - 1.1. Targets of E7 with known function
     - 1.1 A. Human AEBP
     - 1.1. B. Rab-32
     - 1.1 C. SAP-145 and ZNF74
   - 1.2 Targets of E7 with unknown function
2. Interaction of HPV-16 E7 with Skip...... 63
3. Skip acts as co-activator of many diverse promoters......65
4. Skip and Ski cooperate in transcriptional activation...... 66
5. HPV-16 E7 silences the co-activation function of Skip......67
6. Why does E7 inhibit Skip function? 68
7. Mechanism of E7 inactivation of Skip......70
8. Ski interacts with the evolutionarily conserved SNW domain of Skip......71
9. SNW is the key functional domain of Skip...... 73
10. Future prospects......75

Materials and Methods...... 79

References......87

Summary

Cervical cancer is the third most common cancer in women, accounting for 9.8% of all new cancer cases worldwide. Human papillomaviruses (HPVs) play a central role in the etiology of cervical neoplasia. E7 is the major transforming protein of HPVs. The transforming activity of E7 has been attributed in part to its interaction with the retinoblastoma (pRb) tumour suppressor; however, the pRb interaction alone is not sufficient for transformation by E7. In a screen for cellular targets of HPV-16 E7, I identified the Ski interacting protein, Skip, as a new interacting partner of E7. I show that HPV-16 E7 directly associates with Skip via sequences in its carboxy terminal region, and the evolutionarily conserved proline rich sequences (PRS) of the SNW domain of Skip.

Skip has been found to bind to the highly conserved region of v-Ski that is required for its transforming activity, although the mechanism is not yet elucidated. I first investigated the biochemical significance of the Ski- Skip interaction in more detail. Here, I have mapped the interaction site between Ski and Skip. I show that Skip acts as a generalised transcriptional co-activator, using a variety of different promoters. In addition, ectopic expression of Skip and Ski results in synergistic co-activation, which is dependent upon the Skip-Ski protein interaction. The Transcriptional trans-activating activities of Skip were mapped to the evolutionarily conserved core SNW domain of the protein. Moreover, this region was found to be essential for its ability to interact with c-Ski, and this data therefore provides a function for the Skip SNW domain. Taken together, these studies suggest that one of the ways in which Ski brings about transformation is by binding and cooperating with the SNW domain of Skip in transcriptional activation. Interestingly, both E7 and Ski oncoproteins bind to the same conserved SNW domain, suggesting that this is the key functional domain of Skip.

HPV-16 E7 functionally targets Skip *in vivo* and inhibits its transcriptional activation activity. However, E7 does not induce degradation of Skip. Two transformation defective mutants of E7 were identified that failed both to bind Skip and to inhibit its transcriptional activity. These results suggest that inhibition of Skip function may contribute to cell transformation by HPV-16 E7.

INTRODUCTION

1. General Introduction:

Human Papillomaviruses (HPVs) are small double stranded DNA viruses and are associated with several important clinical manifestations in humans. Some of the examples are shown in Table 1. Papillomaviruses are named from the Latin word papilla, which means 'nipple' or 'pustule', to denote their pathogenic activity. Over a hundred different viral types have now been identified (de Villiers 1994; zur Hausen 2000, review). Although most of the HPV induced lesions are of a benign phenotype, increasing evidence suggests that HPVs play a role in the development of more than 10% of all the human cancers worldwide (zur Hausen 1996a; zur Hausen 1996b). The most important of these is cervical cancer (zur Hausen and Schneider 1987; zur Hausen 1991). During the last few decades, much knowledge has been gained on the mechanisms by which these tumour viruses disrupt cell cycle control (Barbosa 1996, review; zur Hausen 1996a; zur Hausen 1996b; Pim et al. 2001). This introduction will therefore give a brief review of HPVs in general, in addition to the data most relevant to the objectives of the research presented in this thesis.

Table 1. HPV types and their association with lesions

Mucosal
- **High-risk:** genital intraepithelial neoplasia and cancers
  - E.g. HPV-16, 18, 31 and 33
- **Low-risk**: benign genital warts
  - E.g. HPV-6 and 11

Cutaneous
- **High-risk**: benign and malignant EV lesions
  - E.g. HPV-5 and 8 (EV: Epidermodysplasia Verruciformis)
- **Cutaneous Low-risk**: benign skin warts
  - E.g. HPV-1, 2 and 4

1. 1. History

The first indications for the existence of viral infectious agents were reported about 100 years ago (Reed 1902; Loeffler and Frosch 1964; Harrison and Wilson 1999, review). An experimental system to study the nature of viruses was developed after isolation of Shope papillomavirus in the 1930s (Shope 1933). This virus was isolated from the cutaneous papillomas of the cottontail rabbit, and provided one of the first experimental examples of a DNA virus with oncogenic properties. (Rous and Beard 1934). The cottontail rabbit papillomavirus (CRPV) has the ability to produce papillomas in domestic rabbits, which can progress to carcinomas (Rous and Beard 1935). Malignant progression of these papillomas can be accelerated by cofactors such as tarring of the skin after infection or additional treatment with chemical carcinogens (Rous and Friedewald 1944). Later, the presence of viral particles in certain human warts could be visualized by electron microscopy and the physical properties of the viral DNA were elucidated (Strauss et al. 1949; Crawford and Crawford 1963). Progress in the characterization of papillomaviruses was, however, impaired because of the lack of a suitable tissue culture system. The virus is an exclusively intra-epithelial pathogen, with an infectious cycle totally dependent upon the growth and differentiation of the keratinocyte. After the development of molecular cloning techniques in the 1970s, a papillomavirus (PV) genome could be cloned, and sequenced, and gene functions began to be elucidated. Further breakthroughs were made after the development of transformation assays for the Bovine Papillomavirus type 1 (BPV 1), which could also induce tumours and served as a prototype for the papillomaviruses (Black et al. 1963; Boiron et al. 1964). These assays allowed the analysis of viral gene functions involved in cellular proliferation (Lowy et al. 1980). The additional isolation of HPV types 6 and 11 from genital warts (Gissmann and zur Hausen 1980; Gissmann et al. 1982) and subsequently HPV 16 and 18 (Dürst et al.

1983; Boshart et al. 1984) from cervical cancer biopsies linked certain HPV types to cancer in humans.

2. Genome Organization:

Despite the great variety of PV types, either of human or animal origin, all the members of this virus group have a remarkably conserved genome which consists of about 8000 bp of closed circular double stranded DNA. The DNA is encapsidated in a virion of icosahedral symmetry composed of 72 protein capsomers, but lacking a lipid-containing envelope (Orth et al. 1977; Chen et al. 1982; Danos et al. 1982; Schwarz et al. 1983). Only one strand is transcribed (Engel et al. 1983; Pfister 1984) and contains 8-10 open reading frames (ORFs) designated as either early (E1 to E7) or late (L1 and L2) (Figure 1), by analogy with other DNA viruses where genes are expressed at different times during the course of a productive infection. Early genes are expressed shortly after infection in the non-productive basal part of the wart and prior to the onset of DNA replication. The products of these genes mediate specific functions controlling replication and expression of viral DNA. Late genes code for structural proteins of viral particles, and are expressed during the final stages of the viral life cycle in the differentiated, productive cells of the wart.

The different ORFs are expressed by a combination of alternative splicing and use of early and late promoters. Alternative splicing might play an important role in the regulation of E6 and E7 proteins of high-risk papillomaviruses. For example, splicing in the E6 ORF to give E6* I-IV, species certainly affects the amount of full length E6 protein that can be translated from an E6 mRNA and may also produce E6 proteins with altered functions (Pim et al. 1997). In addition, this splicing may also regulate the translation of E7 ORF by affecting the translation-initiation at the AUG in the ORF, although opposite results have been reported (Sedman et al. 1991; Stacey et al.

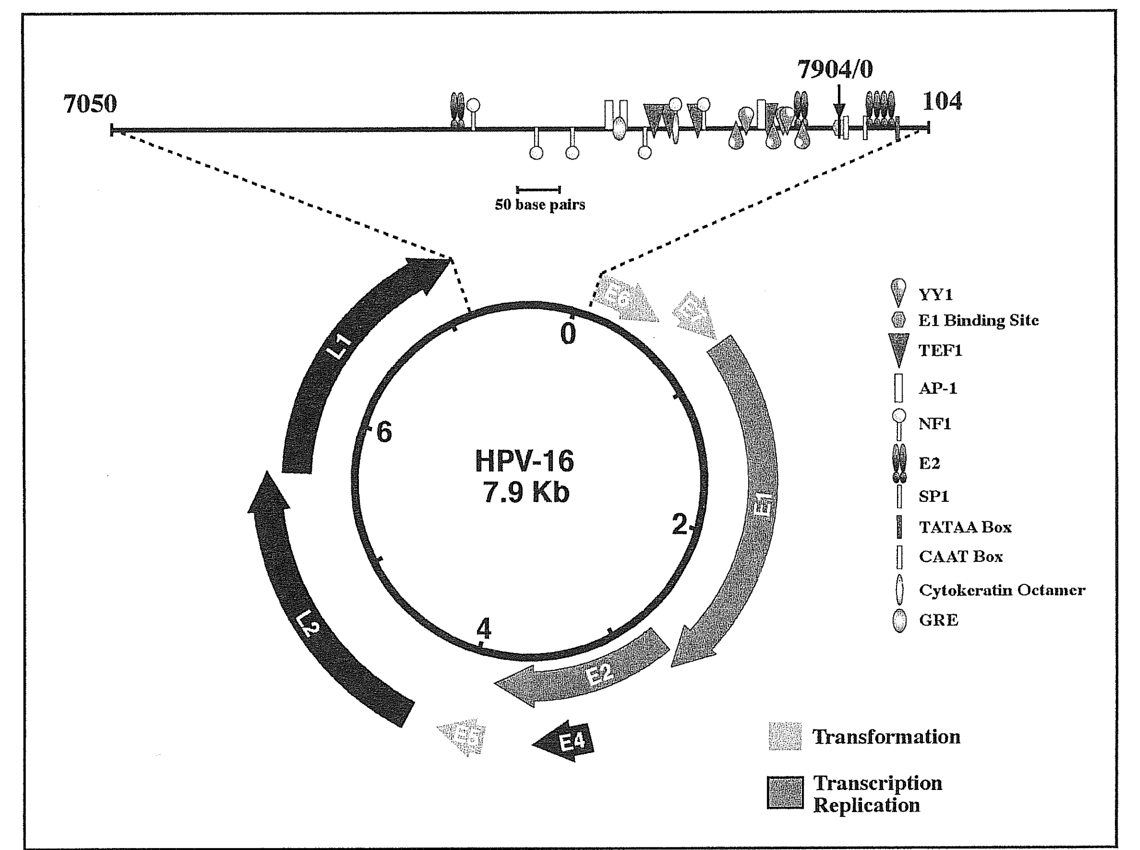


Figure 1. Viral genome organization and function of proteins
The URR (Upstream regulatory region) contains promoters, cellular dependent enhancers and multiple binding sites for the viral E2 protein essential for viral DNA replication and gene expression

1995; Stacey et al. 2000). However, this splicing event does not affect the levels of E7 in differentiating keratinocytes (Cheng et al. 1995).

The non-transcribed upstream regulatory region (URR), which covers about 10% of the genome (Figure 1), contains enhancers and cis-acting sequences essential for the transcription and replication of viral DNA . The transcription of viral genes is tightly regulated in infected cells as different viral RNAs are expressed at different levels within the epithelium. The regulation of viral gene expression is complex and controlled by viral E2 (McBride et al. 1991; Stubenrauch et al. 1998), and cellular transcription factors such as e.g. AP1, NF-1, SP-1 and the glucocorticoid receptor, which act on different enhancer elements within the URR (Gloss et al. 1987; Gloss and Bernard 1990; Thierry et al. 1992; Butz and Hoppe-Seyler 1993; Turek 1994). In contrast, Oct1 and YY1 have been described to negatively interfere with the expression of the early RNAs (Hoppe-Seyler et al. 1991; Bauknecht et al. 1992).

3. Viral life cycle:

Mammalian epidermis consists of proliferating basal cells, which are withdrawn from the cell cycle upon entering the suprabasal layer. Cells then migrate further to the epithelial surface, undergoing a series of morphological and biochemical changes that culminate in their becoming the anucleate keratinocytes that make up the outermost cornified layer. Cells are lost by shedding as a result of abrasion of the epidermal surface (Figure 2) (Byrne 1997). Papillomaviruses infect squamous epithelial cells (Figure 2) although the virus can attach to, and enter, a wide range of cells in cell culture (Roden et al. 1994; Muller et al. 1995).

Viral replication is restricted to keratinocytes as it is dependent on the differentiation of epithelial cells (Olson 1987). For the establishment of a wart or papilloma, basal cells need to be infected since these are the only dividing cells of the squamous

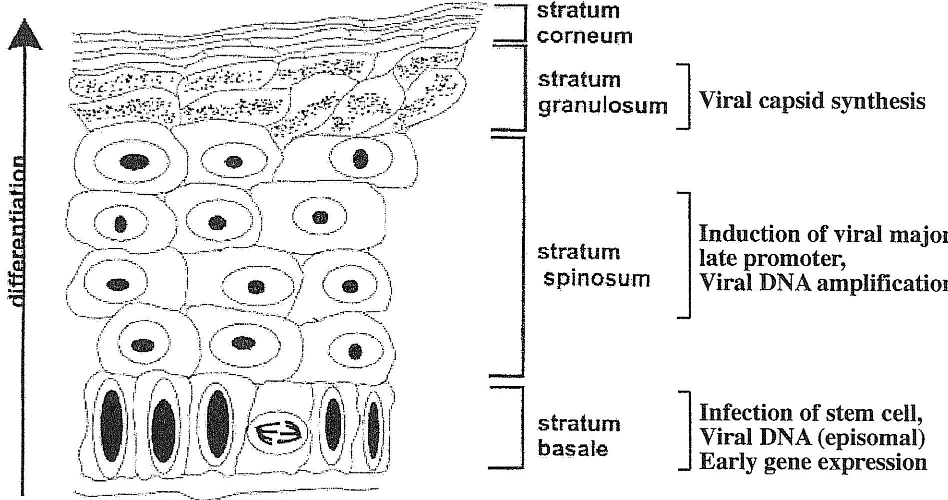


Figure 2. Differentiation dependent functions in HPV-infected epithelial cells

epithelium (Figure 2). The receptor for viral entry is thought to be the alfa-6 integrin (Evander et al. 1997), which in epithelial cells is confined to the basal layer and is present on epithelial stem cells (Li et al. 1988). Following entry into the cell, HPV genomes are established as extra-chromosomal elements in the nucleus. Papillomaviruses are dependent on host replication machinery since they lack their own DNA polymerase. To replicate their viral genome, the infected cells have to be forced to continue DNA replication throughout the suprabasal layer (Figure 2). Papillomavirus early gene products have the ability to ensure cell cycle progression and hence activate the host replication machinery even after the cells have left the basal layer. This continuous proliferation results in the characteristic wart or papilloma from which the virus name is derived. The viral genome, present at low copy number in the basal cells, is then replicated to high copy number in the now proliferating suprabasal cells. Production of late proteins and viral assembly occurs as cells reach the epithelial surface and die (Stoler et al. 1990). The viral life cycle is completed as new infectious virions are released upon normal wear at the epithelial surface.

3. 1. Early genes involved in transcription and replication

Following infection, the first viral genes to be expressed are the viral replication proteins E1 and E2.

The **E1 protein** is the largest ORF of papillomaviruses, it encodes a 68 kD ATP-binding phosphoprotein, which shares structural features with the large T antigen of simian virus 40 (SV40) such as site specific DNA binding, ATPase and helicase activity (Clertant and Seif 1984; Bream et al. 1993; Hughes and Romanos 1993; Seo et al. 1993). The low affinity binding of E1 protein to the origin of replication within

the URR is increased in presence of the E2 protein (Chiang et al. 1992; Seo et al. 1993; Frattini and Laimins 1994).

The **E2 protein** acts as a major viral transcriptional regulator. Viral gene expression is regulated by dimerization of different E2 proteins at specific binding sites on the URR. Transcriptional activation is observed after dimerization of two full-length proteins. Dimerization of a full-length E2 with a truncated form, which lacks the transactivation domain, results in transcriptional repression (McBride et al. 1991). The repression activity of E2 could be due to competitive formation of E2 heterodimers, suggesting that the ratio of two forms is critical for the control of transcription and could have a crucial bearing on the levels of E6 and E7 expression. At low levels, the full length HPV 16 E2 protein can transactivate the p97 promoter in cervical keratinocytes with the up-regulation of levels of viral oncoproteins E6 and E7 expression, whereas increased intracellular levels of E2 result in repression of HPV transcription (Bouvard et al. 1994; Steger and Corbach 1997). In BPV1, E2 mediated transactivation has been shown through the direct association with two components of cellular transcription machinery TFIID and TFIIB (Miller-Rank and Lambert 1995). Besides its role in transcriptional regulation, E2 is involved in viral DNA replication through interaction with E1. Both E1 and E2 bind independently to origin sequences in the URRs of PVs as well as forming a complex with each other (Androphy et al. 1987b; Mohr et al. 1990; Blitz and Laimins 1991; Frattini and Laimins 1994).

Transient replication of HPV genomes requires only E1 and E2 but stable maintenance in normal keratinocytes also requires E6 and E7 expression (Del Vecchio et al. 1992; Stubenrauch et al. 1998; Thomas et al. 1999) (Properties of HPV oncoproteins E5, E6 and E7 will be discussed later in the section 5)

E4: Although designated as an early protein, E4 appears to be expressed as a late gene. Thus far the precise function of E4 is not clear since it seems not to be required for transformation. The E4 protein has been suggested to be involved in morphogenesis because of its high expression in the suprabasal cell layers of infected tissue. It is thought that E4 may play a role in particle maturation and collapse of the keratin cytoskeleton favouring particle release (Doorbar et al. 1991; Roberts et al. 1997).

Late Proteins, L1 and L2: These proteins build the viral capsid of HPV. 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 stabilize the viral particles. Translocation of these two proteins into the nucleus, where the viral assembly takes place (Orth et al. 1977; Zhou et al. 1993), is mediated by nuclear localization sequences (NLS) (Silver 1991; Zhou et al. 1991). HPV L1 contains two NLS sequences at the C-terminal end (Zhou et al. 1991), and alone can assemble into virus-like particles (VLPs), when overexpressed in eukaryotic cells (Kirnbauer et al. 1992). L2 was also reported to to have a NLS, which contributes to its transport into the nucleus after complexing with the L1. In animal PV models, vaccination against L1 and/or L2 viral capsid proteins provides an efficient protection against infection, involving type-specific neutralizing antibodies (Breitburd and Coursaget 1999, review). VLPs present the conformational epitopes required for generating high titre neutralizing antibodies and vaccines based on the use of VLPs obtained by auto-assembly of L1 or L1 and L2 produced by recombinant DNA technology are presently under clinical trials (Breitburd and Coursaget 1999, review; Schiller and Lowy 2001, review).

4. Association of HPV with cancer:

4.1. Epidemiological studies

Cancer of the cervix is the third most common cancer in women worldwide, accounting for 9.8% of all new cancer cases and it ranks second in developing countries (Parkin et al. 1999). More than 99% of women developing the disease harbor the viral DNA, the most frequently found types being HPV 16 and HPV 18, thus they are considered as "high-risk" viral types. Other types, such as HPV 6 and HPV 11, frequently found in benign lesions (condyloma acuminata), are designated as "low risk" types since they are rarely associated with malignant lesions. The viral types causing cervical cancers have also been found associated with the development of squamous cell carcinomas (SCCs) of the anus (Scheurlen et al. 1986; Palmer et al. 1987). HPVs have also been linked to SCC at cutaneous sites in patients with the rare skin disorder, epidermodysplasia verruciformis, and have been associated with the development of SCC in patients undergoing immunosuppression following transplant surgery (Sheil et al. 1985).

In the infection of the cervix, the low-grade lesions are called condyloma or cervical intraepithelial neoplasia (CIN) I (Koss 1987) and can progress to higher-grade CIN II and CIN III lesions (zur Hausen 1985). In CIN III lesions, the normal differentiation capability of the cells is lost completely and cells similar to basal cells (Stratum basale; Figure 2) occupy the complete thickness of the epithelium. The changes in levels of α2, β1 and β4 integrins, CD44, and E-cadherin occur during the evolution of high-grade CIN lesions (Daniel et al. 1997). Increases in the levels of expression of CD44 and E6-E7 transcripts, coupled with changes in cellular localization of the Notch protein were reported to define the transition from CINIII lesions to tumours (Daniel et al. 1997).

4.2. HPV-E6 and E7 are continually expressed and retained in cervical tumours and derived cell lines

Several studies demonstrated that viral DNA was integrated into most of the cervical cancer biopsies or cell lines derived from there (Dürst et al. 1983; Boshart et al. 1984; Schwarz et al. 1985; Smotkin and Wettstein 1986). The integration of DNA in the genome of the host occurs randomly, but integration often results in the disruption of viral DNA with subsequent loss of sequences around the E2 ORF. The URR was found to be intact as well as the E6 and E7 ORFs, which were consistently and actively transcribed in tumours and tumour derived cell lines (Schwarz et al. 1985). The continuous presence of E6 and E7 ORFs in HPV positive malignant lesions and their observed increase of expression after the loss of E2, provided a first indication for a role of these two viral genes in cervical carcinogenesis (Dürst et al. 1983; Stoler et al. 1992). Subsequently, E6 and E7 genes were found to be regularly expressed in cervical cancer cells (Schwarz et al. 1985; Smotkin and Wettstein 1986; Androphy et al. 1987a; Banks et al. 1987).

4. 3. Transformation assays

4. 3.1. Established rodent cells

The first indications that HPVs possessed transforming activity were based on their ability to transform established rodent cells. Transformation of mouse NIH3T3 cells showed that the HPV-16 genome encodes proteins with transforming potential and gives rise to rapidly proliferating foci capable of forming tumours in nude mice (Tsunokawa et al. 1986; Yasumato et al. 1986; Matlashewski et al. 1987a). The E7 open reading frame has been demonstrated to have the most potent transforming activity in these assays (Kanda et al. 1988; Vousden et al. 1988; Barbosa et al. 1991). HPV-16 E7 transfected cells formed rapidly growing colonies in semi-solid medium

(Yutsudo et al. 1988). The interaction of E7 with pRb is essential for the ability of E7 to transform NIH3T3 cells, since mutations in E7 within this region of similarity with SV40/E1a that abolish pRb binding also abolish transformation (Edmonds and Vousden 1989). The E7 oncoprotein has also been shown to have more pronounced transforming activity in Rat-1 cells also, than the E6 protein (Beddel et al. 1989; Sedman et al. 1991).

4. 3.2. Primary rodent cells:

Studies have been conducted mostly on primary baby rat kidney cells (BRK) and primary baby mouse kidney cells (BMK), which are of epithelial origin. E7 requires the presence of a cooperating, activated oncogene like *EJ-ras/v-fos* to bring about full transformation of these cells, unlike Adenovirus E1a, which was found to transform independently (Matlashewski et al. 1987; Crook et al. 1988; Peacock et al. 1990). Further mutational experiments on the HPV-16 genome showed that the principal transforming activity is encoded by the E7 gene (Phelps et al. 1988; Storey et al. 1988) similar to the situation in established rodent cells. In contrast, studies of early gene co-transforming activity in low-risk viruses showed a marked reduction in the efficiency (Storey et al. 1988; Storey et al. 1990) again suggesting that, the transformation assays were a valid representation of the oncogenic potential of these viruses *in vivo*. An interesting observation of cooperativity of HPV E7 and E5 was also seen in inducing proliferation of primary BRK cells (Bouvard et al. 1994b). E7 has been also found to cooperate with HPV-6 E5 in similar cell proliferation assays (Faulkner Valle and Banks 1995). E7 has also been reported to cooperate with insulin-like growth factors in inducing cellular DNA synthesis (Morris et al. 1993). This was thought to explain the cooperation of E7 with E5 protein, which is intimately involved in growth factor signaling.

4. 3.3. Primary human cells

a. Keratinocyte immortalisation

Primary human keratinocytes represent the natural target cells for HPV and are normally very difficult to immortalise. It was thought however that immortalisation studies using these cells might mimic the natural sequence of events of HPV infections *in vivo.* Both E6 and E7 together were found to be essential and sufficient for the keratinocyte immortalisation (Barbosa and Schlegel 1989; Hawley-Nelson et al. 1989; Munger et al. 1989a). However, keratinocytes expressing E6 and E7 proteins have an expanded life span, but are not tumourigenic in nude mice. Only after extensive passage in culture or transfection with activated oncogenes do these immoratlised cells get fully transformed (DiPaolo et al. 1989; Dürst et al. 1989; Hurlin et al. 1991; Pei et al. 1993), further supporting the notion of multi-step progression to malignancy. It is interesting to note that E7 alone can induce the immortalisation of human keratinocytes in the absence of E6, albeit at a low frequency (Hudson et al. 1990; Halbert et al. 1991), further indicating that effective co-operation of the HPV oncogenes is required for full immortalisation of natural target cells.

b. Mammary epithelial cells:

HPV-16 and HPV-18, have both been shown to immortalise mammary epithelial cells (Band et al. 1990). Early studies first indicated that only the E6 open reading frame was responsible for immortalisation of these cells (Band et al. 1991). However, recent studies showed that both E6 and E7 can independently immortalise these cells, and this appears to be dependent on the lineage of the target cells. Thus, early passage

cells were immortalised by HPV-16 E7, whereas late passage cells were immortalised by HPV-16 E6 (Wazer et al. 1995).

In all these transformation assays, the so-called "low risk" types 6 and 11, which are associated with benign lesions, have only limited activity. Thus, the characterization of genital associated HPVs as high-risk and low-risk correlates very well with their transforming activity *in vitro*.

5. Properties of the HPV Oncoproteins

5. 1. HPV E5

The E5 gene was neglected for considerable time, since the portion of the genome encoding the E5 gene is frequently deleted during the development of cervical cancer. However, studies of HPV-18 indicated a transforming activity independent of the E6 and E7 proteins (Beddel et al. 1989), which gave the first indication that E5 might have transforming activity. Later analysis of HPV-16 E5 revealed transforming activity in established murine keratinocytes (Leptak et al. 1991). Although it can cooperate with E7 in transformation assays the ORF is frequently lost in cervical cancers, which indicates that E5 may play a role in early stages of viral infection, but is not required for maintenance of the transformed phenotype (Auvinen et al. 1997, review). It is interesting to note that, although E5 sequences were found to be often lost during the progression to malignancy, the EGFRs are frequently amplified in cervical tumours (Bauknecht et al. 1989; Kohler et al. 1989). Therefore, it may be reasonable to speculate that, loss of E5 could be compensated by the receptor amplification during the development of cervical cancer.

Due to the weak transforming activity of the HPV E5 proteins, most of the work on E5 has been done with BPV-1 E5, which has a far higher transforming potential. The E5 proteins are approximately 10 kD in size, are extremely hydrophobic and localise

to the Golgi apparatus, endoplasmic reticulum and nuclear membranes (Conrad et al. 1993). Stimulation of transforming efficiency of E5 was observed in the presence of activated Epidermal Growth Factor Receptor (EGFR), indicating that E5 may enhance the receptor activity via inhibition of receptor regulation (Martin et al. 1989). Studies have also shown that BPV-1 E5 can activate the turnover of Platelet Derived Growth Factor Receptor (PDGFR) (Petti et al. 1991; Petti and DiMaio 1994). However, several transformation-defective E5 proteins form a complex with PDGFR and induce receptor tyrosine phosphorylation, indicating that PDGFR activation is necessary but not sufficient for E5-mediated cell transformation (Nilson et al. 1995).

HPV16 E5 has also been shown to disturb the EGFR processing in human keratinocytes and this appears to be mediated by a reduction in the acidification of endosomes that are involved in receptor processing (Straight et al. 1993; Straight et al. 1995), probably by a complex formation with the 16kD component of vacuolar H^+ ATPases (Goldstein and Schlegel 1990; Conrad et al. 1993). However, studies with both BPV-1 E5 (Goldstein and Schlegel 1990; Goldstein et al. 1992) and HPV E5 (Faulkner Valle and Banks, 1995; Chen et al., 1996) have produced conflicting reports regarding the correlation of 16kD binding and transforming activity of E5.

As a consequence of E5's ability to stimulate signaling from receptors, there is increased activation of MAP Kinase and a concomitant increase in the levels of c-fos and c-jun, leading to upregulation of the viral URR, which contains recognition motifs for several transcription factors including AP-1, that has shown to be necessary for the viral gene expression (Chan et al. 1990; Chen et al. 1996). E5 has also been shown to upregulate the protein kinase C pathway, which is also involved in viral gene expression (Chen et al. 1996; Crusius et al. 1997). Thus E5 function has been linked directly to the regulation of viral gene expression during the early part of G1 phase of

cell cycle, leading to enhanced cell immortalisation probably by increasing the levels of E6 and E7 expression.

E5 has also been shown to perturb the function of gap junctions, leading to the reduction in cell-cell communication (Oelze et al. 1995), although the actual mechanism is not yet known. This indicates that E5 may have other functions, which might explain its minor transforming activity.

5.2. HPV E6

The E6 protein of HPV 16 consists of 151 amino acids and has four CXXC motifs which allow the formation of zinc binding fingers (Androphy et al. 1987a; Barbosa and Schlegel 1989; Grossman and Laimins 1989). The expression of the HPV E6 gene is required for the continued cell proliferation and maintainance of transformed phenotype in cervical tumours and in cell lines derived from them (von Knebel Doeberitz et al. 1988; Storey et al. 1995; Alvarez-Salas et al. 1998). Inhibition of E6 in cervical tumour cells results in apoptosis (Butz et al. 2000), which makes it a potential therapeutic target.

E6 proteins have been localised to the nuclear, cytoplasmic and membrane fractions of the cell either by cell fractionation or indirect immuno-fluorescence (Liang et al. 1993). Similar to E7 (Phelps et al. 1988), E6 also has intrinsic transcriptional activity and is able to transactivate the adenovirus E2 promoter (Sedman et al. 1991; Desaintes et al. 1992). The first cellular target of E6 to be described was the cellular tumour suppressor protein p53. This is inhibited in over 50% of human cancers, highlighting its critical importance in the host defence and oncogenic transformation. Interestingly, interactions similar to HPV-16 E6 and the p53 (Werness et al. 1990) has been observed for proteins of other DNA tumour viruses: SV40 large T antigen (Lane and Crawford 1979; Linzer and Levine 1979) and Adenovirus E1b 55k (Sarnow et al.

1982). The detection of the interaction of these viral oncoproteins with this tumour suppressor protein led to the postulation that these proteins could interfere with the negative regulatory function of p53. Although all these viral proteins target p53 for inactivation, they do not show any significant similarity at the amino acid level, and their interaction with p53 seems to be quite different. Large T antigen and the E1b inactivate p53 by sequestering it into inactive complexes (Ludlow 1993; White 1995). The E6 oncoprotein on the other hand has been shown to recruit a cellular ubiquitin-protein ligase, E6-AP, to target p53 for ubiquitin-proteosome-mediated degradation (Scheffner et al. 1990; Scheffner et al. 1993). This is true in HPV- positive carcinoma cell lines, where the use of antisense approaches to block E6-AP or use of a dominant negative E6-AP mutant resulted in stabilization of p53 and apoptosis (Beer-Romero et al. 1997; Talis et al. 1998; Butz et al. 2000; Traidej et al. 2000). Although, it is generally assumed that this property of E6 is in part responsible for its oncogenic and antiapoptotic potential, it is now clear that, E6 has p53-independent transforming and apoptotic activities (Pan and Griep 1994; Pim et al. 1994; Pan and Griep 1995; Liu et al. 1999; Thomas et al. 1999). This is borne out by the large number of important cellular partners with which E6 has been reported to interact including c-myc (Gross-Mesilaty et al. 1998), Bak (Thomas and Banks 1998), E6BP (Chen et al. 1995b), hDLG (Lee et al. 1997), IRF-3 (Ronco et al. 1998), E6TP1 (Gao et al. 1999), MAGI (Glaunsinger et al. 2000), hMCM7 (Kühne and Banks 1998; Kukimoto et al. 1998), MUPP-1 (Lee et al. 2000b), paxillin (Tong and Howley 1997), CBP/p300 (Patel et al. 1999; Zimmermann et al. 1999) and hScribble (Nakagawa and Huibregtse 2000). These all represent cellular controls for proliferation, differentiation, apoptosis or DNA replication, and E6 is observed to either degrade or modulate their activities for its biological function.

5. 3. HPV E7

The E7 protein is expressed and continually retained in cervical tumours and the derived cell lines (Smotkin and Wettstein 1986; Seedorf et al. 1987). Inhibition of E7 function, either by blocking expression (von Knebel Doeberitz et al. 1988; Crook et al. 1989; Alvarez-Salas et al. 1998) or the use of single chain antibodies (Wang-Johanning et al. 1998), results in growth arrest. Therefore, E7 represents an ideal target for potential therapeutic intervention.

HPV-16 E7 is a small acidic phospho-protein of approximately 98 amino acids. Amino acid similarity between HPV E7 proteins and other DNA tumour virus transforming proteins, SV40 large T antigen and adenovirus E1a, indicated the usage of similar mechanisms to alter cell proliferation (Phelps et al. 1988). On the basis of homology with the Adenovirus E1a, HPV-16 E7 can be divided into three domains, from conserved domain 1 (CD1) to CD3. (Figure 3). The amino terminal 37 amino acids of HPV 16 E7 have a marked sequence homology to conserved regions 1 and 2 (CR1 and CR2) of the adenovirus E1a, as well as as to the homologous regions found in SV40 large T antigen. CD1 of E7 and E1a are highly conserved but differences in biological functions are observed. E1a has the ability to interact through region 1 with pRb, although with low affinity, and with the cellular protein p300. However, the CD1 of HPV 16 E7 appears to lack these properties, although, deletions or point mutations in HPV 16 E7 CD1 lead to a reduced activity in cellular transformation (Banks et al. 1990; Watanabe et al. 1990; Phelps et al. 1992). HPV 16 E7 CD2 contains the LXCXE domain involved in the interaction with the so-called "pocket proteins", which comprises the retinoblastoma gene product (pRb) and related proteins, p107 and p130 (Dyson et al. 1989; Davies et al. 1993; Hu et al. 1995). Mutational analysis demonstrated a correlation between transformation activity in rodent cells and pRb binding, since mutants unable to bind pRb lost their transforming

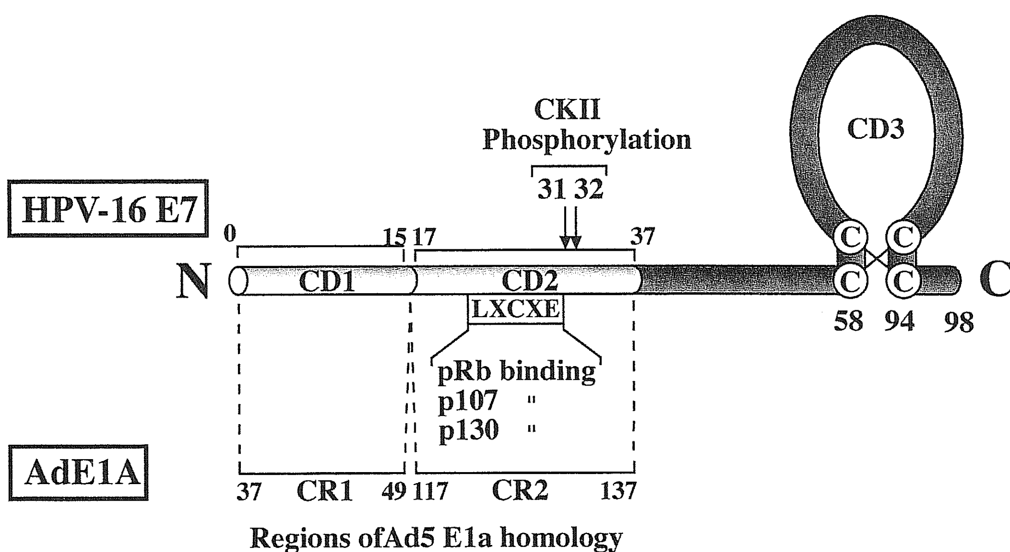


Figure 3. Schematic representation of of HPV-16 E7 and homlogy with Adenovirus E1A.
Regions showing homology with the AdE1A, from conserved domain (CD1) to CD3 are indicated. The Casein Kinase II phosphorylation site is also shown.

potential (Munger et al. 1991; Heck et al. 1992; Phelps et al. 1992). The "high-risk" HPV-16 and 18 E7 proteins have a higher affinity for pRb than the E7 proteins of the "low-risk" types HPV-6 and 11 (Munger et al. 1989b; Heck et al. 1992), which results from a difference of one amino acid (aspartic acid 21 versus glycine 22 in 6 and 11 E7). Substitution of the glycine 22 with aspartic acid in HPV-6 E7 revealed features similar to these of "high-risk" HPV-16 E7, conferring greater affinity for pRb and the ability to co-operate with activated ras in the transformation of primary rodent cells (Heck et al. 1992; Sang and Barbosa 1992). The LXCXE domain within E7 is not solely responsible for pRb binding activity (Jones et al. 1990); a low affinity-binding site is present in the C-terminal domain (CD3) of the molecule (Patrick et al. 1994). Region 3 (CD3) of HPV-16 E7 has very little sequence similarity with E1A CR3 except for two CXXC motifs present in the carboxy terminal half of E7, which are involved in zinc binding (Barbosa et al. 1990; McIntyre et al. 1993) and dimerization (McIntyre et al. 1993; Zwerschke et al. 1996) (Figure 3). The distance between the CXXC is tightly conserved within the E7 proteins and shows some similarity with those found in E6, indicating that there may have been a gene duplication at some stage of viral evolution (Cole and Danos 1987) (Figure 3). The metabolic stability of E7 has been determined by pulse-chase experiments (Smotkin and Wettstein 1987) and the half-life calculated was 55 minutes in CaSki cells and 70 minutes in SiHa cells.

The E7 protein is normally phosphorylated (Smotkin and Wettstein 1987) and it is done largely by casein kinase II (CKII) (Firzlaff et al. 1989; Barbosa et al. 1990). Similarly large T antigen of SV40 (Tegtmeyer et al. 1977), large and middle antigens of polyomavirus (Schaffhausen and Benzamin 1979), and E1a of adenovirus are phosphorylated by CKII (Vousden and Jat 1989). In HPV-16 E7, serine 31 and serine 32 are the two amino acids capable of being phosphorylated since the double mutants

encoding arginine 31 and proline 32 are not detectably phoshphorylated (Barbosa et al. 1990). Furthermore, the arginine-31/proline-32 double mutant, which also produced a stable E7 protein, had a marked reduction in biological activity, though retaining the wild type levels of binding with pRb (Barbosa et al. 1990). Deletions of the analogous sequences of E1a have been reported to retain pRb binding by that protein (Whyte et al. 1989). Similarly, an analogous double mutation within the large T antigen had only a limited effect on its transforming activity (Schneider and Fanning 1988). Therefore, pRb binding and phosphorylation of E7 by CKII are considered as independent activities, both of which are required for efficient transformation by E7. HPV-18 E7 believed to have the most oncogenic potential (Kurman et al. 1988; Schlegel et al. 1988; Arends et al. 1993) and interestingly was found to be phosphorylated more rapidly than the HPV-16 E7. In comparison, HPV-6 E7 mostly associated with the benign lesions, showed the slowest rate of phosphate incorporation (Barbosa et al. 1990).

The E7 protein has been found in a variety of locations within the cell, including the cytoplasm (Smotkin and Wettstein 1987), nucleus (Sato et al. 1989; Greenfield et al. 1991; Smith-McCune et al. 1999) and nucleoli (Zatespina et al. 1997), and amino acid residues 16 to 41 of E7 have been reported to be responsible for the transport of E7 into the nucleus. It is interesting to speculate that these different observations might suggest that E7 shuttles between different parts of the cell and this might be related to cell differentiation or post-translational modifications.

One of the main functions of E7 is intimately linked to deregulation of the cell cycle mechanisms, considering its interactions with the pocket proteins, HDACs, cyclin A, Cyclin E, cdk inhibitors $p21^{WAF1}/p27^{CIP1}$ and the AP-1 family of transcription factors (Dyson et al. 1989; Dyson et al. 1992; Davies et al. 1993; Tommasino et al. 1993; Zerfass et al. 1995a; Zerfass et al. 1995b; Antinore et al. 1996; McIntyre et al. 1996;

Zerfass-Thome et al. 1996). Interactions of E7 with the cellular proteins and their biological relevance will be discussed in more detail in section 7.

6. HPV E6 and E7 oncoproteins target the products of two tumour suppressor genes, p53 and pRb, respectively

The ability of the viral proteins E6 and E7 to induce cell cycle progression of the infected cell appears to be mediated by their association with two tumour suppressor genes, p53 and pRb respectively. Both proteins play a key role in regulating the fundamental events during the life cycle of the cell. Thus, the human papillomaviruses have evolved two strategies to alter the cellular pathways, with a consequent loss of control of cell cycle and apoptosis, by their interactions with the main tumour suppressor proteins p53 and pRb. Therefore, a brief introduction of p53 and pRb would help in correct understanding of the mechanisms how these viral oncoproteins work in inducing malignant lesions in the human body.

6.1. p53

Inactivation of tumour suppressor genes is a common event in the development of many types of cancer. Molecular analysis of tumours identified genetic alterations in the p53 in more than half of all cancers arising from a wide spectrum of tissues (Furihata et al. 1995, review). p53 is a short-lived, sequence-specific transcription factor that is activated and stabilized in response to wide range of cellular-stresses, including DNA damage and oncogenic activation (Levine 1997; Giaccia and Kastan 1998). Once activated, p53 is able to coordinate complex cellular responses aimed at preventing unrepaired DNA damage from turning into permanent mutations. Depending on the stress and cell type, the response evoked by p53 activation can be as diverse as reversible cell-cycle arrest, irreversible senescent-like status or apoptosis.

The efficiency of p53 in controlling cell growth is indispensable to prevent malignant progression, but this activity must be tightly restrained in normal cells to allow physiological cell proliferation and development. Several studies have demonstrated that p53 is tightly regulated through a complex series of events including translationtal regulation, interactions with regulatory proteins such as MDM2, and a series of posttranslationtal modifications that include multiple phosphorylation and acetylation (Anderson et al. 1998; Giaccia and Kastan 1998; Meek 1999). As a transcription factor, p53 either activates (El-Deiry et al. 1993; Miyashita and Reed 1995; Polyak et al. 1997) or represses (Seto et al. 1992; Horikoshi et al. 1995) genes resulting in the inhibition of entry into S phase or the induction of apoptosis (Canman and Kastan 1995). The p53 induced G1 arrest is mediated by transactivation of cyclin dependent kinase (cdk) inhibitor $p21^{CIP1/WAF1}$ (Harper et al. 1993) and is required to provide the cell with enough time to repair the DNA damage before entering S phase (El-Deiry et al. 1993). p53 dependent apoptosis is an important mechanism by which tranformation is suppressed and this may be achieved by p53 either through transcriptional activation or direct protein signaling or both (Bates and Vousden 1996, review). Despite the importance of p53 for such crucial processes, the protein seems to be dispensible for normal cell function. Knock-out mice, lacking p53, developed with out any apparent effect suggesting that p53 may become critically limiting when normal growth control is lost (Oren 1992, review). This is in agreement with the observation that lack of p53 in knockout mice resulted in higher rates of tumour growth in relatively early stages of life (Donehower et al. 1992). These results strengthened the image of p53 as a damage control protein and monitor of genomic integrity.

6.2. Retinoblastoma gene

The retinoblastoma gene (Rb-1) product was discovered from studies of the childhood disease retinoblastoma. Karyotopic analysis showed loss of both alleles of the retinoblastoma gene on chromosome 13q14 (Godbout et al. 1993). The isolation of the gene, followed by studies on retinoblastoma samples demonstrated the loss or inactivation of the gene rb-1 (Friend et al. 1987; Lee et al. 1987). Further reports then confirmed the loss of Rb-1 in tumours other than retinoblastoma (Ewen 1994, review). For example, the cdks that switch off Rb are hyperactive in many tumours (Weinberg 1995, review; Grana et al. 1998; Mulligan and Jacks 1998, review). Indeed, it has been suggested that the regulatory pathways involving Rb may be compromised in all human malignancies (Weinberg 1995).

The rb-1 gene product, pRb/p105^{rb} spans 928 amino acids and has a predicted molecular mass of 106 kD (Lee et al. 1987). The protein is organised into at least three discrete structural domains, which as shown in Figure 4A. The N-terminal domain is involved in *in vitro* oligomerisation. The domains A (aa 378-572) and B (646-772) together are referred to as the "pocket domains" since the sequence is conserved among this family of proteins, including p107 and p130 and this is throught to be major functional domain of pRb. An intact pocket, as well as much of of the adjacent carboxyl terminus, is required for pRb to bind E2F transcription factor. The nuclear localisation sequence and DNA-binding activity are located at the C-terminal domain, although no DNA sequence specificity has so for been shown (Ewen 1994; Riley et al. 1994, review).

The Rb protein functions as a signal transducer, connecting the cell cycle with transcriptional control. The connection of pRb with the cell cycle progression was provided by the observation that this protein undergoes cell cycle dependent phosphorylation (Buchkovich et al. 1989; Chen et al. 1989). Additionally, it could be

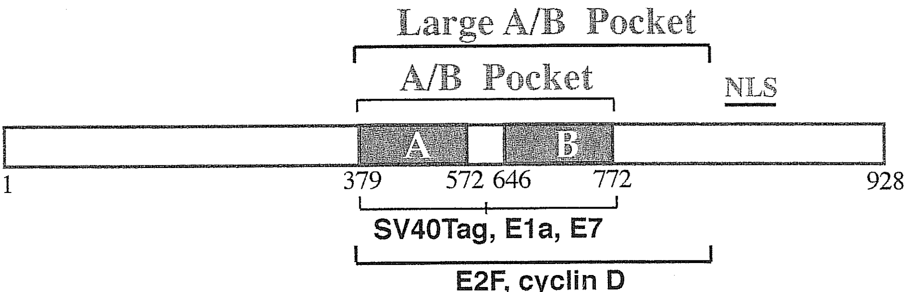


Figure 4. A. Scheme of Rb protein. The structural domain mostly involved in binding proteins of viral as well as cellular origin is the A/B pocket. Domains A and B are separated by a spacer region. pRb contains a nuclear localisation sequence(NLS) within its C-terminal domain

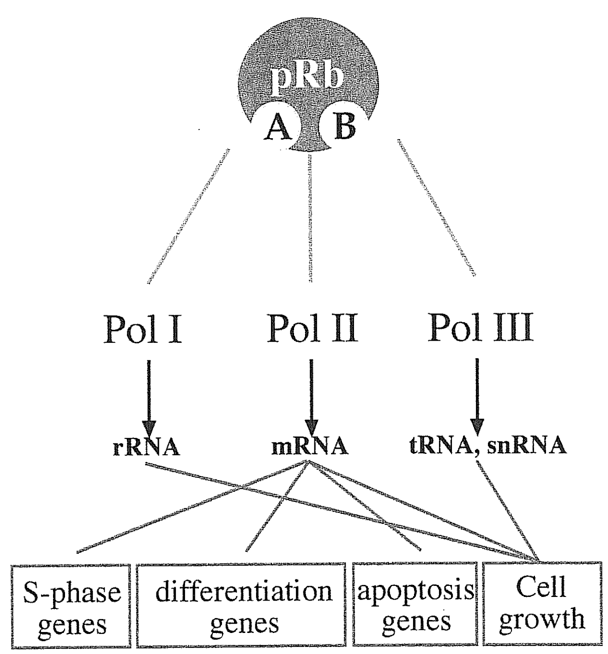


Figure 4. B. pRb regulates major cellular processes.
Through association with a variety of cellular transcription factors pRb can either stimulate or repress genes required for the onset of major cellular processes such as cell division, cell growth, differentiation and programmed cell death (updated from Herwig and Strauss, 1997)

demonstrated that hypophosphorylated, active pRb, inhibits DNA synthesis in early G1, but not in late G1 or early S-phase. So, pRb seems to act at a specific restriction point (R) in the early G1. Passage through this point allows cells to commit DNA synthesis and cell division (Riley et al. 1994, review). Since the hypophosphorylated form of pRb appears to be functional in cell cycle progression, oncoproteins of DNA tumour viruses, the SV40 Large Tag (DeCaprio et al. 1988), the AdE1a (Whyte et al. 1989), and HPV-16 E7 (Dyson et al. 1989; Munger et al. 1989b) all bind preferentially to this form of pRb (Weinberg 1995, review). Thus, inactivation of pRb by either loss or mutation, binding to DNA tumour virus antigens or phosphorylation mediated by cyclin-dependent kinase (cdk) (Sherr 1994, review) turned out to have equivalent effects: release of E2F. However, the activity of pRb appears not to be limited to its growth suppression during the cell cycle and increasing evidence suggests a role in regulation of other major cellular processes, such as differentiation and cell death (Herwig and Strauss 1997) (Figure 4B). Through its association with variety of cellular proteins of transcriptional machinery, pRb can either stimulate or repress RNA polymerase (Pol) II transcription (Kouzarides 1995; Weinberg 1995, review), tRNA synthesis by Pol III (White et al. 1996) and rRNA synthesis by Pol I (Cavanaugh et al. 1995), the latter through its association with the upstream binding factor (UBF). For several Pol II- transcribed genes, Rb has been shown to inhibit expression by recruiting histone deacetylases, which are thought to decrease promoter accessibility (Brehm et al. 1998; Magnaghi-Jaulin et al. 1998). A recent study provided the evidence that Rb disrupts the interaction between TFIIIB and TFIIIC2 which is required for most class III-transcribed genes, thus repressing Pol III transcription (Sutcliffe et al. 2000). In addition, analysis of Rb-deficient Saos2 cells and primary fibroblasts from $Rb^{-/-}$ mice demonstrates five-fold higher Pol III transcriptional activity in the absence of functional Rb protein (White et al. 1996;

Larminie et al. 1997). Rb-induced repression of pol III activity is also alleviated by mutations in the Rb pocket domain that occur naturally in tumours, and by viral transforming proteins that bind and inactivate Rb, implying that repression of Pol III transcription is a mechanism for pRb-induced growth arrest (Sutcliffe et al. 2000).

6.3. Role of p53 and pRb in G/S progression

The cell cycle is devided into four phases. The periods associated with DNA synthesis (S phase) and mitosis (M phase) are separated by gaps of varying length called G1 and G2. Several control mechanisms ensure an ordered progression of the cell cycle. The order and timing of cell cycle events is considered to be very critical for accurate transmission of genetic information. A number of biochemical pathways or checkpoints have evolved to ensure that initiation of a particular cell cycle event is dependent on the successful and accurate completion of one before. For example, mitosis after DNA replication.

pRb and its relatives p107 (Ewen et al. 1991) and p130 (Hannon et al. 1993; Li et al. 1993), the so-called 'pocket proteins', have been identified as key regulators for the pathway controlling the commitment to enter S phase. pRb controls proliferation by sequestering, and thereby inactivating, a set of regulatory proteins that favour cell proliferation. The best-characterized protein to be complexed by pRb is E2F (Bagchi et al. 1991). E2F/DRTF-1 (differentiation regulated transcription factor) was initially identified as a mammalian DNA binding protein required for the E1A dependent transcription of the adenovirus E2 gene (Bagchi et al. 1990). E2F comprises a group of closely related proteins (E2F: 1-5), that heterodimerize with another set of cellular proteins (DP: 1-3) in various combinations to form functionally active DNA binding complexes (Lam and La Thangue 1994, review). The dimerization of DP and E2F family members synergistically increases their DNA binding and their transcriptional

activity and it is required for stable interaction with pRb (Herwig and Strauss 1997, review). Phosphorylation of pRb plays an important role in the association or dissociation of E2F and pRb. A family of protein kinases can phosphorylate members of the pRb family and release the transcriptionally active E2F/DP heterodimers, permitting S phase entry (Beijersbergen and Bernards 1996). These protein kinases need to bind a regulatory subunit (cyclin) to become active; hence they are called cyclin-dependent kinases (CDKs). The activity of CDKs is also regulated by several other mechanisms (Pines 1993b, review). Besides complex formation of the cyclin and catalytic CDK subunit, phosphorylation by CDK activating kinase (CAK) and dephosphorylation of the N-terminal catalytic site is essential for kinase activity. Furthermore, kinase activity is regulated upon synthesis or degradation of CDK inhibitors (CDIs). CDIs bind specifically to the CDKs either alone or as cyclin-CDK complex. CDIs may function as intrinsic components of the cell cycle engine controlling cell cycle check points, but may also be activated in response to extra cellular signals like DNA damage (Grana and Reddy 1995). The increase of active p53 after DNA damage leads to induction of a CDI, $p21^{CIP1/WAF1}$. Activation of $p21^{CIP1/WAF1}$ results in inhibition of the CDK-cyclin kinase activity, thereby preventing phosphorylation of pRb. Hypophosphorylated pRb in its active form, remains in complex with E2F1-DP1 and prevents S-phase entry. The G1 arrest facilitates correction of the acquired DNA lesion and allows progression of DNA synthesis after repair. Depending on the amount of DNA damage or when cells fail to undergo G1 arrest in the presence of p53, apoptosis occurs as a default mechanism (Hansen and Oren 1997, review).

7. Interactions of E7 with the cellular proteins:

Interactions of DNA tumour virus oncoproteins with cellular proteins has proved to be critical to our understanding of cell cycle regulation. E.g. SV40 Large T ag, E1a, E6 and E7 interactions with p53, pocket proteins, CBP/p300 etc. A major aim has been elucidating how the viral oncoproteins work and this has been helped greatly by looking for their potential cellular interacting partners. In fact, these studies have provided us with the vital information regarding the mechanisms of viral mediated cell transformation, regulation of cell cycle and cell proliferation, differentiation and apoptosis. A summary of major interactions of HPV-16 E7 with the cellular proteins is shown in Figure 5.

7. 1. Disruption of cell cycle control through its interaction with the pocket proteins

Expression of HPV 16 E7 in primary cells results in a diverse and distinct set of changes in cellular behaviour. Rodent cells in G_0 re-enter the cell cycle and the proliferating capacity of human keratinocytes increases. E7 expression causes these cells to have a reduced growth factor requirement, a decreased contact inhibition and to be less sensitive to anti-proliferative signals (White et al. 1994). The decision whether a cell proceeds through another division is made during G1. Since HPV-16 E7 can override these checkpoints it seems that inhibitory signals can be neutralised or bypassed.

E7 associates with the pRb tumour suppressor (Dyson et al. 1989), which plays an important role in cell cycle control (Buchkovich et al. 1989; DeCaprio et al. 1989). This interaction occurs mainly with the active, hypophosphorylated form of pRb (Imai et al. 1991), which interestingly is the form of pRb predominantly found in differentiating keratinocytes (Nead et al. 1998), and is associated with the E2F family

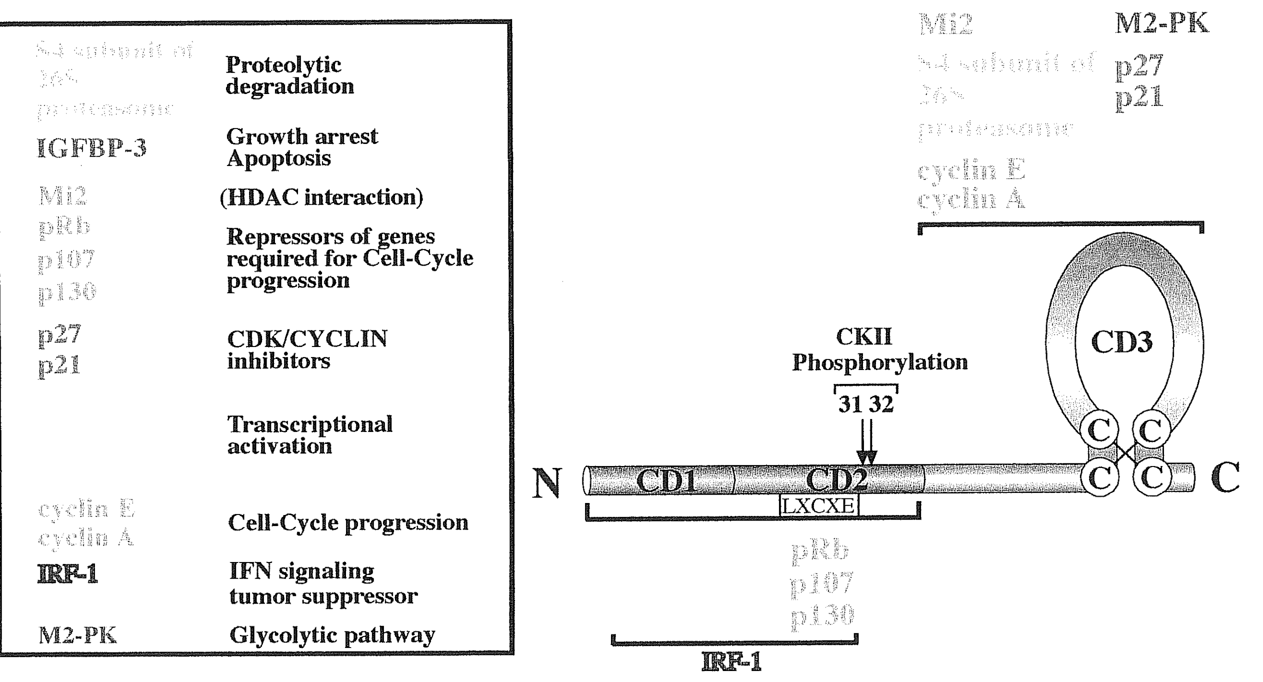


Figure 5. A summary of interactions of E7 with cellular proteins.
IGFBP-3: Insulin-like Growth factor binding protein, IRF-1: Interferon regulatory factor-1, MPP2: M phase phosphoprotein 2, TBP: Tata box binding protein, M2-PK: M2 pyruvate kinase

proteins (Chellappan et al. 1991) (Figure 6). As a consequence of the binding of E7 to pRb, the transcriptionally active proteins of the E2F family are released from pRb (Chellappan et al. 1992; Pagano et al. 1992; Morris et al. 1993; Scheffner et al. 1994, review). Thus, in the presence of E7, the progression of the cell cycle is independent of activation of CDK and phoshphorylation of pRb (Figure 6). Because the critical target of CD4/6-cyclin D appears to be pRb, the interaction between E7 and pRb obviates the requirement for phosphorylation of pRb by CD4/6-cyclin-D and thereby eliminates the cell-cycle control by $p16^{INK4a}$ (Khleif et al. 1996). E7 proteins bind to the pocket domain of pRb encompassing amino acids 394-571 and 649-772 (Hu et al. 1990; Kaelin et al. 1990), which has been found to be mutated in many tumours. But these mutations are not seen in cervical tumours (Scheffner et al. 1991), indicating that E7 function with respect to pRb is analogous to an inactivating mutation.

The pRb binding region on E7 has been mapped to amino acids 20-30 by mutational analysis. The principal element consists of the "LXCXE" motif, which is enough for binding to pRb (Dyson et al. 1989; Munger et al. 1989b). This observation has been further confirmed from recent studies, which defined the crystal structure of a nine-residue E7 peptide containing the "LXCXE" motif bound to the retinoblastoma pocket (Lee et al. 1998). However, binding of the CD2 of E7 to pRb alone is not sufficient to release E2F nor is it sufficient to inhibit pRb DNA binding activity. In fact, sequences in the carboxy terminal region of E7 have been also found to be essential for binding to the pRb gene product, which together gives rise to high affinity binding of E7 to pRb. This leads to the release of free E2F (Huang et al. 1993; Patrick et al. 1994), which then stimulates the E2F responsive genes involved in cell cycle regulation and DNA synthesis (Bandara et al. 1991; Phelps et al. 1991), including DNA Polymerase alpha (Polα), thymidine kinase, dihydrofolate reductase (DHFR), Proliferating cell nuclear antigen (PCNA), cyclin A, cyclin E, B-myb and c-jun (Nevins 1992, review;

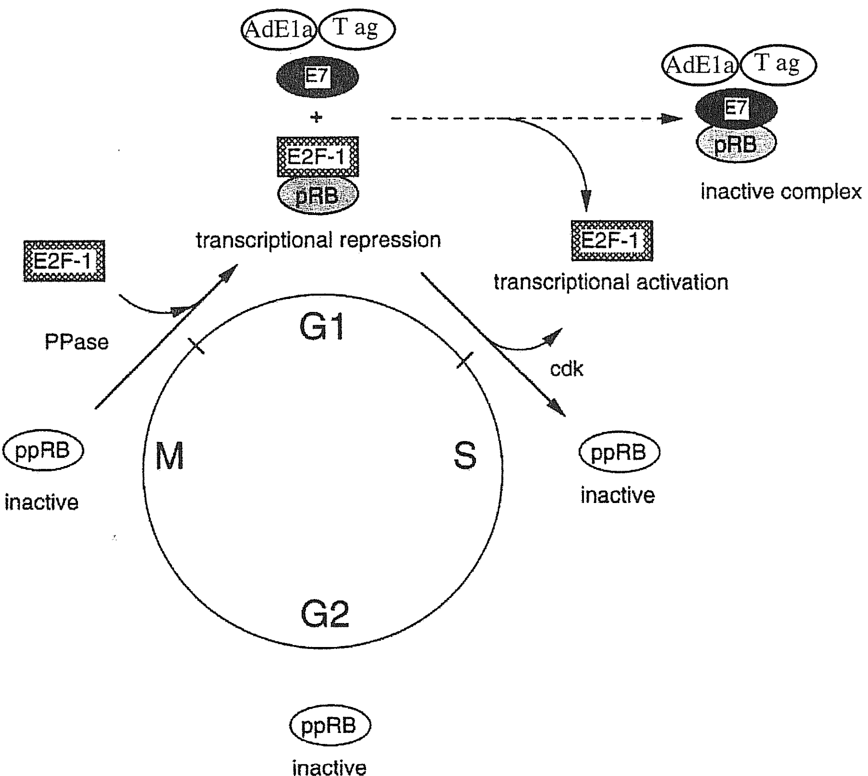


Figure 6. E7 inhibits pRb function (as well as related proteins p107 and p130) by forming a complex.
pRb is differentially phosphorylated during the cell cycle and the underphosphorylated form is detectable only in the Go/G1 phase. This underphosphorylated form is the active form of pRb, acting as inhibitor of the cell cycle. Phosphorylation of pRb by cdks, makes pRb inactive for its cell cycle regulatory functions. The underphosphorylated form of pRb binds to the cellular transcription factor E2F1 and inhibits its function to activate transcription. Phosphorylation of pRb or complex formation with E7/AdE1a/T ag results in the release of free E2F-1, which then upregulates the cellular genes involved in cellular DNA synthesis and progression into S phase of the cell cycle.
(updated from Scheffner et al. 1994).

Lam and La Thangue 1994, review). However, whether the upregulation of PCNA in a E7 dependent manner is the direct result of transcriptional activation by E2F or by another protein is not yet known (Huang and Prystowsky 1996). Stimulation of DNA replication in growth arrested rodent cells can be induced by E7 alone, without any extra stimuli (Sato et al. 1989b; Banks et al. 1990). This activity seems to correlate with the ability of E7 to bind pRb (Banks et al. 1990) and release E2F (Pagano et al. 1992; Morris et al. 1993). The release of E2F by E7 is associated with the induction of active p53, which controls the CDK inhibitor $p21^{CIP1/WAF1}$. Interestingly, expression of E7 in normal diploid human fibroblasts (NDFs) gives rise to an increase in the levels of p53 (Jones et al. 1997a), and it does this by inhibiting the interaction of p53 and MDM2 (Seavey et al. 1999), unlike E1a, which stabilises p53 also by upregulating the p19ARF protein (de Stanchina et al. 1998), which inhibits the ability of MDM2 to ubiquitinate p53 (Zindy et al. 1998). E7 was found to stabilize p53 in mouse embryo fibroblasts lacking $p19^{ARF}$ (Seavey et al. 1999). However, E7-expressing human fibroblasts continue to divide even though E7 abrogates the ability of MDM2 and p53 to bind. Furthermore, NDFs expressing E7 did not exhibit an increased sensitivity to UV light (Seavey et al. 1999), which has been reported to induce p53-mediated apoptosis in NDFs (Ford and Hanawalt 1997). Therefore, these results suggested that, in addition to inhibiting the ability of MDM2 to regulate p53, E7 must block signaling steps downstream of p53 to allow cell division. Thus, E7 can overcome atleast the p53 induced growth arrest, but induces apoptosis (Jones et al. 1997a; Stoppler et al. 1998) unless E6 is present to counteract this effect.

E7 also binds p107 and p130, the other two pocket proteins (Dyson et al. 1992) which are associated with the regulation of cell cycle progression and which interact with the E2F in a way similar to that of pRb (Dyson et al. 1992; Cobrink et al. 1993; Schwarz et al. 1993). However, recent studies have shown that pocket proteins regulate the cell

cycle progression not only through the E2F family of proteins, but also by actively repressing the transcription of genes involved in G1 to S-phase transition (Nevins 1992, review). While cells are still in G1 phase, pRb actively down-regulates the genes essential for G2 by recruiting a histone deacetylase (Brehm et al. 1998; Magnaghi-Jaulin et al. 1998) and forms a complex of E2F: pRb: HDAC1 to suppress the E2F1 responsive promoters. It is assumed that HDAC1 is responsible for converting the chromatin from a transcriptionally active form to a transcriptionally repressive form. HPV-16 E7 can disrupt this complex (Brehm et al. 1998), by directly binding Mi2β, a component of NURD histone deacetylase complex, through its zinc finger domain (Brehm et al. 1999), suggesting that removal of HDAC1 from the vicinity of the promoter is required for stimulation of E2F responsive promoters, in addition to the release of free E2F. This is the first indication that E7 might manipulate gene expression by interfering with chromatin remodeling and histone deacetylation, though E1A has been associated with the opposite enzymatic activity, in the form of CBP/p300 acetyltransferase (Bannister and Kouzarides 1996; Ogryzko et al. 1996) for its immortalisation capacity (Lundblad et al. 1995).

Most interestingly, HPV 16 E7 binding to pRb results in an increased degradation of pRb. In both human keratinocytes (Demers et al. 1994) and mammary epithelial cells (Wazer et al. 1995) reduction in the pRb protein levels have been observed in the presence of E7. Later, it was shown that E7 induces the degradation of pRb (and also possibly p107) through the ubiquitin proteasome pathway (Boyer et al. 1996; Jones and Munger 1997; Jones et al. 1997a). At present, the mechanism by which this occurs is unclear, since no ubiquitin ligase equivalent to E6-AP has been found to be involved. However, one possible link is through the interaction of E7 with the S4 subunit of 26S proteasome (Berezutskaya and Bagchi 1997), thereby stimulating the ATPase activity of S4 enzyme, which is associated in the 26S proteasome assembly.

In addition, E7 itself is found to be a target of ubiquitination at the amino-terminal residue in a novel way followed by degradation through the 26S proteosome (Reinstein et al. 2000), indicating that this might have a link to the E7 degradation of pRb.

The region of E7 interacting with the pRb is similar to that through which it binds to the other two pocket proteins p107 and p130. However, much importance has been given to pRb binding in the cellular transformation, since it is a tumour suppressor protein (Davies et al. 1993) and as yet there is no indication that p107 or p130 are tumour suppressors. The association of E7 with p107 forms part of the cyclin-cdk complex, which could be found during G1/S phase transition or S phase, depending on whether cyclin E or cyclin A is part of the complex. However, unlike the situation with pRb, the E7-p107 interaction does not dissociate E2F (Arroyo et al. 1993; Lam et al. 1994). Moreover, p107 is one of the targets of the associated kinase activity found within these complexes, suggesting that altered phosphorylation of p107 may be one of the mechanisms by which E7 might abrogate p107 function. Very little information is available so far about the functional interactions of E7 with p130, the third member of the pRb family (Cobrink et al. 1993; Mayol et al. 1993).

7. 2. Interaction of E7 with the cyclin/cdk2 inhibitors

Several studies have been performed to investigate how E7 can promote DNA synthesis while maintaining keratinocyte differentiation. E7 achieves this by affecting the function of cyclin/cdk2 inhibitors. Serum withdrawal or loss of cell adhesion is often accompanied by increased levels of $p27^{KIP1}$ and both antiproliferative states can be overcome by E7. Moreover, both $p27^{KIP1}$ and $p21^{CIP1}$ are transiently upregulated during keratinocyte differentiation and this is independent of the presence of p53 (Missero et al. 1996). In particular, during terminal differentiation, $p21^{CIP1}$ is

upregulated in a p53-independent manner by a number of other transcription factors, including AP2, BRCA1 and the CCAAT-enhancer-binding protein β (C/EBP β), in order to mediate G1 arrest (Chinery et al. 1997; Somasundaram et al. 1997; Zeng et al. 1997). A direct interaction between E1A and the C-terminus of $p27^{KIP1}$ allows E1A to sequester $p27^{KIP1}$ and prevent its association with CDK-cyclin complexes (Mal 1996). Similarly, the E7 interaction with $p27^{KIP1}$ was demonstrated both in a *in vitro* reconstituted system and *in vivo* in both yeast and mammalian cells. This interaction requires its sequences in the carboxy terminal portion of E7 and, as a consequence, E7 antagonises the ability of $p27^{KIP1}$ to inhibit cyclin E associated kinase activity and can overcome the $p27^{KIP1}$ inhibition of cyclin A gene expression (Zerfass-Thome et al. 1996). This finding tends to suggest that the ability of E7 to override G1 arrest is mediated in part by binding to and subsequent inactivation of the cdk inhibitor $p27^{KIP1}$, even though the exact mechanism needs to be determined.

A similar kind of direct interaction of E7 has been also found to take place with $p21^{CIP1}$. When p21 is induced it can inhibit both cyclin/cdk2 activity and PCNA-dependent DNA replication by its interaction with cyclin/cdk2 and PCNA via two separate domains (Chen et al. 1995a; Luo et al. 1995). HPV-16 E7 protein inhibits $p21^{CIP1}$-mediated growth arrest *in vivo*, despite the high levels of $p21^{CIP1}$ after DNA damage (Funk et al. 1997). E7 does this by preventing $p21^{CIP1}$ inhibition of cyclin E/cdk2 activity and by blocking p21 inhibition of PCNA-dependent DNA replication (Funk et al. 1997; Jones et al. 1997), thus providing a means to ensure the activity of G1-phase CDK-cyclin complexes and, further, to promote DNA replication directly. Studies showed that E7 caused no detectable difference in the amount of $p21^{CIP1}$ associated with inactive CDK complexes (compared with that associated with CDK complexes activated by E7) (Funk et al. 1997; Jones et al. 1997), and quaternary CDK-cyclin-$p21^{CIP1}$-E7 complexes were observed. E7 from the low-risk group of

viruses binds less strongly to p21 than high-risk. So, E7 from the low-risk group of HPVs were found to have a much-reduced ability to relieve p21^{CIP1}-mediated cell-cycle arrest *in vivo* (Demers et al. 1994). This suggests that there is a complementarity between the ability of E7 to block p53-independent p21 function and the ability of E6 to inhibit p53-dependent p21 function. This is probably because E7 has to overcome p21^{CIP1} activity for effective entry into the cell cycle, whereas E6 might be targeting the apoptotic functions of p53. Thus, the inhibition of p21 expression may be a by-product of E6 mediated degradation of p53. However, the functional interplay between p21^{CIP1} and E7 appears quite complex, since it appears that E7 not only inactivates p21^{CIP1} by direct complex formation, but also controls the intracellular level of this growth suppressive protein. Recently, it was shown that HPV-18 E7, induces very high levels of p21^{CIP1} by posttranslational mechanisms, and these cells, although expressing E7, fail to reactivate DNA synthesis (Jian et al. 1998). Hence, more work is required to precisely define the role of this interaction for cell transformation *in vivo*.

7. 3. Interaction with cyclins and cyclin/cdk complexes

Cyclins were proposed as critical targets of restriction point (mid-G1-phase) control in cell cycle (Pines 1993a, review). Overexpression of cyclin E can shorten the G1 phase and reduce growth factor dependency (Ohtsubo and Roberts 1993; Resnitzky et al. 1994; Wimmel et al. 1994), and its functional inactivation interferes with S phase entry. Cyclin A is believed to participate in DNA replication (Girard et al. 1991) and may have a role in transcription control during S phase (Lees et al. 1992; Krek et al. 1994). Expression of E7 in NIH 3T3 cells was reported to induce constitutive expression of the cyclin A and cyclin E in the absence of external growth factors

(Zerfass et al. 1995b). Similarly, E7 expressing human epithelial and fibroblast cells showed elevated levels of cyclin E and entered S phase early (Martin et al. 1998). This suggests that E7 can shortcut the regulatory cascade of cyclin gene expression. But the conclusions about the mechanisms by which the steady state levels of cyclin E protein were increased are contradictory. In NIH3T3 cells expressing HPV-16 E7 (Zerfass et al. 1995b), E2F binding sites in the promoter of cyclin E were necessary for induction, and mutants of E7 that failed to bind Rb were not able to stimulate cyclin E expression (Zerfass et al. 1995b). $Rb^{-/-}$ murine fibroblasts were also shown to have increased levels of cyclin E (Herrera et al. 1996), confirming the prediction that disruption of Rb-E2F pathway will lead to increased transcription of E2F-responsive genes. In contrast, in the case of primary human cells, elevation of cyclin E levels required both the CD1 and CD2 domains (Martin et al. 1998), which may point to differences in the regulation of cyclin E between primary human cells and established murine cell lines. Similarly, cyclin A upregulation also requires sequences in both CD1 and CD2 domains of E7. Although, cyclin A upregulation is thought to be dependent on cyclin E expression, it is interesting to note that in serum starved cells, where cyclin E levels are low, E7 expression alone could lead to upregulation of cyclin A promoter, indicating that E7 has multiple routes for disrupting the cyclin regulation. Cyclin E expression was deregulated both transcriptionally and posttranscriptionally and persisted at high levels in S and G2/M. Transit through G1 was shortened by the premature activation of their associated kinase activities (Zerfass et al. 1995b; Martin et al. 1998). Furthermore, through p107 a direct interaction of E7 with cyclin A and E (and other associated kinases) could be shown which result in redirecting the activity of these complexes (Tommasino et al. 1993; McIntyre et al. 1996). More recently, Cdc25A levels were found be high in E7-expressing cell lines and, furthermore, theCdc25A promoter was transcriptionally activated by HPV-16 E7

(Katich et al. 2001). Cdc25A is a tyrosine phosphatase that is involved in the regulation of the G1/S transition by activating cyclin E/cdk2 and cyclin A/Cdk2 complexes through removal of inhibitory phosphorylations. These data collectively suggest that the ability of E7 to regulate cyclins involves activities in addition to the release of E2F.

7. 4. Interaction with elements of the cellular transcriptional machinery

It is becoming now clear that there are other transcriptional activities of E7 in addition to the upregulation of E2F responsitve promoters (Phelps et al. 1988; Phelps et al. 1991; Jansen-Durr 1996, review). It was found that E7 can bind to the core component of TFIID transcription factor complex, the TATA Box Binding Protein (TBP) (Massimi et al. 1996) and to a TBP associated factor (TAF), TAF110 (Mazzarelli et al. 1995). TFIID plays a role in transducing the activation signal from upstream regulatory proteins to the basal machinery by directly interacting with transcriptional activators (Verrijzer and Tjian 1996). The association with TBP was found to be regulated by CKII phosphorylation of E7, which is an interesting observation, since the mutants shown to be defective for CKII phosphorylation have reduced transformation activity (Banks et al. 1990; Barbosa et al. 1990). E7 is phosphorylated by CKII at serine 31 and serine 32, whereas TBP binding seems to occur in the carboxy terminus, in the cysteine loop of E7. Mutations in the cysteine loop of E7 result in significant reduction in the transformation activity (Massimi et al. 1997). These observations suggest that E7-TBP interaction might alter the transcription of genes involved in transformation other than those that are regulated by E2F. Indeed, there is evidence that, certain cellular genes are repressed by E7. It was shown that E7 can block the ability of p53 to stimulate transcription (Massimi and Banks 1997), involving the interaction of E7 with TBP. It was also shown that the gene encoding

smooth muscle α-actin is downregulated in E7-expressing embryonic fibroblasts, and transcriptional repression of this gene by E7 was also observed in transient transfection experiments (Nishida et al. 1995). Alternatively, this interaction could play a role in the activation of promoters by p107-E2F-E7 complexes, since E7 has been also found in these complexes at the appropriate E2F driven promoters (Arroyo et al. 1993). Furthermore, similar to AdE1a and large T ag of SV40, HPV-16 E7 is also found to activate Pol III transcription and TFIIIB, for its immortalizing activity, since it is evident that a broad range of transformed cell types display abnormally elevated levels of Pol III transcripts (White 1998a, review; White 1998b), and this is dependent on its ability to bind Rb and to overcome its repressive effect (Larminie et al. 1999). HPV-16 E7 has also been found to interact with the fork head domain transcription factor M phase phosphoprotein 2 (MPP2) and stimulates MPP2- specific transcriptional activity (Luscher-Firzlaff et al. 1999). This interaction is functionally relevant for the transforming activity of high risk E7, since MPP2 enhances the E7/Ha-ras co-transformation of rat embryo firbroblasts and neither non-transforming mutants of HPV-16 E7 nor low risk HPV-6 E7, were able to stimulate MPP2-specific transcriptional activation.

In addition, E7 has been found to interact with AP-1 family of transcription factors: c-Jun, JunB, JunD and c-Fos *in vitro*, and the interaction between E7 and c-Jun has been also found *in vivo* by coprecipitation experiments and a yeast two-hybrid assay (Antinore et al. 1996). c-Jun has been shown to bind within the CD3 domain of E7 and this interaction leads to the upregulation of c-Jun responsive promoters. HPV E5 proteins have also been shown to induce c-Fos and c-Jun expression and this could explain the cooperation between E5 and E7 in mitogenesis (Leechanachai et al. 1992; Bouvard et al. 1994b; Crusius et al. 1997). In addition, dominant negative c-Jun has been shown to abolish the cooperation between E7 and EJ-ras in primary rodent cell

transformation assays (Antinore et al. 1996), as well as to suppress the anchorage independent growth of HPV transformed keratinocytes (Li et al. 1998). 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). Taken together, these studies indicate that the upregulation of c-Jun responsive promoters by HPV-16 E7 contributes to the transforming potential of the protein. Additional transcription factors such as ATF and Oct-1 may also be activated by E7, since E7 can complement certain E1a mutants defective for transactivation of adenovirus early genes (Wong and Ziff 1996), although no further studies have been done to investigate these aspects.

More recently, HPV-16 E7 was also reported to repress the transcription of fibronectin, a key component of the extracellular matrix. This repression, detected in several HPV-positive nontumorigenic and tumorigenic cell lines, was abolished when the Cys-X-X-Cys repeats in E7 were disrupted (Rey et al. 2000b). Furthermore, E7 was also reported to regulate the transcription of cellular genes encoding the calcium binding protein S100P and ADP/ATP carrier proteins (Hellung Schonning et al. 2000). Expression of S100P mRNA was down-regulated by E7 protein induction. S100P belongs to the family of proteins associated with cell differentiation and malignancy, when these proteins are most frequently up-regulated (Schafer and Heizmann 1996) and with chemotoxis (Newton and Hogg 1998). The importance of this second E7-regulated gene, the ADP/ATP carrier protein gene, in the control of cell growth is unknown. Studies reported that this protein interacts with Bax and mediates permeabilization of the mitochondria as a first step in an apoptotic pathway (Marzo et al. 1998). Furthermore, as the S100 proteins have also been described to promote apoptosis (Mariggio et al. 1994; Hu and Van Eldik 1996), the differential regulation of these two proteins by E7 could be important for its role in the modulation of apoptosis.

7. 5. Association with IGFBP-3

Immortalisation of mammalian cells is considered to the first step in tumourigenesis (Yeager et al. 1998), which abrogates a cellular senescence program that is characterized by irreversible cell cycle exit and results in tissue culture, in cells retaining viability after extended passaging. It is assumed that p16/pRb and the ARF/p53/p21 pathways play key roles in establishing cellular senescence (Stein and Dulic 1995, review; Sharpless and DePinho 1999). E7 is generally thought to override cellular senescence, through abrogating the p16/pRb pathway, since E7-expressing cells were found to be refractory to growth inhibition by the cyclin-dependent kinase inhibitor p16INK4 (Lukas et al. 1994; Lukas et al. 1995) and also by inactivating the growth-inhibitory gene p21WAF1 (Funk et al. 1997; Jones et al. 1997), the levels of which are also considerably increased in senescent cells (Stein and Dulic 1995, review). In the light of the above findings, the recent report about the E7 interaction with and inhibition of, growth inhibitory Insulin-like growth factor binding protein-3 (IGFBP-3) (Mannhardt et al. 2000), which is found to be over-expressed in senescent cells (Goldstein et al. 1991; Grigoriev et al. 1995; Wang et al. 1996) is of obvious significance. IGFBP-3 can block the proliferation of various cell types by a variety of pathways and also induces apoptosis (Rajah et al. 1997; Sharpless and DePinho 1999). The IGFBP-3 gene is induced by both p53 dependent (Buckbinder et al. 1995; Rajah et al. 1997; Butt et al. 1999, review) and p53-independent mechanisms (Edmondson et al. 1999). Expression of E7 *in vivo* is found to suppress the ability of IGFBP-3 to induce apoptosis, which also correlates with the ability of E7 to trigger the proteolytic degradation of IGFBP-3 (Mannhardt et al. 2000). The E7 induced degradation of IGFBP-3 was found to be sensitive to proteasome inhibitors such as LLnL, which blocks the protease activity associated with the 26S proteasome (Rock et al. 1994),

Although IGFBP-3 is found to bind only CD3 of E7, both CD1 and CD3 were found to be essential for degrading the IGFBP-3, thereby blocking IGFBP-3 dependent apoptosis, since both mutants p2Pro (CD1) and Δ79-83 (CD3) were severely impaired in these functions. Since, both of these mutants of E7 are defective for transformation, it is reasonable to assume that inactivation of IGFBP-3 by HPV-16 E7 is important for its transforming activity.

7. 6. Association of E7 with the carbohydrate metabolism

Changes in carbohydrate metabolism are frequently observed in tumour cells and represent one of the earliest discernible events in tumorigenesis (Weinhouse 1972, review; Mazurek et al. 1997). Reduced glycogen storage is regularly observed in early lesions of the cervix, a finding that has been used for clinical diagnosis of cervical dysplasia for many years (Galloway 1934). The glycolytic phosphometabolites, which are necessary for the biosynthesis of nucleic acids, phospholipids, and complex carbohydrates, are up-regulated in the G1 phase of the cell cycle (Mazurek et al. 1997) and constant high levels of phosphometabolites have been detected in rapidly proliferating tumour cells (Mathupala et al. 1997). Interestingly, HPV E7 has also been shown to modulate the phosphometabolite levels through an interaction with M2-pyruvate kinase (M2-PK). As a consequence of this association E7 lowers the affinity of M2-PK for phosphoenol-pyruvate, and thus slows the influx of substrates into the tricarboxylic, citric acid cycle (Zwerschke et al. 1999). This leads to accumulation of upstream phosphometabolites, which serve as precursors to amino acids and nucleotides. The pool of these precursors is low in resting cells, but its expansion is necessary during rapid cell division, which is induced by E7. This function of HPV-16 E7 has also been attributed to contribute to its transforming

potential, since both HPV-11 E7 and a transformation deficient mutant of 16 E7 showed considerably reduced binding with M2-PK (Zwerschke et al. 1999). Recent findings further extends these results showing that, E7 also targets another metabolic enzyme, acid α-glucosidase. This interaction occurs through the C terminal region of E7, and induces the catalytic activity of acid α-glucosidase both *in-vitro* and *in vivo* (Zwerschke et al. 2000). Expression of E7 in 14/2 cells resulted in a seven-fold increase in the substrate affinity of this enzyme. Taken together, these observations suggest that, E7 also has the potential to directly affect carbohydrate metabolism during the induction of malignancy.

7. 7. Interaction of E7 with immune modulating molecules

Many viral pathogens have evolved various strategies for inhibiting the expression of immune-modulating molecules on infected cells to avoid T lymphocyte attack (Ploegh 1998). AdE1a has been shown to inhibit the transcription of MCP-1, a chemo-attractant molecule responsible for the attraction of T lymphocyte to their target cells (Timmers et al. 1989). Molecular epidemiological studies have suggested that HPV-induced cervical cancer might be closely related to escape from the host immune surveillance. In HPV-infected cervical cancer tissue the expression of MHC class molecules and the transporter associated with the antigen processing subunit1 (TAP1), which are essential for T lymphocyte recognition of antigen are markedly downregulated (Cromme et al. 1993; Cromme et al. 1994). In addition, MCP-1 is expressed much less in the stroma surrounding the carcinoma cells (Riethdorf et al. 1996; Kleine-Lowinski et al. 1999). Recent studies demonstrated that expression of HPV-16 and 18 E7, like Ad 12 E1a, resulted in the repression of the MHC class I heavy chain promoter. In addition, HPV-18 E7 repressed a bi-directional promoter

that regulates expression of the genes encoding the TAP1 and a proteasome subunit, low molecular weight protein 2 (LMP2). These immune modulatory molecules are transcriptionally induced either by IFN-activated STAT or indirectly by IFN-induced IRF-1 (Chatterjee-Kishore et al. 1998). Thus, the inactivation of either STAT or IRF-1 could be one probable mechanism through which HPV escapes host immune surveillance. HPV-16 E7 has also been implicated in the immune evasion by inducing E7-specific cytotoxic T cell tolerance (Borchers et al. 1999) and by abrogating IFN-α-mediated signaling (Barnard and McMillan 1999), though the mechanism was not identified. Furthermore, E7 was found to abrogate the activation of an IRF-1 ISRE reporter by IFN-γ (Perea et al. 1997). In addition, the recent observation of HPV-16 E7 association with IRF-1 (Park et al. 2000; Perea et al. 2000) might possibly provide a step towards understanding the evasion of immune mechanisms by E7. Upon expression of E7 in 14/2 BRK cells containing dexamethasone-inducible HPV-16 E7 gene, a large inhibition of IRF-1 DNA binding activity was observed, which could lead to the impairment of IFN response in HPV-infected cells (Perea et al. 1997). In agreement with this observation, E7 was found to bind IRF-1 through the amino-terminal pRb binding domain, but to require both the pRb binding domain as well as the carboxy terminal zinc finger HDAC1 binding domain (Brehm et al. 1999) for inhibiting the IRF-1 mediated activation of IFN-β promoter in NIH 3T3 cells, thus recruiting HDAC1 to the IFN-β promoter for the repression of IRF-1 (Park et al. 2000). This is an interesting finding since IRF-1 has also been attributed to have tumour suppressor activity (Kirchhoff et al. 1993; Tanaka et al. 1994) associated with the antiproliferative effect of IFNs in addition to the immune promoting role. Interestingly, HPV-16 E6 was also reported to bind with IRF-3, another member of the IRF family and to inactivate its transactivation function (Ronco et al. 1998),

suggesting that HPV has evolved a dual strategy for inhibiting the critical mediators of IFN signaling for the cervical carcinogenesis. These observations could be relevant for the development of peptide drugs to break either the E7-IRF1 or E6-IRF3 interaction as therapeutic agents for the treatment of cervical cancer.

Taken together all these studies show that the major role of E7 is to create conditions which are favourable for viral replication in the normally quiescent differentiating keratinocytes. This involves induction of cell cycle, DNA replication, alteration of cellular metabolism and escape from host defence mechanisms. It is therefore not surprising that on occasions where viral replication is blocked, due to as yet unidentified events, the processes leading to immortalization and continued proliferation of the infected keratinocytes can be triggered by the presence of the E7 protein.

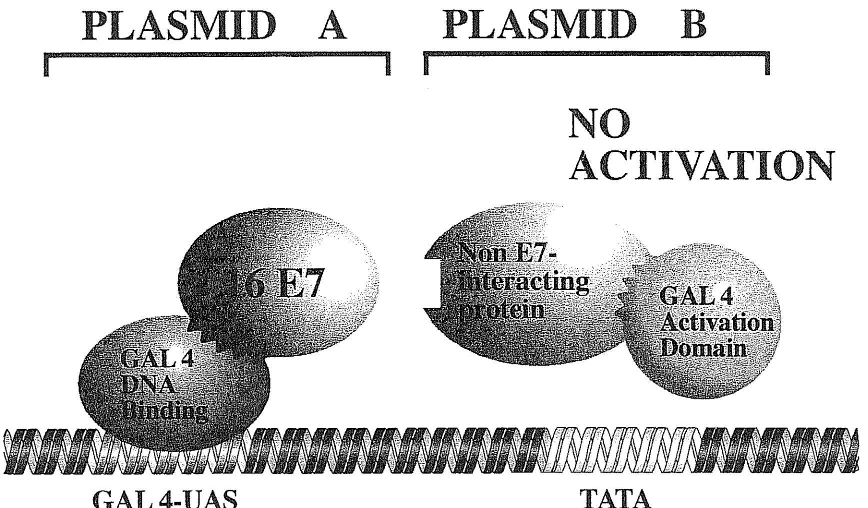
Objectives of Study

A major transforming activity of HPV-16 E7 has been attributed to its inactivation of pRb (Chellappan et al. 1992) and the consequent release of free E2F favouring viral DNA replication (Jansen-Durr 1996, review). Several findings suggest, however that, pocket proteins are not the only cellular targets of E7 required for its transforming activity. For example, a pRb binding-defective mutant of E7 can still immortalise primary human keratinocytes in cooperation with E6 (Jewers et al. 1992). In addition, deletion mutants of either CD1 or the carboxyl-terminal region of E7 markedly reduce its transforming capacity, while still retaining the ability to bind pRb (Banks et al. 1990; Chesters et al. 1990; Phelps et al. 1992; Huang et al. 1993; Wu et al. 1993; Brokaw et al. 1994). Finally, a recent study has demonstrated that HPV E7's stimulation of proliferation does not correlate directly with pRb binding (Caldeira et al. 2000). These observations imply that regions outside the Rb-binding domain must be important for the transforming potential of HPV 16 E7. However, how these regions contribute to transformation is poorly understood. The yeast two-hybrid system has proven to be very useful in identifying novel protein-protein interactions. In an attempt to discover new cellular target proteins of E7, I have performed a two-hybrid screen, using HPV-16 E7 as bait (Figure 7).

RESULTS

1. Identification of new interacting partners of HPV-16 E7.

I used a yeast two-hybrid assay to identify novel cDNA clones encoding proteins, which interact with HPV-16 E7. The HPV-16 E7 oncoprotein was fused in-frame to the GAL4 DNA binding domain in the vector pAS1 (containing a Trp1 marker), and this was used to screen a lymphoid cDNA library that was constructed in pACT (containing a Leu2 marker) (Figure 7) (Durfee et al. 1993). In brief, the cDNA library plasmid was transformed into the yeast strain Y190 (His3) containing the pAS1-HPV-16 E7. E7-interacting clones were selected based on their ability to grow on His^-, in the presence of 3-aminotriazol (3-AT), and on their ability to activate the lacZ reporter. Out of a total of 2.5×10^6 transformant clones screened, I streaked 337 single colonies on selective medium, and 202 of them were found to be positive in the subsequent ß-galactosidase assay. DNA from all the positive clones (pACT-new targets) was isolated by growing them on SC-Leu, rescued in *E. coli.* The plasmid DNA was then purified from these clones and restriction analysis performed to detect the sizes of inserts. First, I sequenced a set of 50 clones by the dideoxy chain termination method (Sanger et al. 1977) and used them as probes for southern blotting to eliminate the repetitive clones. At the same time, all the clones were retransformed into yeast Y187 (MAT a) and Leu^- colonies were crossed to Y190 (MAT α) strain containing selected E7 deletion mutants in pAS-E7 together with additional GAL4-DNA binding domain fusion constructs as controls. This secondary screening with E7 identified a total of 22 unique putative clones. The results of a ß-galactosidase assay from the crosses together with the E7 deletion mutants are shown in Figure 8. None of the candidates showed any interaction either with pAS1 alone or



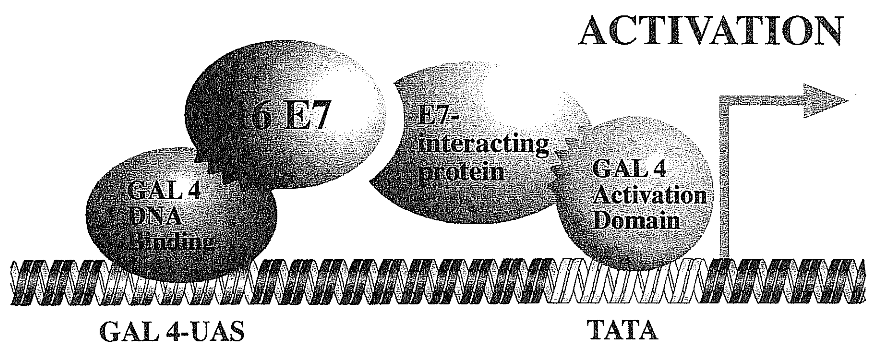


Figure 7. Strategy of two-hybrid screening

with the negative control baits pAS-HPV18 E6 or pAS-HPV18 E2. However, strong interactions with the wild type HPV-16 E7 were obtained suggesting that the new interactions identified are specific to E7.

2. Targets

Of the total of twenty-two cellular targets identified from the two-hybrid screen of E7, ten of them showed no homology with genes of known function from the database (Figure 8), and these may still be potentially interesting. Below is a brief introduction to some of the putative E7 targets of known function; these are listed in Figure 8.

43. human AEBP

This cDNA clone, encoding an 845-amino acid protein is a human homologue of mouse adipocyte transcription factor AEBP1 (He et al. 1995), except that it has additional 105 amino acids in the N terminus containing a nuclear targeting signal (Nakai and Kanehisa 1992) (Ohno et al. 1996). The mouse homologue of AEBP was found to have carboxypeptidase activity associated with transcriptional repression during adipocyte differentiation (He et al. 1995). AEBP is also found to be expressed in osteoblastic cell line but expression is shut off in the final calcification phase, suggesting a transcriptional repressive effect on genes for bone formation (Ohno et al. 1996).

Potential targets (known)

Target identifed	pAS1 Vector	wt E7	E7 1-42	E7 27-98	P2 Pro	11E7	16E2	18E6
43. AEBP	-	++	-	+	++	-	-	-
50. Skip	-	++	-	++	++	+/-	-	-
58. Rab-32	-	++	++	-	+/-	+	-	-
65. SAP-145	-	++	+	++	++	-	-	-
127. Zn Fing. P.	-	++	-	++	++	++	-	-

Potential targets (unknown)

Target identifed	pAS1 Vector	wt E7	E7 1-42	E7 27-98	P2 Pro	11E7	16E2	18E6
1. # 4/76	-	++	-	++	++	-	-	-
2. # 5/163/175	-	++	-	++	++	-	-	-
3. #30/78	-	++	-	++	++	+/-	-	NA
4. # 40/128/220	-	++	-	++	+	-	-	-
5. # 83/119	-	++	-	++	++	+/-	-	-
6. # 126	-	++	+	-	+	NA	-	-
7. # 141/187	-	++	-	+	++	-	-	-
8. # 148	-	++	-	++	++	-	-	-
9. # 193	-	++	-	+	++	-	NA	-
10. # 199	-	++	NA	++	++	-	-	-

Figure 8. Some of the putative targets of E7 identified from the two-hybrid screen
Mutational analysis E7 with each of the target from the backscreenings is also shown

 # 58. Rab 32

The Rab proteins belong to the Ras superfamily of small GTPases. These are a group of GTP-binding proteins which are implicated in the targeting of different transport vesicles within the cell, and are needed for the maintenance and integrity of organelles (Armstrong 2000, review). Initially, Rab proteins were thought to be primarily involved in vesicle docking as regulators of SNARE (soluble NSF-attachment protein receptors) pairing. However, recent findings indicate that their function in vesicle trafficking can go beyond this role, and a number of proteins, unrelated to each other, have been identified as putative rab effectors. Thus rab proteins might control several distinct pathways needed for vesicle movement and vesicle docking, placing them as the master regulators of membrane traffic (Novick and Zerial 1997, review).

#65. SAP-145 (Human spliceosome associated protein)

SAP-145 is the subunit of mammalian SF3b, which is a U2 snRNP (small nuclear ribonucleoprotein)- associated protein complex essential for spliceosome assembly (Gozani et al. 1996). Sequence independent binding of the highly conserved SF3a/SF3b subunits upstream of the branch site in functional A complexes is essential for the anchoring of U2 snRNP to pre-mRNA (Gozani et al. 1996) and thus are critical for mRNA splicing. Recently, cyclin E has been associated with the components of pre-mRNA splicing machinery in mammalian cells (Seghezzi et al. 1998), suggesting that pre-mRNA splicing may be linked to the cell cycle machinery.

 # 127. Zinc finger protein, ZNF74

ZNF74 encodes a RNA binding protein, tightly associated with the nuclear matrix, that belongs to the large subfamily of Cys2-His2 (C2H2) zinc finger proteins containing a Kruppel-associated box (KRAB) repressor motif (Grondin et al. 1996). The zinc finger domain mediates a dual function, being responsible both for the RNA binding properties and for the nuclear targeting of ZNF74 protein (Grondin et al. 1996). ZNF74 was found to interact, via its zinc finger domain with the hyperphosphorylated, largest subunit of RNA ploymerase II (pol II_o), but not with the hypophosphorylated form and it was found to be co-localized with pol II and the SC35 splicing factor (Grondin et al. 1997). Taken together, the ZNF74 sublocalization in nuclear domains enriched in pre-mRNA maturating factors, its RNA binding activity, and its direct phosphodependent interaction with pol II_o, a form of the RNA polymerase functionally associated with pre-mRNA processing, suggest a role for this member of the KRAB multifinger protein family in RNA processing (Grondin et al. 1997).

#50. Skip (Ski interacting protein)

Skip was discovered as Ski interacting protein in a two-hybrid screen using v-Ski as bait, and Skip interacts with both the cellular and viral forms of Ski (Dahl et al. 1998). Interestingly, Skip was found to interact with a highly conserved region of Ski required for its transforming activity (Dahl et al. 1998), suggesting that this interaction is important for the ability of Ski to transform cells. The oncogene v-Ski was originally identified in avian Sloan Kettering viruses and was found to transform chicken embryo fibroblasts (Li et al. 1986). The cellular homologue, c-Ski, has been identified in several

species, including human, chicken and Xenopus (Nomura et al. 1989; Sutrave and Hughes 1989; Sleeman and Laskey 1993). The v-Ski protein lacks a 292 amino acid region from the carboxy terminus of c-Ski, but retains the N-proximal cysteine region (Stavnezer et al. 1989). This region is responsible for cellular transformation and the myogenic activities of Ski (Zheng et al. 1997). Overexpression of c-Ski or v-Ski induces either transformation or muscle differentiation of quail embryo fibroblasts, depending on the growth conditions (Colmenares and Stavnezer 1989; Colmenares et al. 1991). Ski overexpression also causes postnatal hypertrophy of type II fast muscle fibers in transgenic mice (Sutrave et al. 1990). Moreover, germline inactivation in mice demonstrates that loss of Ski function results in decreased myofiber development in addition to other abnormalities (Berk et al. 1997).

3. E7 interacts with Skip through its carboxy terminal domain

I selected Skip (#50) for further analysis, since E7 and the known binding partner of Skip, v-Ski, seem to share many characteristics. Both are viral transforming proteins and have been found to inhibit pRb function for some of their transforming activities (Dyson et al. 1989; Munger et al. 1989b; Tokitou et al. 1999). Therefore, I reasoned that the interaction between HPV-16 E7 and Skip might be of biological significance to E7 function. The two-hybrid screen with E7 identified three clones of the same cDNA clone encoding Ski.

To further confirm a specific interaction between Skip and HPV-16 E7, the isolated clones (pACT-Skip) were rescued in *E. coli* and retransformed into yeast containing pAS-E7 and selected E7 deletion mutants, together with additional GAL4-DNA binding

domain fusion constructs as controls. The results of the ß-galactosidase assay from the back-screenings are shown in Figure 9. The pAS-16 E7 did not show any interaction with pACT alone, but again showed strong interaction with pACT-Skip. Specificity controls included testing pACT-Skip against pAS-HPV18 E6, pAS-HPV18 E2 and the pAS1 parental vector. Both pAS-HPV18 E6 and pAS-HPV18 E2, which had been used earlier as baits in two-hybrid screens (Kühne and Banks 1998), failed to show any interaction with Skip. The ability of the low risk type HPV-11 E7 protein (pAS-11 E7) to interact with Skip was also assessed and, as can be seen, the interaction was significantly weaker than that with HPV-16 E7 (Figure 9).

In order to identify the region of HPV-16 E7 required for the interaction with Skip, two deletion constructs of E7, encompassing amino acids 1-42 and 27-97, were also included in this assay. As can be seen from Figure 9, the amino-terminal region of E7 (amino acids 1-42) did not show any interaction, whereas the carboxy terminal portion (amino acids 27-97) showed wild type levels of interaction. This implies that the portion of E7 from amino acid 27 onwards is important for the interaction with Skip.

4. The interaction between HPV-16 E7 and Skip is direct

To further confirm the specificity of the E7/Skip interaction observed in yeast, I next performed *in vitro* GST pull-down assays. Bacterially expressed GST protein or GST-16 E7 fusion protein were immobilised on Glutathione-agarose and were then mixed with *in vitro* translated [^{35}S] methionine labeled Skip protein. After extensive washing, the bound proteins were analysed by SDS-PAGE and autoradiography. As shown in Figure 10, approximately 25% of input Skip protein was specifically retained on the E7-conjugated

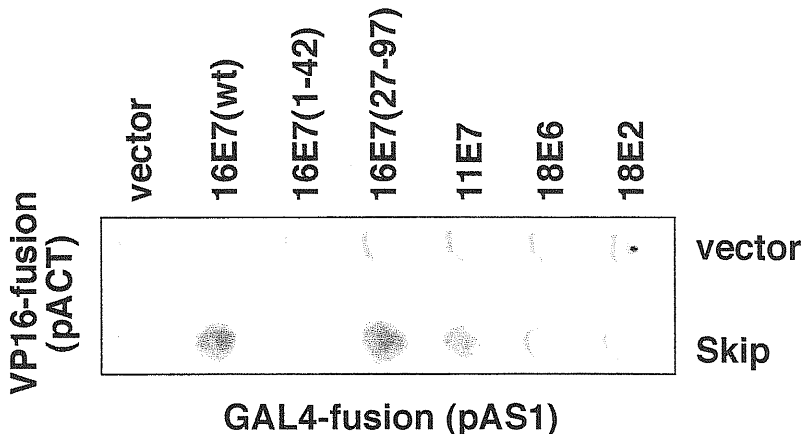


Figure 9. HPV-16 E7 specifically interacts with Skip in yeast
The strength of interaction is analysed by β-galactosidase assay

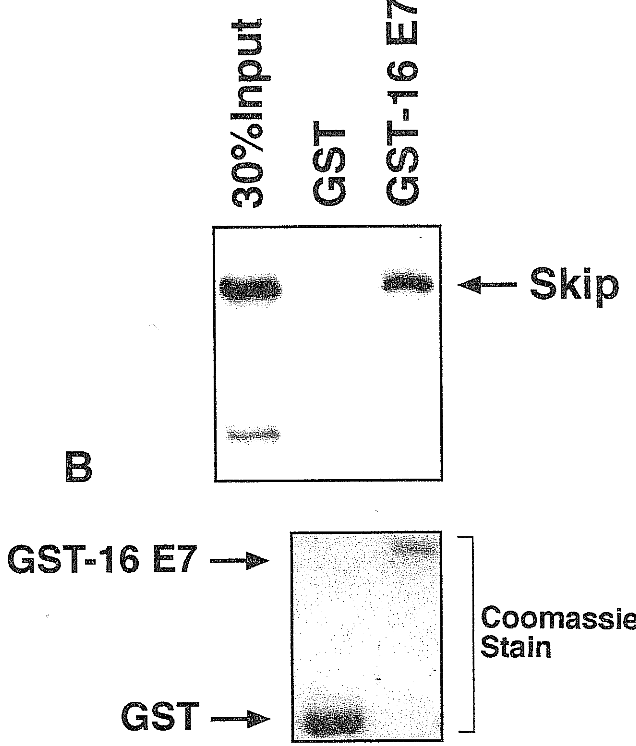


Figure 10. Interaction of HPV-16 E7 with Skip *in vitro*
A GST-pull down assay was performed by incubating purified GST-E7 protein with *in vitro* translated Skip together for 1h at room temperature. After extensive washing, bound proteins were analysed by SDS-PAGE and autoradiography (Panel A).
Panel B shows the Coomassie Blue stain of the gel used in the binding assay.

agarose beads, further confirming the validity of the interaction seen in yeast. However, neither the *in vivo* interaction in yeast nor the GST-pull down assay using *in vitro* translated Skip rules out the possibility that this interaction occurs via an intermediate protein. To investigate this, HPV-16 E7 was expressed as a six histidine-containing fusion protein (His_6) and purified by Ni^{2+} -NTA agarose column chromatography. A GST-pull down assay was then performed using recombinant His_6-16 E7 with GST or GST-Skip fusion protein. Following extensive washing, the amount of E7 retained was determined by electrophoresis and western blotting with an anti HPV-16 E7 antibody. The results obtained are shown in Figure 11. As can be seen, quantitative recovery of the E7 protein was obtained on the GST-Skip agarose resin (lanes 2 and 3), whereas no binding to GST alone (lane 1) was detected. The direct interaction between recombinant His_6-16 E7 and GST-Rb served as a positive control (lane 4). These results demonstrate that the interaction between E7 and Skip is direct and is not mediated by any other protein.

5. Identification of the Skip binding domain on HPV-16 E7.

To determine whether the ability of E7 to bind Skip might be relevant for its oncogenic potential, several transformation-defective mutants of E7 were analyzed for their ability to interact with Skip. The structure of the HPV-16 E7 and the mutants used in the study are shown schematically in Figure 12A. I first performed GST-pull down assays using *in vitro* translated, [^{35}S] methionine labeled Skip protein, together with GST-mutant E7 fusion proteins. As can be seen in Figure 12B, GST-16 E7 wild type was able to bind Skip with high affinity, whereas GST alone did not. However, both the amino terminal

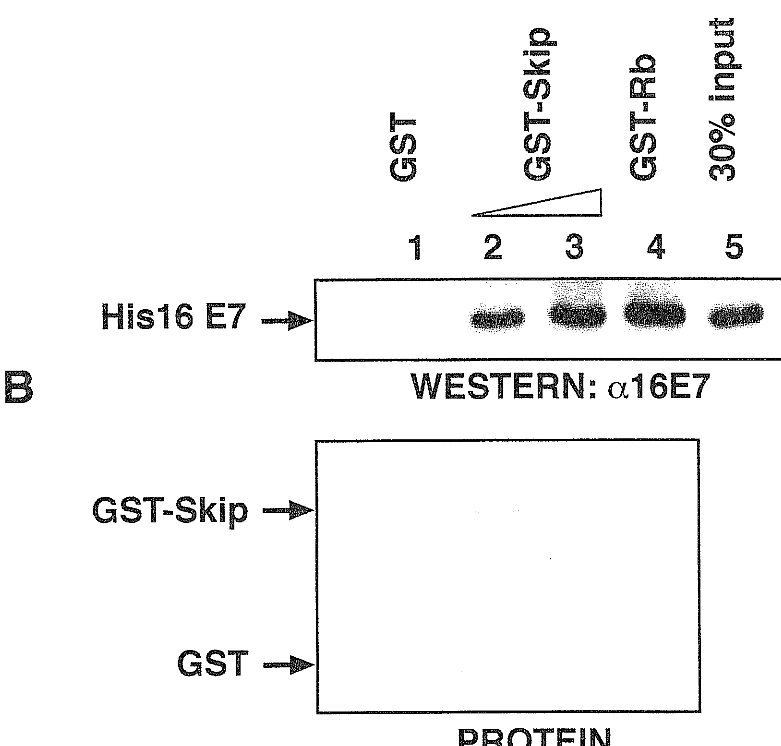


Figure 11. The interaction between HPV-16 E7 and Skip is direct

Panel A. GST-Skip and GST-Rb fusion proteins and GST alone were purified on glutathione agarose columns. These were then incubated with 40 ng of recombinant purified His6-16 E7 at room temperature for 1 h. After extensive washing bound E7 was assessed by SDS-PAGE and Western blot analysis with an antiserum raised against HPV-16 E7.

Panel B shows the Ponceau protein stain of the membrane used in the binding assay.

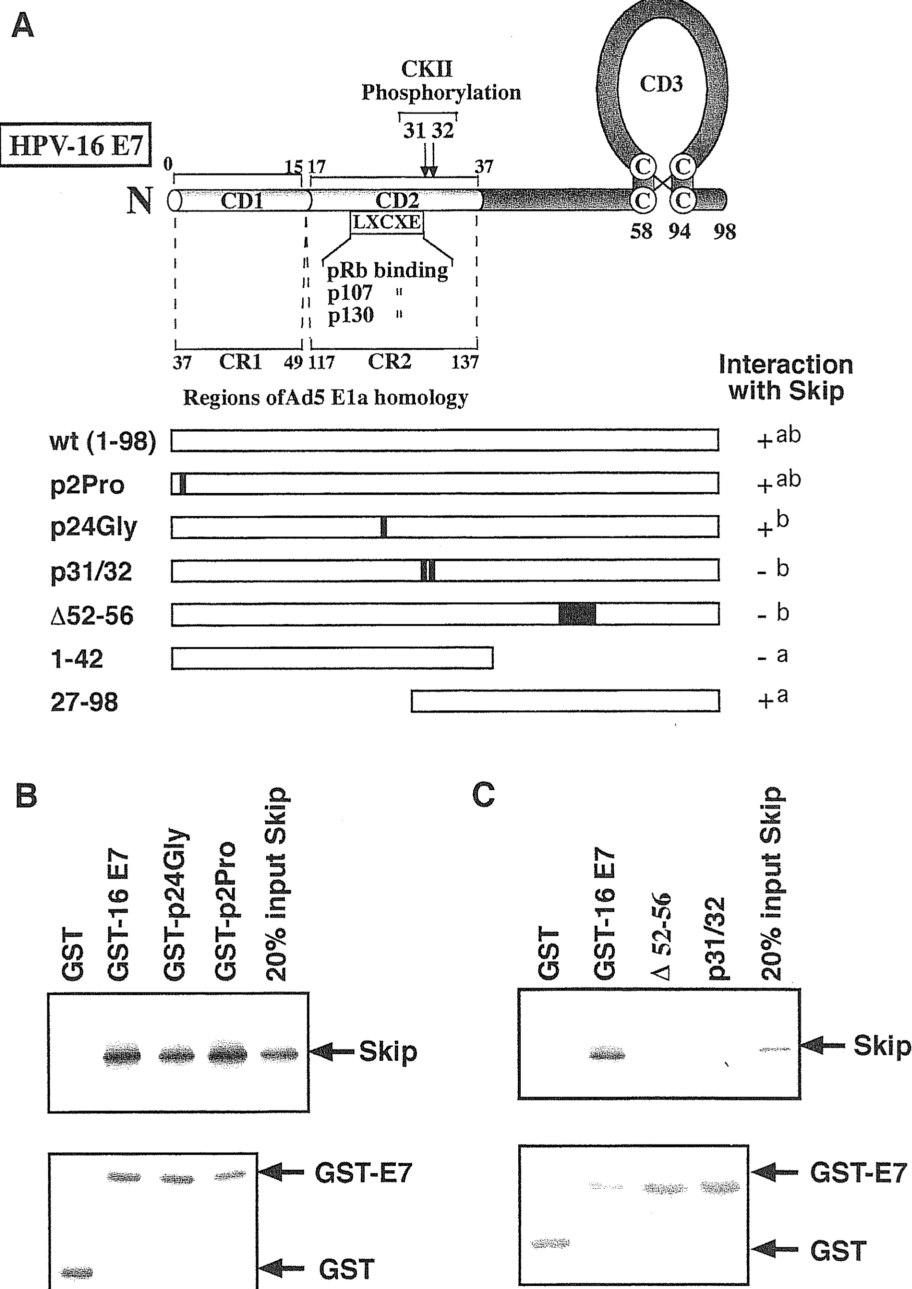


Figure 12. Mapping the Skip interaction domain on HPV-16 E7

Panel A shows a schematic diagram of the E7 structure and deletion mutants of E7 used in the analysis. Numbers refer to the amino acid residues and the domains sharing homology with the AdE1a is shown. Also shown is the summary of the interactions with Skip, where **a** denotes interaction seen in the two-hybrid screen, and **b** indicates the interactions observed in the in vitro binding assays. **Panel B** shows the results from a representative GST pull down assay with the E7 amino-terminal mutants. Purified GST fusion proteins on glutathione resin were incubated with the in vitro translated Skip for 1h at room temperature. After extensive washing bound proteins were analysed by SDS PAGE and autoradiography. **Panel C** shows the results from a representative GST pull down assay from the E7 carboxy-terminal mutants. The lower Panels of B and C show the Coomassie Blue stain of the gels used in the binding assay.

mutants, p24Gly (pRb-binding defective) and p2Pro showed wild type binding affinity, confirming that the interaction is not through the amino-terminal region of E7 and is independent of the pRb/E7 interaction. In contrast, mutants p31/32 and Δ52-56 showed little or no binding with Skip (Figure 12C). The p31/32 (Arg/Pro) lies within the CKII phosphorylation site in E7 and Δ52-56 is a small deletion within the carboxy terminal loop. Both mutants exhibit greatly reduced transforming activity in primary BRKs (Baby Rat Kidney cells) in comparision with the wild type E7 (Figure 13) (Barbosa et al. 1990; Massimi 1999), suggesting that its interaction with Skip may contribute to E7's oncogenic activity. In those assays, BRKs from 9-day-old Wistar rats were transfected with EJ-ras and pSV2neo together with either the wild type E7 or the mutant versions of E7. After two weeks of selection in G418, the cells were fixed, stained and colonies were counted. As can be seen from the figure 13, which depicts the collated results obtained from many independent assays, the mutants p31/32 (Arg/Pro) and Δ52-56 showed at least a three-fold reduction in the number of G418 resistant colonies compared with the wild type.

6. Identification of the E7 binding motif on Skip.

Having identified the binding region of Skip on E7, I next wanted to identify the potential motifs of Skip involved in the interaction with E7. The region of amino acids from 176-333 encompasses the SNW domain and is highly conserved in all the Skip homologues in different species (Figure 14), including *Drosophila melanogaster* (Wieland et al. 1992), *Dictyostelium discoideum* (Folk et al. 1996) and *Saccharomyces cerevisiae* (Diehl and Pringle 1991; Harris et al. 1992). Therefore, I first made a series of Skip deletion constructs, taking the conserved domains into consideration, which are shown

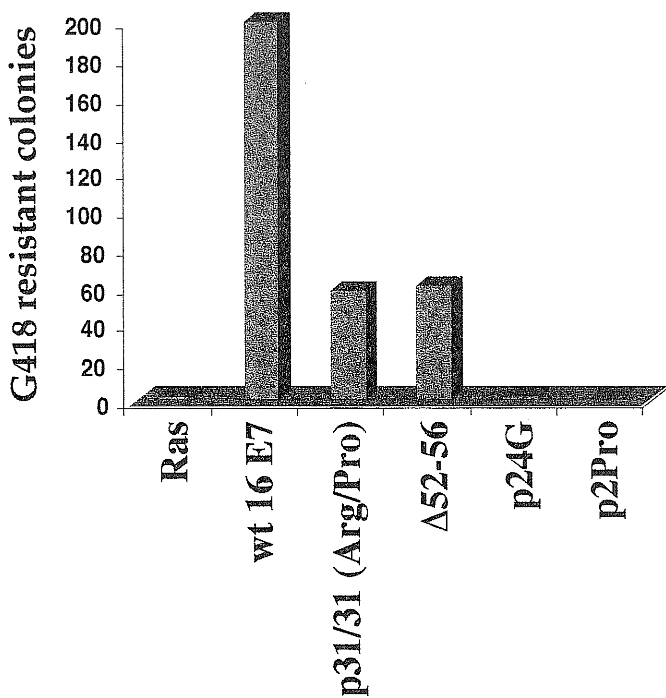


Figure 13
Transforming activity of E7 mutants in BRKs (primary baby rat kidney cells)

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   176 .          .          .          .          .          . 232
1. YIRYTPSQQGVAFNSGAK-QRVIRMVEMQKDPMEPPRFKINKKIPRGPP--SPPAPVMH
2. YIRYTPSQQGDTFNSGAK-QRVIRMVEAQLDPMEPPKFRINKKIPRGPP--SPPAPVLH
3. YIRYTPSQQN--GAAGSQ-QRIIRMVEEQKDPMEPPKFKINQKIPRAPP--SPPAPVMH
4. YIRYTPSNQ--MGQALSK-QRIIKMVTAEQDPMEPPKFR-HKKVPRGPP--SPPPPVLH
5. YIKYTPSNQLGSNNGSALNSKIVRMVDVAQDPLEPPKYKIKKKIMEHG---SPPAPVMH
6. YVTETHQAP---ANLLLNNSHHIEVVSKQMDPLLPRFVGKKARKVVAPTENDEVVPVLH
  233      .          .          .          .          .291
1 SP-SRKMTVKEQQEWKIPPCISNWKNAKGYTIPLDKRLAADGRGLQTVHINENFAKLAEA
2 SP-SRKVTVKEQKEWKIPPCISNWKNAKGYTIPLDKRLAADGRGLQQVHINEKFAKMAEA
3 SP-PRKMTAKDQNDWKIPPCISNWKNPKGFTVGLDKRLAADGRGLQQTHINENFAKLADA
4 SP-PRKVSAQEQQDWQIPPSISNWKNPKGYTIPLDKRLAADGRGLNDVEINDGFAKFSEA
5 SP-TRKLSVQDQQDWTIPPCVSNWKNPKGFAISIDKRLVSNGGGLQDVEINDKFAHFTQA
6 MDGSNDRGEADPNEWKIPAAVSNWKNPNGYTVALERRVG-KALDNENNTINDGFMKLSEA
  292       .          .          .          .   336
1 LYIADRKAREAVEMRAQVERKMAQKEKEKHEEKLREMAQKARERRA
2 LYIADRKAREAVEARSQLEKKLAQKEKEKKEDMLRMMAQRAREERA
3 LYIADRKAREEVETRAQLERRVAQNKKSEQEAKMAEAAAKARQERS
4 LYTVERQAREEVRYRAIMRQKMAEKEKQEKEQRLFMLAQKAREDRM
5 LYIAESNAREEVSARAELERKLAQKEKERKQDMLRKLAEDVRNERS
6 LENADKKARQEIRSKMELKRLAMEQEMLAKESKLKELSQRARYHNG
```

Figure 14. Comparison of the human Skip sequence (Q13573) with its homologues from 1) D. melanogaster (P39736), 2) *C. elegans* (Q22836), 3) S. pombe (Q09882), 4) D. discoideum (P54705) and 5) S. cerevisiae (P28004) illustrating the high degree of amino acid (aa) conservation. Red indicates aa identical in all the homologues, **blue** indicates aa conserved in charge and **green** indicates aa having similar polarity. The E7 binding motif rich in prolines is shown in yellow shading. The alignment was generated using ClustalIW at http://au.expasy.org/

schematically in Figure 15A. The various Skip deletion mutants were translated *in vitro* and labeled with [^{35}S] methionine. GST-pull down assays were done using a GST-16 E7 fusion protein bound to glutathione-agarose. The results obtained are shown in Figure 15B, C and D. As can be seen, wild type (wt) Skip showed strong binding to GST-16 E7 (Figure 15A, B and C, lane 1). In addition, mutants 1-353 (Figure 15B, lane 2), 171-536 (Figure 15C, lane 2) and 179-353 (Figure 15C lane 3) showed wt levels of interaction, whereas the carboxy terminal mutant containing amino acid residues 350-536, and the amino terminal mutant containing amino acids 1-179 failed to bind (Figure 15A, lanes 3 and 4). Interestingly, the mutant comprising the amino acids 220-398 retained a substantial binding in the GST-pull down assay (Figure 15B, lane 5), suggesting that this region is important for the interaction with 16 E7. To further define the E7 binding motifs on Skip, additional deletion constructs of Skip were made and GST-pull down assays were done, and the results obtained are shown in Figure 15D. Strong binding was seen with wt Skip and only very little binding was obtained with the 1-179 mutant. In addition, mutant 1-219 showed a minimal binding to E7 (Figure 15D, lane 3). Most interestingly, the mutant 1-236, which contains the proline rich sequence (PRS), retained wild type levels of binding (lane 4), thus demonstrating that the PRS is the region of Skip bound by E7. Mutant 1-292, which also contains the PRS, also showed wild type binding activity to E7 (lane 5). These results suggest that Skip's PRS within the SNW domain is the binding motif for E7.

15A

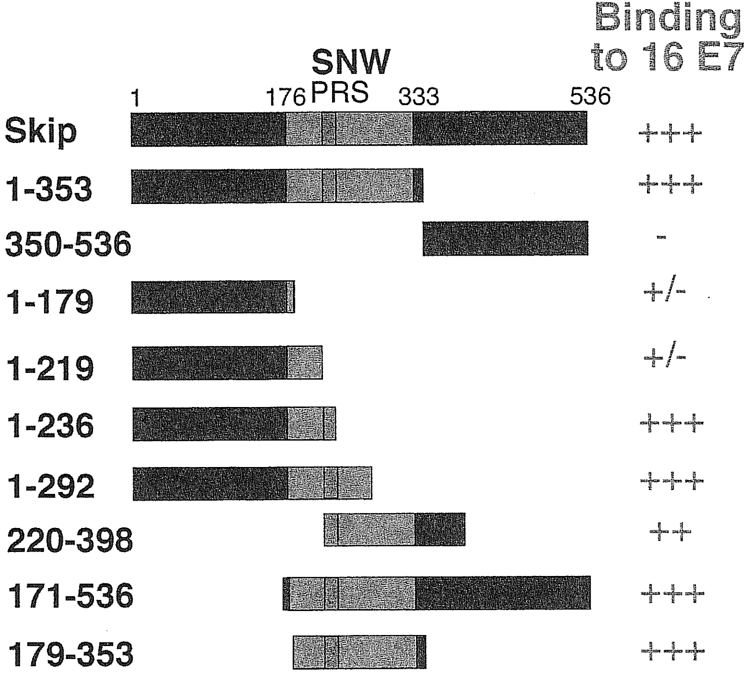

Figure 15A. Schematic diagram of the Skip deletion mutants used in the analysis. Numbers refer to the amino acid residues and the central red boxed region corresponds to the highly conserved SNW region, where homology with Skip proteins derived from other species is very high. Proline Rich Sequence (PRS) within the SNW domain is also shown. The relative strength of binding of each protein to E7 is also shown, together with the mean percentage binding from at least three separate assays, where +++ and +/- indicates 20-30% and 1-2% binding respectively. See Panels B and C for the results from representative GST pull-down assays.

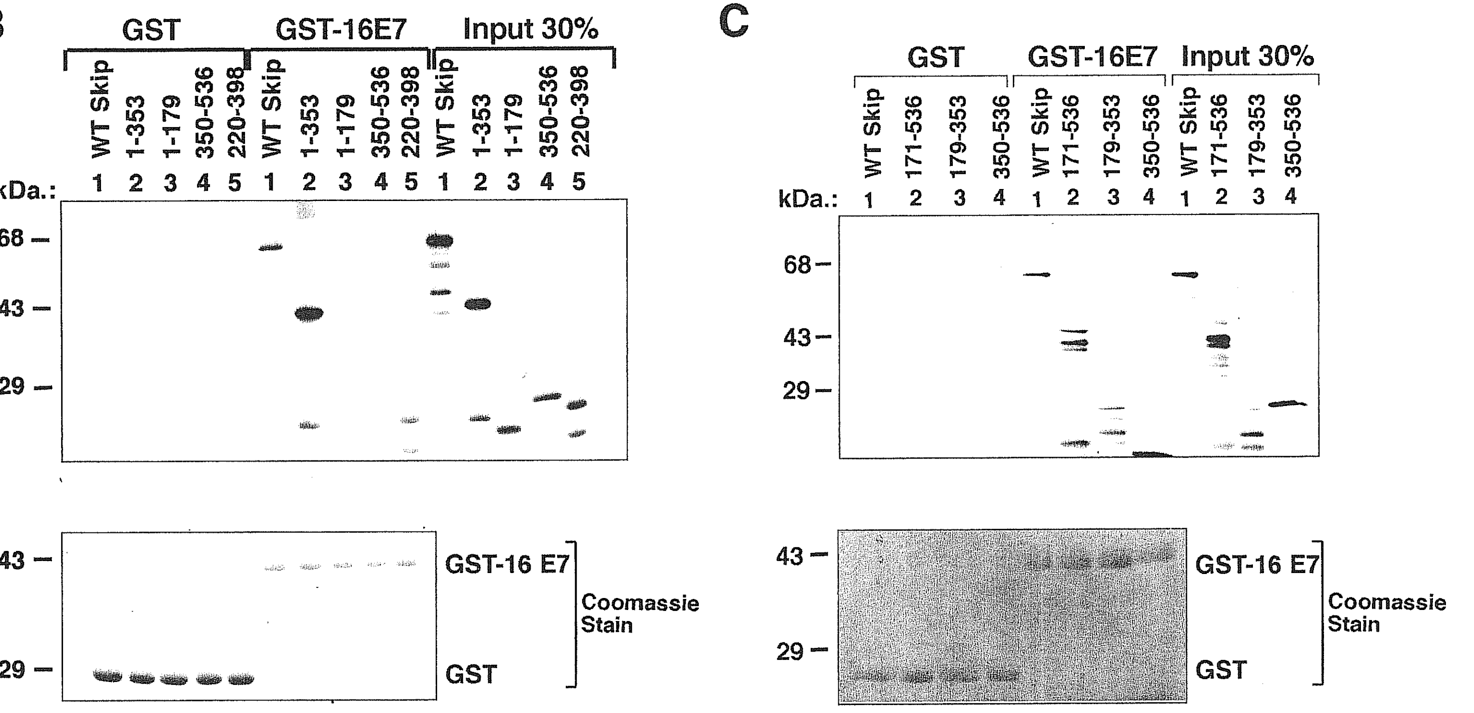

Figure 15. Mapping the HPV-16 E7 interaction domain on Skip.

Panels B and C show the results from representative GST pull-down assays. Purified GST or GST-E7 on glutathione resin were incubated with the in vitro translated Skip deletion mutants for 1h at room temperature. After extensive washing bound proteins were analysed by SDS PAGE and autoradiography. Lower panels of B and C show the Coomassie Blue stain of the gels used in the binding assay.

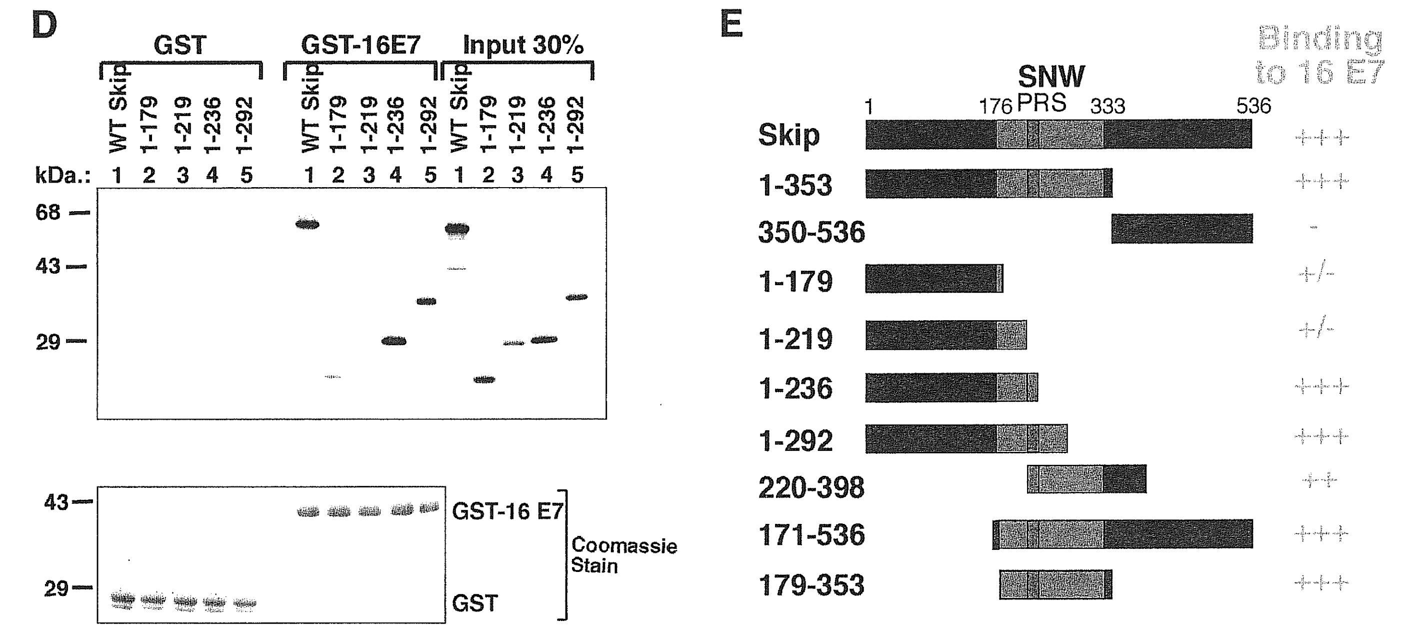

Figure 15. Mapping the HPV-16 E7 interaction domain on Skip.

Panels D show the results from representative GST pull-down assays. Purified GST or GST-E7 on glutathione resin were incubated with the in vitro translated Skip deletion mutants for 1h at room temperature. After extensive washing bound proteins were analysed by SDS PAGE and autoradiography. Lower panel of D show the Coomassie Blue stain of the gels used in the binding assay. extensive washing bound proteins were analysed by SDS PAGE and autoradiography.
Panel E shows a schematic diagram of the Skip deletion mutants together with the summary of interactions. The relative strength of binding of each protein to HPV-16 E7 is also shown, together with the mean percentage binding from at least three separate assays, where +++ and +/- indicate 20-30% and 1-2% binding respectively.

7. Skip stimulates the activation of diverse viral and cellular promoters

The *D. melanogaster* homologue of Skip, Bx42, is found associated with chromatin in transcriptionally active puffs of salivary glands (Saumweber et al. 1990; Wieland et al. 1992), suggesting a role in the regulation of transcription. Recently, Skip has been shown to have a role in the EBNA2 (Epstein-Barr virus encoded latency protein) activation of CBF1-repressed promoters (Zhou et al. 2000a). Contacts with both CBF1 and Skip were shown to be important for the effective targeting of EBNA2 to DNA. Skip was also shown to interact with the ankyrin repeat domain of NotchIC to facilitate NotchIC function in the activation of downstream target genes of the notch signaling pathway (Zhou et al. 2000b). More recently, Skip has also been shown to interact with Smad proteins to augment TGF-β dependent transcription (Leong et al. 2001).

Ski activates transcription of muscle specific and certain viral promoters (Kelder et al. 1997), and has also been found to enhance transcriptional activation by binding nuclear factor I (NF1) sequences (Tarapore et al. 1997). In addition, Ski was also found to be a component of the histone deacetylase (HDAC1) complex (Nomura et al. 1999; Tokitou et al. 1999) and was shown to interact with Smad proteins and to regulate Transforming Growth Factor-β (TGFβ) signaling (Sun et al. 1999a; Sun et al. 1999b). Despite these observations, the molecular mechanism by which Ski transforms cells and regulates differentiation remains unknown, as is the role of Skip in these processes.

E7 has also been found to transcriptionally activate E2F-responsive promoters by dissociating the Rb/E2F complex (Phelps et al. 1988; Jansen-Durr 1996, review) and several studies have also described E2F-independent transcriptional activities of E7. Therefore, considering the clear association of Skip, Ski and E7 with diverse

transcriptional activities, I was interested in determining how the E7-Skip interaction might function with respect to the transcriptional activity. However, in order to do this it was first necessary to identify the appropriate promoters that would be Skip responsive. Therefore, I first analysed the transcriptional activities of Skip on a series of different reporter constructs containing both cellular and viral promoters. These are shown schematically in Figure 16 and include the Adenovirus E2 promoter (AdE2) (Murthy et al. 1985), the HSV TK promoter (pBLCAT2) (Luckow and Schutz 1987) and the cellular, p53 responsive, p21 promoter (El-Deiry et al. 1993). U2OS cells were transfected with the different reporter constructs together with increasing amounts of Skip expression plasmid. After 48h the cells were harvested and CAT assays were performed. The results obtained are shown in Figure 17. It is clear from this analysis that Skip is a potent transcriptional activator of the viral and cellular promoters tested, showing strong, dose-dependent activation of the AdE2 promoter, the HSV TK promoter, and the p53 responsive p21 promoter (Figure 17). In contrast, no increase in transcription is seen using the heterologous GAL4 promoter, which cannot function without the GAL4 activation domain in mammalian cells, nor when using the pBLCAT3 reporter (Luckow and Schutz 1987), which lacks a TK promoter (Figure 17). This would suggest that Skip can behave as a generalized transcriptional co-activator, since increased transcriptional activity was only obtained with Skip on already active promoters. As can be seen from Figure 18, no transcriptional activity was observed on the p53 responsive p21 promoter in p53-negative Saos-2 cells, indicating further that Skip acts as a co-activator.

To further investigate the potential of Skip to act as a co-activator, promoter activation assays were performed in the presence of the HPV-16 E2 transactivator (Phelps and

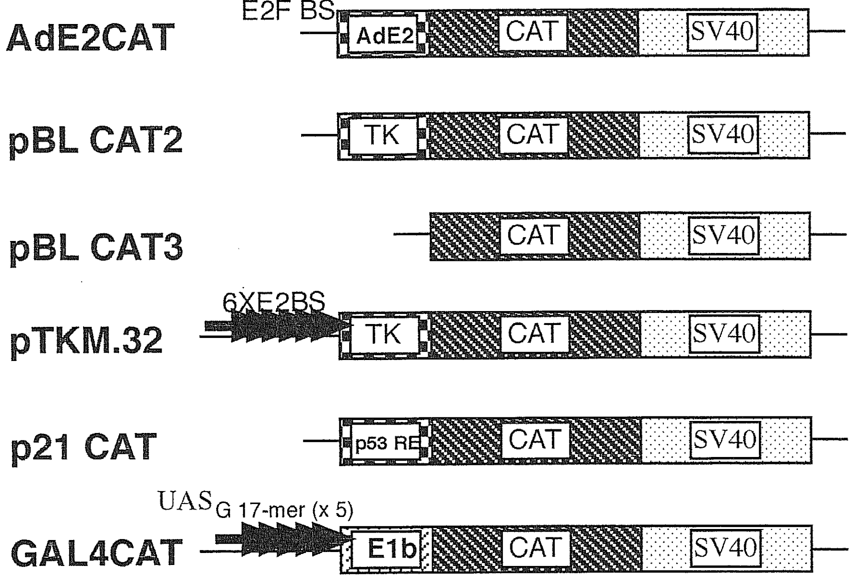

Figure 16. CAT reporter constructs used in the study
The promoter and SV40 polyadenylation signal is shown. BS refers to DNA binding site, RE refers to responsive element, 6xE2BS means 6 HPV-16 E2 binding sites. GAL4 CAT contains five consensus GAL4 binding sites, which is represented as UAS G17-mer (x 5).

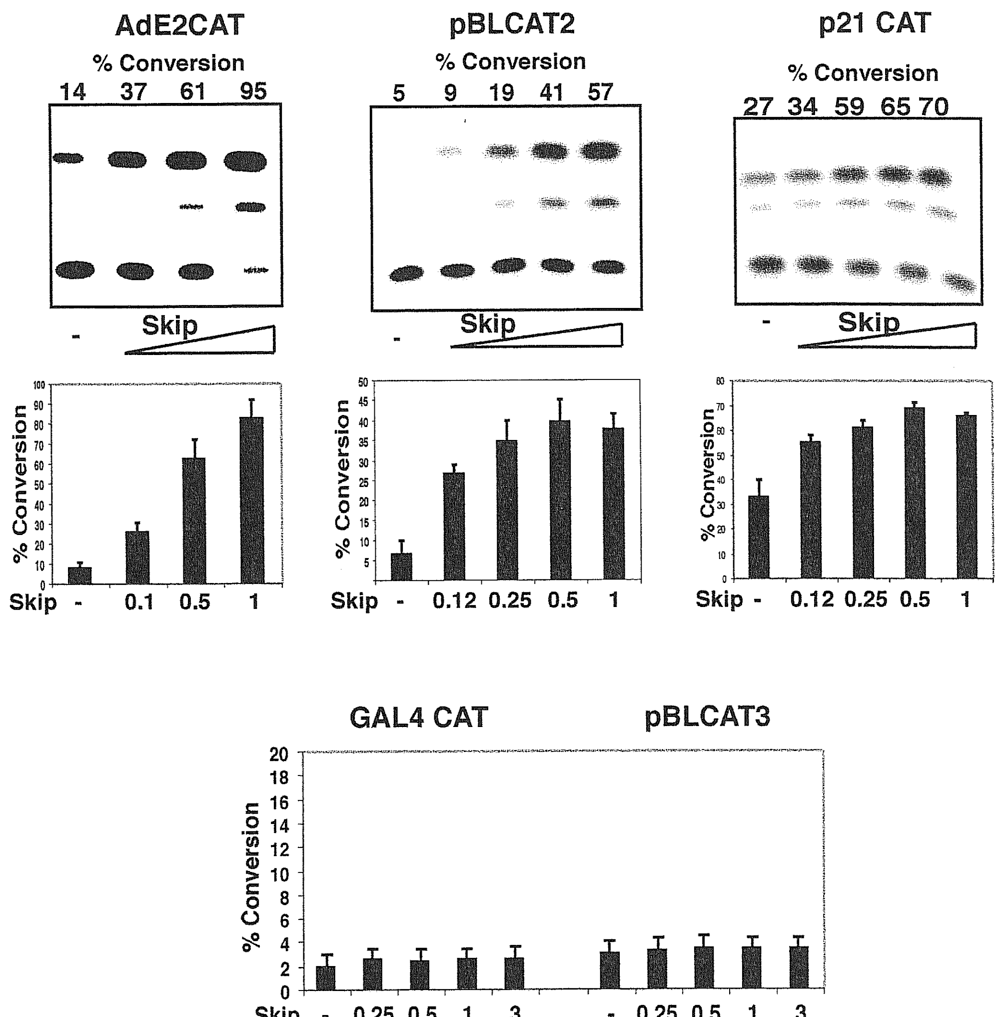

Figure 17. Skip stimulates the activation of diverse viral and cellular promoters U2OS cells were transfected with the indicated reporter plasmids together with increasing amounts of the Skip expression plasmid as shown (concentrations are shown in μg). After 48h the cells were harvested and CAT assays performed. In each case a representative CAT assay is shown, together with the collated results from at least three independent transfections. Standard deviations are shown.

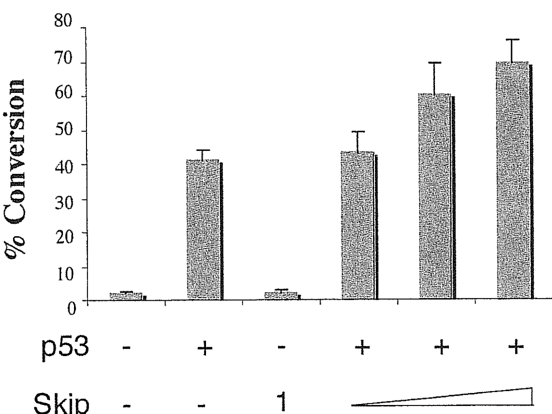

Figure 18. Skip alone does not activate the p53 responsive p21 promoter in Saos-2 cells.
Saos-2 cells were transfected with p21 reporter plasmid together with p53 (2 μg) and Skip expression plasmids as indicated (0.5, 1 and 3μg). The graph shows the collated results from atleast three independent transfections. Standard deviations are shown.

Howley 1987; Bouvard et al. 1994). Cells were transfected with an E2-responsive construct, containing six E2 DNA binding sites upstream of the HSV TK promoter (pTKM.32) (Figure 16) with constructs expressing Skip and HPV 16 E2 in various combinations. After 48 h the cells were harvested and CAT assays were performed. The results obtained are shown in Figure 19. Skip activated the pTKM.32 promoter on its own which is consistent with the results obtained showing Skip activation of the HSV TK promoter (Figure 17). However, as can be seen in Figure 19, HPV-16 E2 transactivation activity is dramatically increased in a dose-dependent manner by increasing amounts of Skip. These results provide further evidence that Skip acts as a transcriptional co-activator on a diverse range of promoters.

8. Cellular Ski and Skip cooperate in transcriptional transactivation

Since Skip was originally identified as a Ski interacting protein and as Ski itself has been shown to have intrinsic transcriptional activity, I next wanted to investigate the effect of Ski on Skip's transcriptional activation activity. In order to do this, I chose to analyse the effects of Ski upon Skip activation of the HSV TK promoter (pBLCAT2). U2OS cells were transiently transfected with the pBLCAT2 reporter plus constructs expressing Skip or c-Ski, alone or in combination, and CAT assays were performed after 48 h. The results are shown in Figure 20. As can be seen, expression of Skip alone results in a significant increase in promoter activity. In contrast, expression of Ski alone has minimal effect. Strikingly, co-expression of Ski and Skip results in a dramatic increase in the HSV TK promoter activity, suggesting that the Skip-Ski complex can act synergistically to activate the TK promoter. Having observed the co-operation between Skip and Ski in the

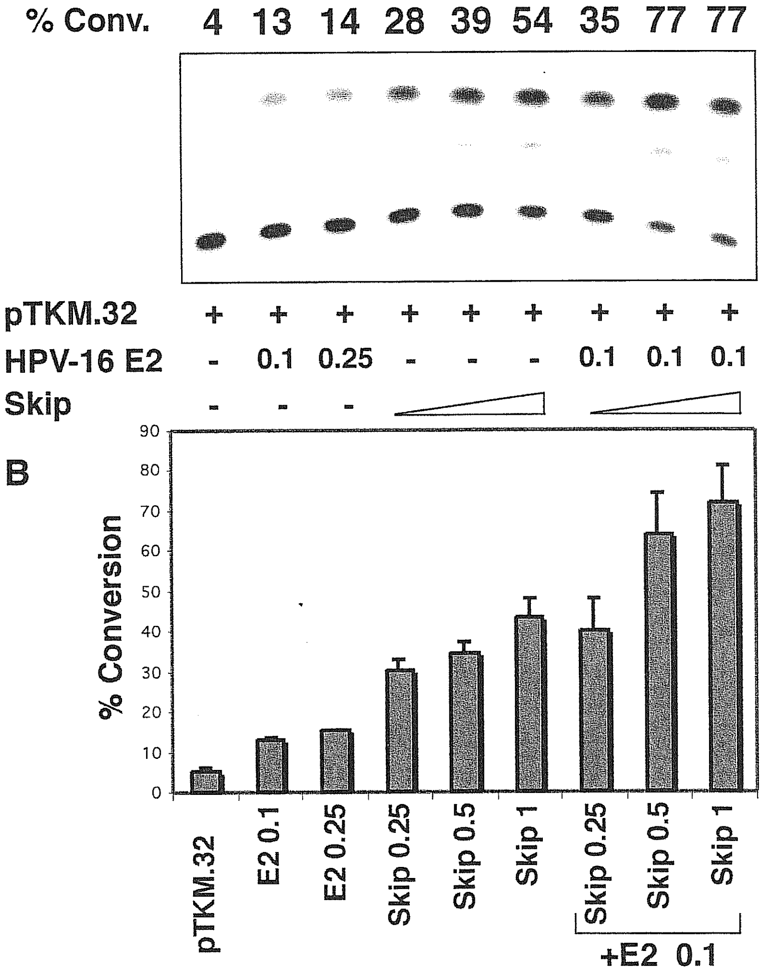

Figure 19. Skip is a transcriptional co-activator.
U2OS cells were transfected with the HPV-16 E2 responsive pTKM.32 CAT reporter plasmid together with 0.1μg or 0.25μg of pJ4Ω.16E2, as indicated. For assessing Skip co-activation, the input of pJ4Ω.16E2 was fixed at 0.1μg and increasing amounts of Skip expression plasmid were used as indicated (concentrations are shown in μg). After 48h the cells were harvested and CAT assays performed.
The upper panel shows the results from a representative assay and the lower panel shows the collated results from at least three independent transfections. Standard deviations are shown.

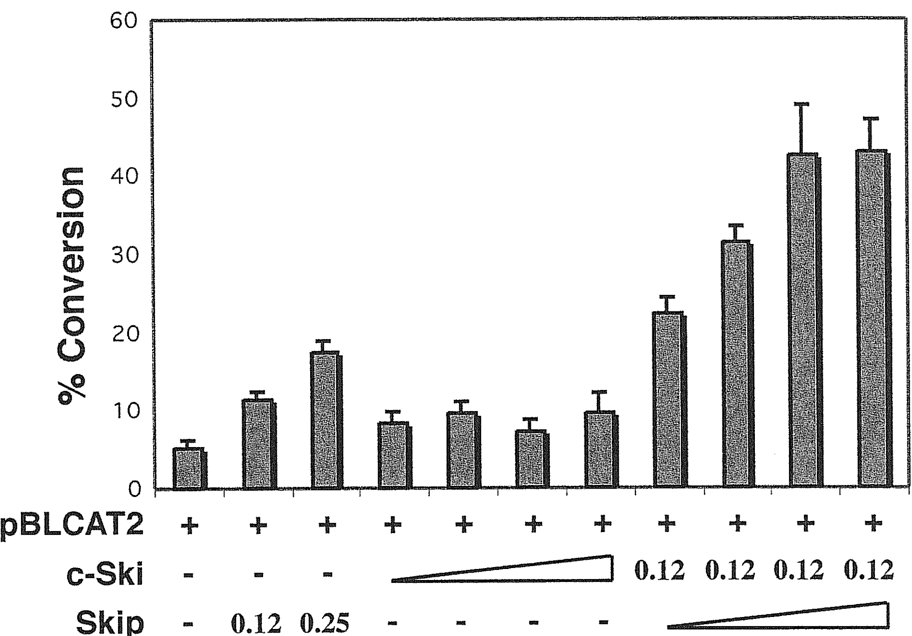

Figure 20. Skip and c-Ski cooperate in transcriptional activation of the HSV TK promoter. U2OS cells were transfected with the pBLCAT2 reporter plasmid together with the combinations of Skip and c-Ski expression plasmids indicated (concentrations are in μg). The concentrations of c-Ski expression plasmid used were 0.125, 0.25, 0.5 and 1 μg. The graph shows the collated results from atleast three independent transfections. Standard deviations are shown. Standard deviations are shown.

activation of the HSV TK promoter in U2OS cells, I next wanted to investigate their effects in an another cell line and on another promoter. Therefore, I repeated the assays using the AdE2 promoter in Saos2 cells. As can be seen in the Figure 21, Skip and Ski separately transactivated the AdE2 promoter and the results seen with Ski are in agreement with the previous observations of vSki-induced enhancement of E2F1 (Tokitou et al. 1999). However, transcriptional cooperation between Skip and Ski was again observed, suggesting that this activity is neither cell type nor promoter dependent.

9. HPV E7 abrogates the transactivation function of a Skip-Ski complex.

I next proceeded to investigate the effects of HPV-16 E7 upon Skip's transcriptional activity. To do this, U20S cells were co-transfected with the HSV TK promoter construct, together with constructs expressing Skip, Ski and increasing concentrations of the HPV-16 E7 expression construct. As can be seen in Figure 22, HPV-16 E7 induced a dose-dependent inhibition of the Skip-Ski transactivation activity. In contrast, a parallel assay using the E7 responsive AdE2 promoter, in the presence of E7 alone, showed marked transcriptional transactivation, in agreement with previous observations (Phelps et al. 1988), demonstrating the functionality of the E7 protein used in the assay.

In order to determine whether pRb was involved in this activity of E7, the transcriptional inhibition assay was also performed in Saos-2 cells, which lack any functional pRb. In this case the AdE2 promoter was used, which is unresponsive to E7 in these cells due to this lack of pRb. As can be seen from Figure 23, increasing amounts of E7 again dramatically suppressed the transactivation activity of the Skip-Ski complex. These

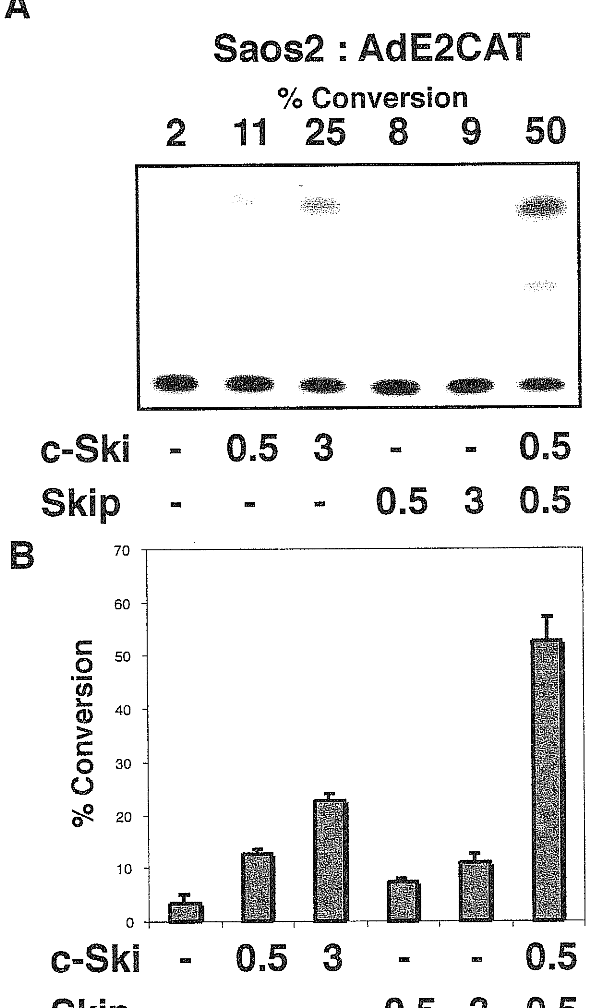

Figure 21.
Skip and c-Ski act synergistically to activate the AdE2 promoter. Saos 2 cells were transfected with the AdE2 reporter plasmid together with the combinations of Skip and c-Ski expression plasmids indicated (concentrations are in μg). After 48h the cells were harvested and CAT assays performed. The graph shows the collated results from at least three independent transfections. Standard deviations are shown.

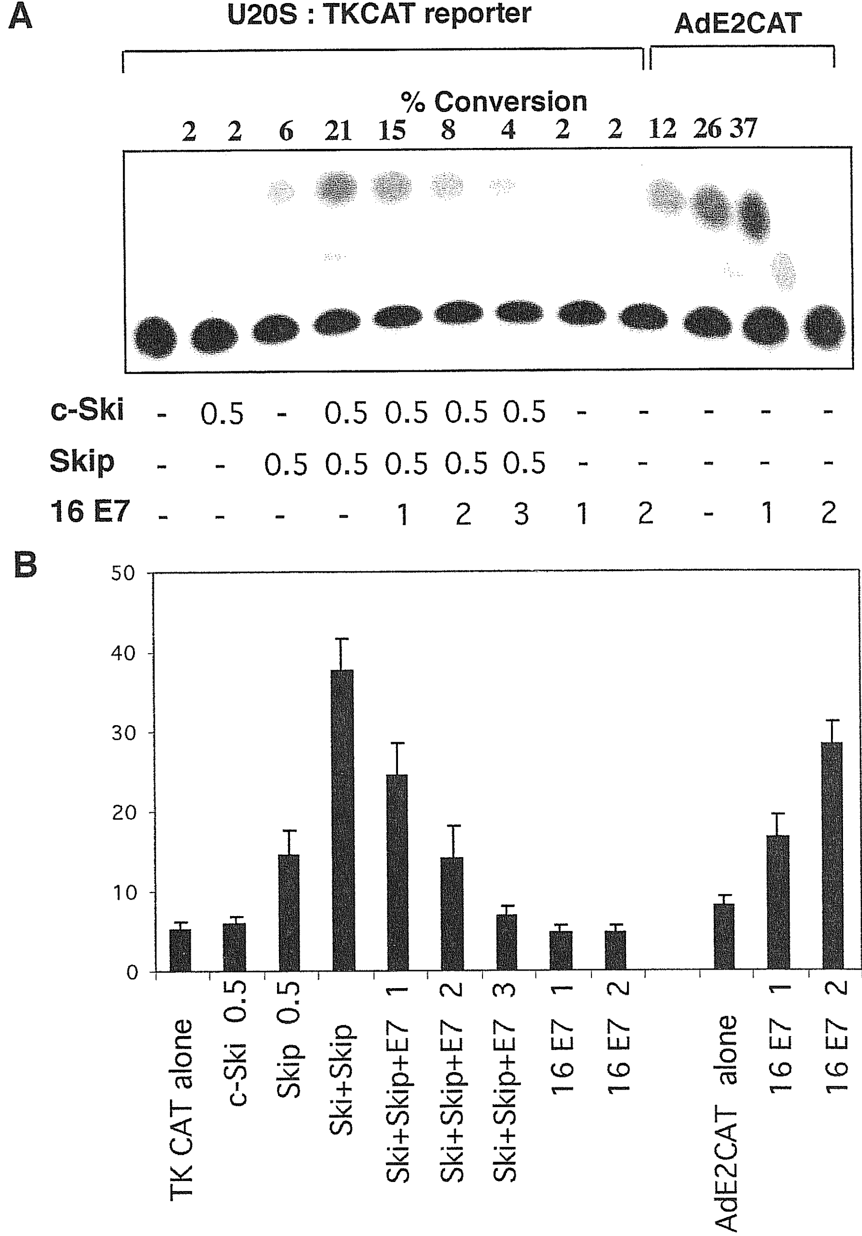

Figure 22. HPV-16 E7 inhibits Skip transcriptional activaty. U2OS cells were transfected with either pBLCAT2 (containing the HSV TK promoter) or AdE2CAT reporter plasmids, together with the combinations of Skip, c-Ski and E7 expression plasmids indicated (concentrations are in μg). After 48 h the cells were harvested and CAT assays performed. The upper panel shows the results from a representative assay and the lower panel shows the collated results from at least three independent transfections. Standard deviations are shown.

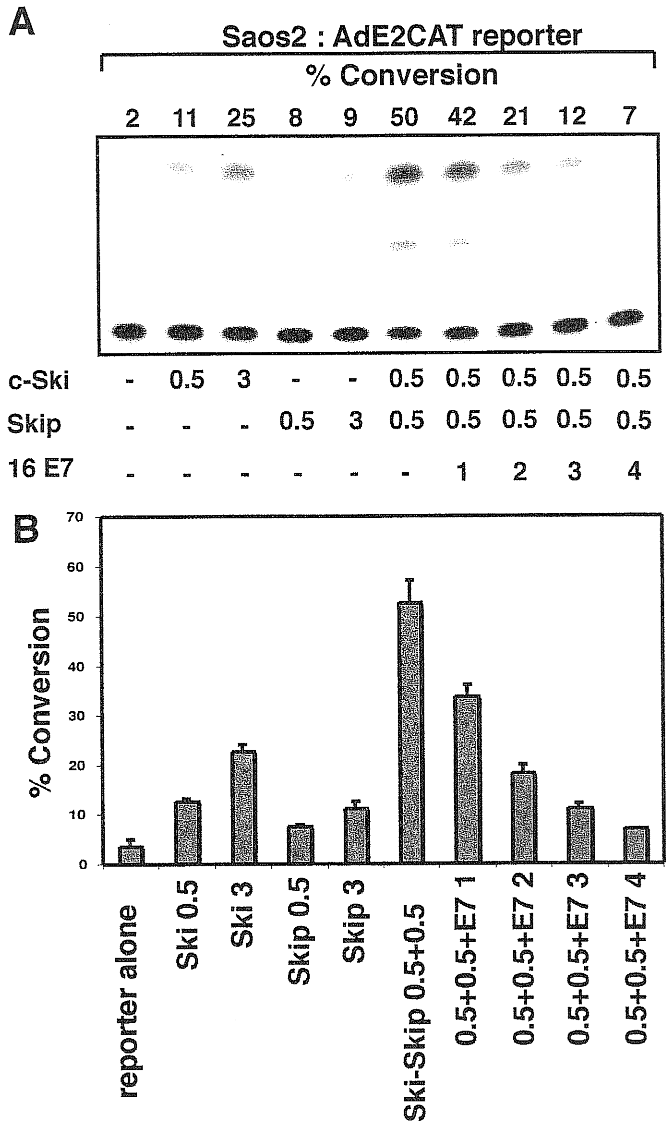

Figure 23. HPV-16 E7 repression of Skip transcriptional activation is pRb independent. Saos-2 cells were transfected with 2 μg of the AdE2 CAT reporter construct together with the indicated amounts of Skip, c-Ski and E7 expression plasmids (concentrations are in μg). After 48 h the cells were harvested and CAT assays performed. The upper panel shows the results from a representative assay and the lower panel shows the collated results from at least three independent transfections. Standard deviations are shown.

results demonstrate that one consequence of the E7-Skip interaction is the inhibition of the ability of Skip to activate the expression from certain target promoters.

Having determined that HPV 16 E7 abrogates the trans-activation function of a Skip-Ski complex and having identified mutants of E7, which fail to interact with Skip, I next proceeded to investigate whether inhibition of Skip-Ski mediated transactivation was dependent upon the ability of E7 to bind Skip. Transcriptional activation assays were done in U2OS cells using the HSV TK CAT reporter. As shown in Figure 24, wild type E7 strongly repressed the Skip+Ski-mediated transactivation of this promoter. Interestingly, the p31/32 mutant failed to suppress Skip activation of the HSV TK promoter. Likewise, mutant Δ52-56 was also reduced in its ability to repress the Skip+Ski mediated trans-activation. Both of these mutants of E7 are defective in their abilities to interact with Skip, therefore these results demonstrated that the ability of E7 to repress Skip-Ski transcription correlates with the ability of E7 to bind Skip.

I then wanted to explore the possible mechanisms of inactivation of Skip by E7. First, I investigated, whether E7 induces degradation of Skip in a similar manner to to its effect on pRb (Boyer et al. 1996; Jones et al. 1997a). Therefore I first analyzed the expression levels of E7 and Rb in HPV negative (HaCat) and positive (Caski and SiHa) keratinocyte cell lines, and the result is shown in Figure 25. As can be seen Rb showed lower steady state levels in HPV infected cells, whereas there are no significant differences between steady state expression levels of Skip in HPV positive and negative cells, suggesting that, the effect of E7 on Skip is not due to proteolysis induced by E7. This hypothesis was further confirmed by the results from the transient expression experiments in U20S cells (Figure 26). Plasmid constructs expressing HA-Skip and E7 were co-transfected into the

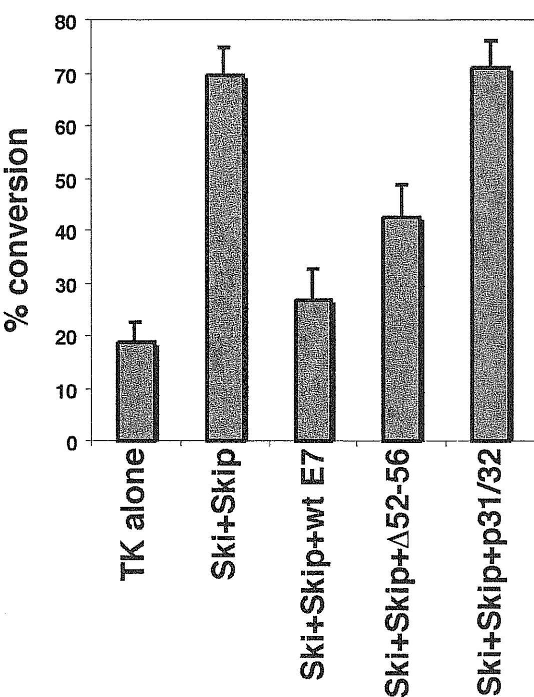

Figure 24. Functional analysis of E7 mutants negative for Skip binding

U2OS cells were transfected with 1μg of pBL CAT2 reporter plasmid (containing the HSV TK promoter) together with the combinations of Skip (0.5μg), Ski (0.5μg) and E7 (3μg) expression plasmids. After 48 h the cells were harvested and CAT assays performed. The graph shows the collated results from at least three independent transfections. Standard deviations are shown.

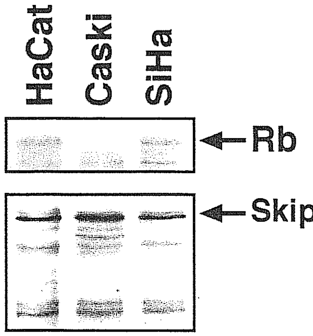

Figure 25. Expression levels of Rb and Skip in HPV positive (Caski, SiHa) and negative cell lines (HaCat).
Total cell extracts from human keratinocyte cell lines were separated on SDS-PAGE gel and analayzed by western blotting with antibodies against Rb (C15) and Skip (α1-219). Equal amounts of protein, as determined by Bradford assay were loaded.

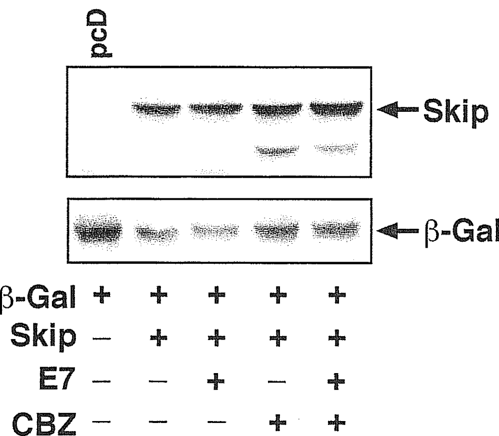

Figure 26. Western blot analysis of cells after transient transfection with HPV-16 E7.
U2OS cells co-transfected with Skip, E7 and β-Gal expression plasmids as indicated. Total cell protein was analyzed on SDS-PAGE. Western blots of proteins were probed with α-HA and β-Gal antibodies to determine the expression levels.

U2OS cells and Skip levels were assessed by western blotting and probing with an α-HA antibody. The results again do not suggest significant regulation of Skip protein levels by E7. Next, I investigated whether E7 can physically disrupt the interaction between Skip and Ski and so inactivate Skip. As can be seen from the Figure 27, *in vitro* translated (IVT) Skip protein specifically associated with GST-Ski on beads. However, increasing concentrations of IVT E7 could not disrupt the interaction between Skip and Ski.

10. Identification of the Ski interacting domain on Skip

Taking the evolutionary conserved nature of the PRS/SNW domain, together with the fact that this region is also targeted by E7, suggests that this most likely represents an important functional domain of the protein. An obvious candidate protein for binding to this domain on Skip is Ski. Therefore, since the binding site of Ski on Skip has not yet been defined, I proceeded to investigate the ability of the different Skip mutants to interact with Ski. The various Skip deletion mutants were translated *in vitro* and GST pull-down assays were performed using a GST c-Ski fusion protein bound to glutathione-coupled agarose. The results obtained are shown in Figure 28. As can be seen, the wild type (wt) Skip showed strong binding to c-Ski, in agreement with previous studies (Dahl et al. 1998). In addition, mutant 1-353 showed wt levels of interaction, whereas the carboxy terminal mutant containing amino acid residues 350-536 failed to bind and only weak binding was obtained with the mutants spanning residues 1-179. Interestingly, the mutant spanning residues 1-292 retained wt type binding with Ski. These results clearly show that the region of Skip required for interaction with Ski lies principally between

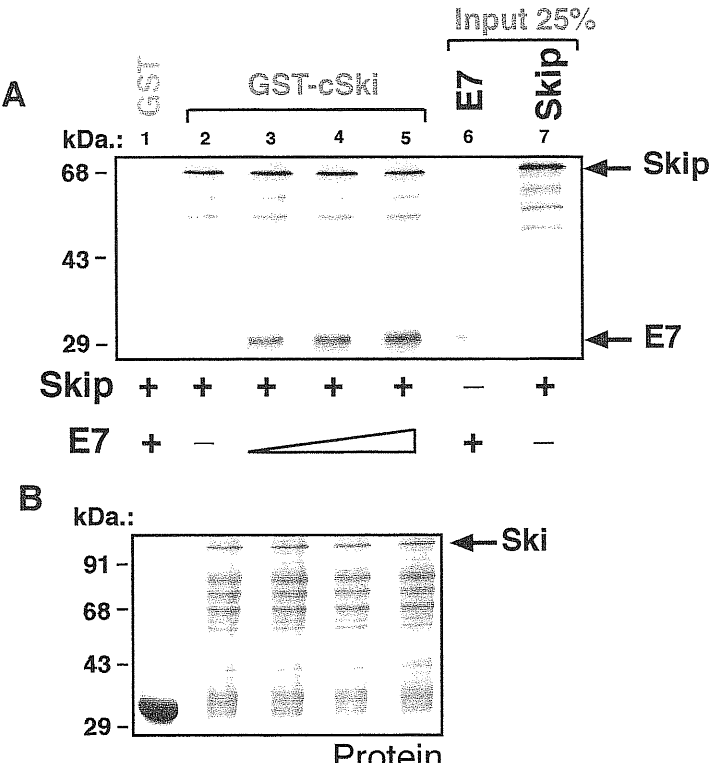

Figure 27. E7 does not dissociate the Skip-Ski complex
Panel A shows the results from a representative GST pull down assay. Purified GST or GST-c-Ski on glutathione resin were incubated with the in vitro translated Skip and/or E7 for 1h at room temperature. After extensive washing bound proteins were analysed by SDS PAGE and autoradiography.
Panel B shows the coomassie stain of the gel used in the binding assay.

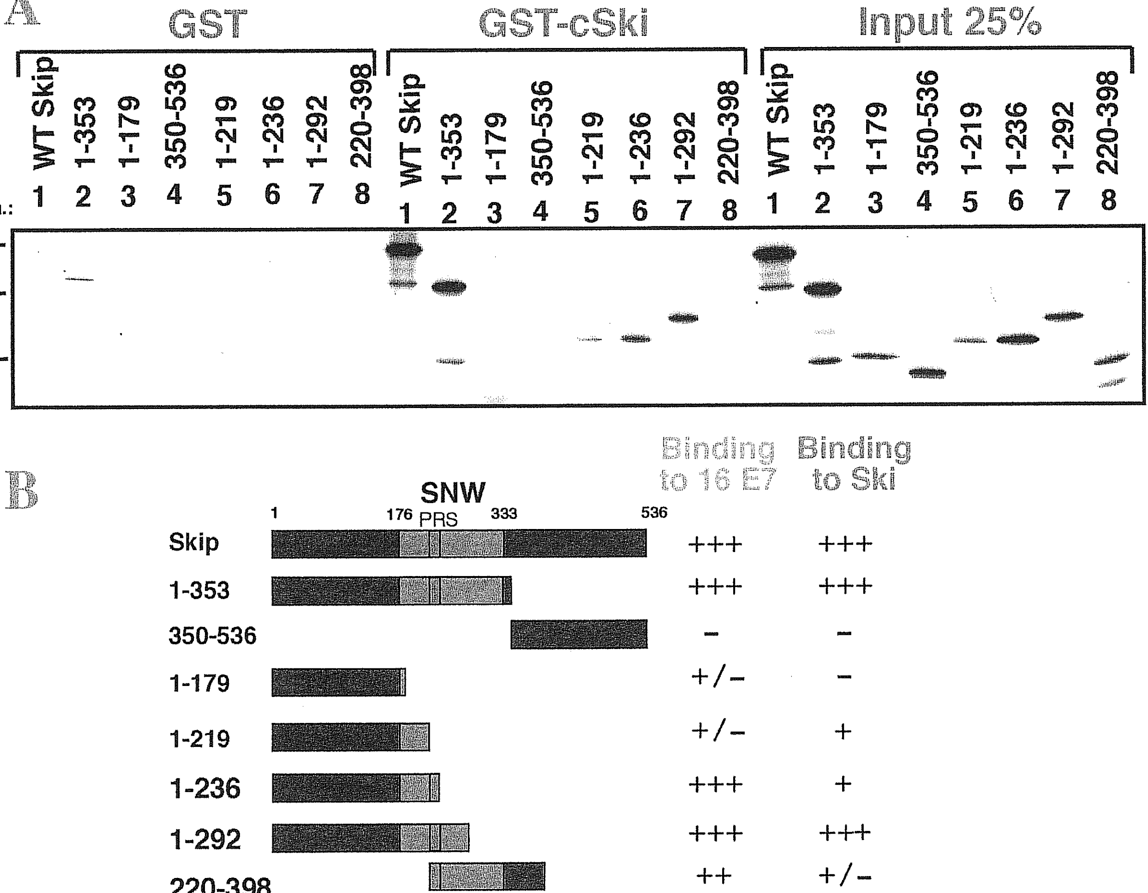

Figure 28. Mapping the c-Ski interaction domain on Skip.
Panel A shows the results from a representative GST pull down assay. Purified GST or GST-c-Ski on glutathione resin were incubated with the in vitro translated Skip deletion mutants for 1h at room temperature. After extensive washing bound proteins were analysed by SDS PAGE and autoradiography.
Panel B shows a schematic diagram of the Skip deletion mutants used in the analysis. Numbers refer to the amino acid residues and the central red boxed region corresponds to the highly conserved SNW region where homology with Skip proteins derived from other species is greater than 90%. The relative strength of binding of each protein to HPV-16 E7 and c-Ski is also shown, together with the mean percentage binding from at least three separate assays, where +++ and +/- indicate 20-30% and 1-2% binding respectively.

residues 179 to 292 and lies within the SNW domain, which is defined by amino acids from 176 to 333.

11. Characterization of SNW domain of Skip

Having characterized Skip as a transcriptional co-activator, I next wanted to investigate whether the region of Skip required for binding E7 and Ski, was also required for its transcriptional activity. In order to ensure that the mutant Skip proteins were stably expressed, cells were transfected with each of the expression plasmids, and mutant Skip expression assessed by western blotting. The results obtained are shown in Figure 29. As can be seen, the Skip mutants were all expressed and produced comparable levels of protein. Next, I analyzed the behaviour of these mutants in the reporter assay. U2OS cells were transiently transfected with the mutants of Skip together with the pBLCAT2 reporter, in the presence or absence of c-Ski. Cells were harvested after 48 h and transcriptional assays were performed: the results obtained are shown in Figure 30. Strikingly, the region of Skip found to be responsible for binding Ski is also essential for the ability of Skip to activate transcription. Thus, the construct containing amino acid residues 1-353 behaves very similarly to the wt Skip in both transcriptional activation and in its ability to synergise with c-Ski. In contrast, the mutant comprising amino acid residues 350-536, which is completely defective in its ability to bind c-Ski, is also defective in its ability to activate transcription and to synergise with c-Ski. Interestingly, the construct containing amino acid residues 1-179, which showed weak binding to c-Ski, also retains both weak transcriptional activating activity and ability to synergize with c-Ski.

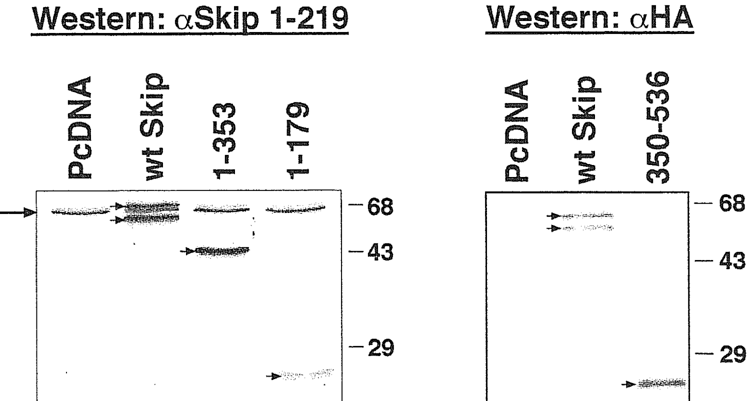

Figure 29. Expression levels of Skip proteins in U2OS.
Total protein extracts were prepared, transferred to nitrocellulose membrane and incubated with either anti-Skip polyclonal antibody or with an anti HA-tag monoclonal antibody. Staining was done with ECL detection system. Large arrow indicates the position of endogenous Skip protein.

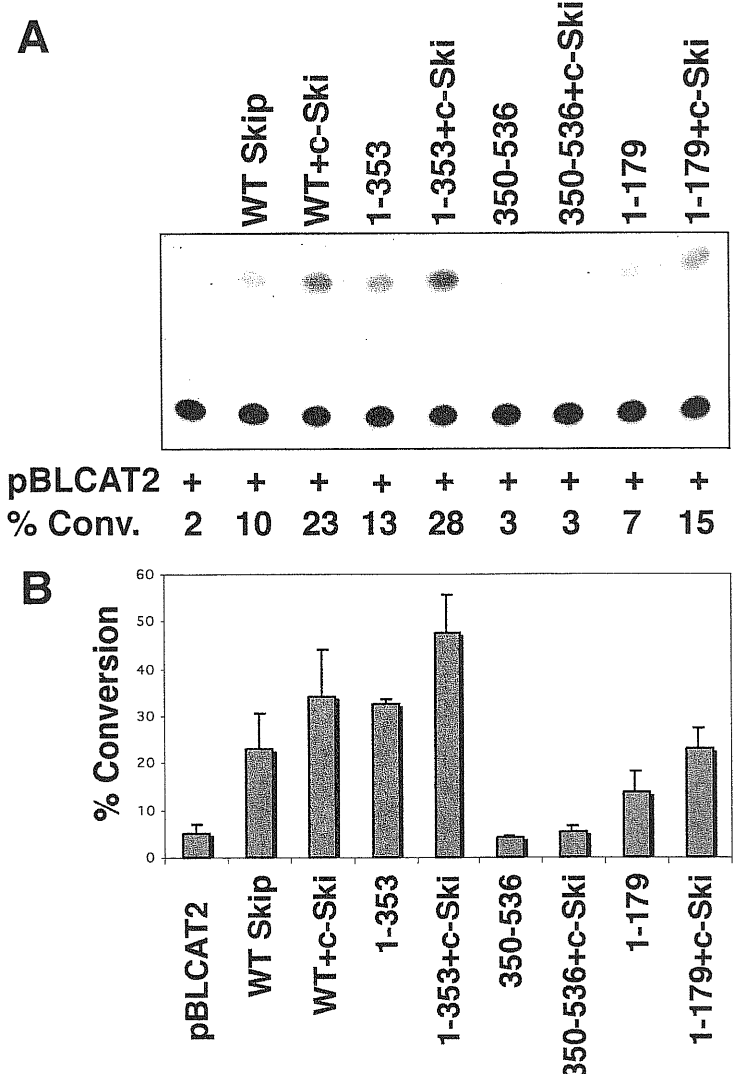

Figure 30. The c-Ski binding region of Skip is required for its ability to stimulate the HSV TK promoter. U2OS cells were transfected with the pBLCAT2 reporter construct together with 0.25 μg of the different deletion constructs of Skip. In addition 0.5 μg of c-Ski expression plasmid was also included as indicated. The upper panel shows the results from a representative assay and the lower panel shows the collated results from at least three separate transfections. Standard deviations are shown.

DISCUSSION

The E7 oncoprotein of the high-risk human papillomavirus type 16, which is etiologically associated with uterine cervical cancer, is a potent immortalizing and transforming agent. Several studies suggest that the protein exerts most of its oncogenic functions by interacting physically with key cellular regulatory proteins resulting in the modulation of their normal activity. E7 interacts with hypophosphorylated pRb causing disruption of the growth suppressive pRb/E2F complexes (Chellappan et al. 1992), with a consequent release of E2F, which then upregulates the genes involved in G1/S cell cycle transition (Phelps et al. 1988; Jansen-Durr 1996, review). However, a large amount of evidence has indicated that inactivation of pRb is necessary, but not sufficient, for transformation (Edmonds and Vousden 1989; Banks et al. 1990; Chesters et al. 1990; Jewers et al. 1992; Phelps et al. 1992; Huang et al. 1993; Wu et al. 1993; Brokaw et al. 1994), suggesting that, in addition to pRb, other cellular targets of E7 are required for its full transforming activity. Therefore, the precise molecular events that underly the transforming and immortalizing activities of E7 are still obscure.

1. Identification of novel binding partners of HPV-16 E7

Here, in the first part of the thesis, I focused on identifying novel binding partners of HPV-16 E7 by applying a yeast two-hybrid screen. I used an improved version of the yeast two-hybrid method developed by Stephen Elledge (Durfee et al. 1993). The full-length E7 gene fused to the GAL4 DNA binding domain was used as bait. Intrinsic transcriptional activation of the E7 bait was overcome by using 50 mM 3-aminotriazol

(3AT), an inhibitor of the HIS3 selection marker. False positives has been recurring problem of the two-hybrid system. To eliminate the false positives, I generated a strain that has lost the pAS plasmid but retains the library plasmid. This was done by selecting them on SC-leu. The Leu^+ were then backscreened with the pAS-E7 construct. Positive clones were then immediately tested for β-galactosidase activity. This screen yielded clones containing Actin (Nishida et al. 1995; Rey et al. 2000a) and ADP/ATP carrier protein genes (Hellung Schonning et al. 2000), already known targets of E7, further increasing our confidence in the validity of this screen. No cDNA clone encoding for example pRb, TBP and c-jun were present in the screen among the positive clones. There are a number of possible reasons why a two-hybrid screen is not 100% effective in isolating all binding partners. In this case, the B-lymphocyte cDNA library used in the screen was constructed with a poly (A), so it may not have extended to the amino terminal sequences of some of the known targets. In addition, certain interactions may not manifest themselves in yeast if the interaction is lethal to the strain used for any reason. It is also known that some true positive interactions may not be seen in yeast screens due to structural reasons of incompatibility of bait and prey with the GAL4 DNA binding or GAL4 activation domains.

This E7 screen discovered a set of twenty two potential candidates; the specificity of interactions of some of them are shown in Figure 8. All the candidates were identified more than once (Figure 31).

Target Identity	No. of times found	Function	Key features
Skip	3	Transcription	SNWKN
SAP-145	8	Splicing	+ U2 SnRNP
Zn Finger P	3	Splicing	Zinc Finger
Rab - 32	5	Vesicle transport	GTP-binding
AEBP	4	Transcription	carboxy-peptidase
Actin	7	cytoskeleton	
ATP/ADP Carrier protein	8	apoptosis?	carrier protein
4/76	6		Cdk2 site
5/163/175	3		SNWKX
30/78	3		SNWKX
40	10		Cdk2 site
83/119	4		Cdk2 site
141/187	4		SNWKX/Cdk2 site
172	6		SNWKX
199	NA		Cdk2 site

Figure 31. A summary of some of the functional domains found in the targets of E7 identified from the two-hybrid screen.
NA: means not assessed

1.1. Targets of E7 with known function

1.1 A. Human AEBP

This cDNA encodes a transcription factor and a northern hybridization of AEBP expressing osteoblastic cell line showed that its expression is shut off in the final calcification stage (Ohno et al. 1996). Mouse adipocyte transcription factor belongs to a family of carboxypeptidase E genes since it is found to have a carboxypeptidase activity, which is essential for its transcriptional repression activity (He et al. 1995). However, at present nothing is known about its role if any, in the keratinocyte differentiation. However, should this protein turn out to be relevant for keratinocyte differentiation, then it would be oviously be of great interest to pursue the potential interaction with HPV E7.

1.1 B. Rab-32

It is known that HPV E6 interacts with E6TP1, a Rap GTPase and targets it for degradation (Gao et al. 1999) and this activity of E6 has been shown to correlate with its ability to immortalize human mammary epithelial cells (Gao et al. 2001). Therefore, it is also interesting to speculate that E7 may also target vesicle transport mechanisms through its association with Rab-32, should this interaction be verified. This belongs to a group of proteins belonging to the ras family of GTPases (Armstrong 2000). This would suggest that HPVs might have evolved two different strategies to regulate small G protein signaling.

1.1 C. SAP-145 and ZNF74

The interactions of E7 with the components of splicing machinery namely, SAP-145, a component of mammalian SF3b, a U2 snRNP associated protein (Gozani et al. 1996) and ZNF74, which was found to be colocalized with the SC35 splicing factor (Grondin et al. 1997) might possibly point to new mechanisms of HPV E7 mediated regulation of mRNA splicing in the cell cycle. This is particularly interesting taking into account of the recent report of cyclin E association with pre-mRNA splicing machinery, thereby suggesting that splicing is subject to cell cycle regulation (Seghezzi et al. 1998). There is also precedent for pre-mRNA splicing to be activated by other viruses, in particular adenoviruses (Muhlemann et al. 2000). The complexicity of RNA splicing in HPVs suggest that, HPVs must impinge on the splicing pathways, in order to subvert the host cell RNA biosynthetic machinery to facilitate their own replication. Therefore, this potential interaction also offers a mechanism whereby E7 may contribute to the regulation of HPV splicing during viral infection.

1.2 Targets of E7 with unknown function

In addition to the targets whose function is known, the ten new candidates that did not show homology to any gene of known function from the human genome database, hold potential promise for future studies. Five of the new E7 targets (#4, 40, 83, 141 and 199) showed a conserved motif STPXKR, which is a potential cyclin phosphorylation site, suggesting that these proteins are cell cycle regulated, which is obviously very interesting. Four of the candidates (#5, 30, 141 and 172) showed a conserved SNWKX motif which interestingly is also conserved in Skip. This suggests that SNWKX, which

lies in the SNW domain of Skip is important contact site for E7 and suggests that E7 may be targeting all these candidates by a conserved mechanism similar to its inactivation of Skip. A summary of some of the targets together with the prediction of their possible functional motives is shown in Figure 31.

2. Interaction of HPV-16 E7 with Skip

In the second part of thesis, I analyzed the interaction between E7 and Skip and I present evidence that Skip is a new cellular target of HPV-16 E7. The Skip protein was found to interact with a highly conserved domain of v-Ski, an oncogene of avian Sloan Kettering viruses (Dahl et al. 1998), which was found to transform chicken embryo fibroblasts (Stavnezer et al. 1981). Interestingly, this highly conserved region of Ski comprising amino acid residues from 79-185, which includes an amino proximal cysteine rich region and amphipathic helices, was found to be required for its transforming activity (Colmenares et al. 1991; Dahl et al. 1998). It has been suggested that, the transforming activity of v-Ski is partly due to its interaction with the pRb tumour suppressor protein and the consequent inhibition of pRb-mediated transcriptional repression (Tokitou et al. 1999). The conservation of properties between HPV E7 and v-Ski oncoproteins prompted me to select Skip for further analysis. Furthermore, Skip has also been shown to be essential for the Epstein-Barr virus-encoded latency protein (EBNA2)-mediated immortalisation of B-lymphocytes (Zhou et al. 2000a). Skip interacts with the CR5 of EBNA2, which is important for its transforming activity and plays a role in EBNA2 activation of CBF1-repressed promoters (Zhou et al. 2000a).

The interaction of E7 with Skip, identified from the yeast two-hybrid screen, was confirmed first by a GST-pull down assay using *in vitro* translated Skip, and then by a direct interaction assay using recombinant His_6-16 E7. The weak interaction of non-oncogenic HPV-11 E7 with Skip suggested that HPV-16 E7's interaction with Skip might be high-risk virus specific, and hence related to its oncogenic function. This was confirmed by a mutational analysis of E7 using a number of transformation-defective mutants. The ability of E7 to interact with Skip was drastically reduced when mutations were introduced either into the CKII recognition site (mutant p31/32) (Barbosa et al. 1990) or into the C-terminus of E7 (Δ52-56) (Massimi 1999). This suggests a number of possibilities: either that the binding domain encompasses a long stretch of amino acids from 31-56 or that Skip has multiple binding sites on E7, in a manner similar to the E7-Rb interaction. The binding of the CD2 (20-30) of E7 alone to pRb is not sufficient to release E2F nor inhibit pRb DNA binding activity. Indeed, also the sequences in the carboxy terminal region of E7 have been also found to be essential for binding to the pRb gene product, which together gives rise to high affinity binding of E7 to pRb (Huang et al., 1993; Patrick et al., 1994). In addition, it is also possible that the interaction site is conformational rather than linear. Obviously additional analyses are required to elucidate between these different possibilities. The striking observation to be noted is that both these mutants are also severely reduced in their ability to transform cells. Unfortunately, I was unable to detect an *in vivo* interaction between Skip and E7 using conventional coimmunoprecipitation/immunoblot analysis. There are a number of possible explanations for this. The worst case scenario might suggest that the Skip-E7 interactions was a false positive from the two-hybrid screen. However, the extensive mutational

analysis showing specificity of binding with respect to both proteins, coupled with the *in vivo* functional assays (See below in section 5) argue strongly that the interaction is real and specific. It is also interesting to note that the region of E7 bound by Skip spans the site of CKII phosphorylation, and recent studies have shown that E7 is differentially phosphorylated during the cell cycle (Massimi and Banks 2000). Therefore, this raises the intriguing possibility that the E7-Skip interaction could be cell cycle regulated, with specific association being obtained at only certain points during the cell cycle. Further studies are necessary to investigate this fully, including co-immunoprecipitation experiments using synchronized cultures of cells, as opposed to the asynchronous populations that I have used so far.

Similarity searches of the Skip sequence within the database using clustalIW revealed that, a conserved region called SNW, spanning amio acids from 176-333 exists in homologues from different species (Figure 14). Therefore, I constructed a series of truncations of Skip taking the conserved nature of this motif into consideration. The first set of interaction assays with E7 (Figure 15B and C) suggested that, the region from 179-353 is indeed important for binding E7. I then made further constructs of Skip in this region to more accurately map the amino acid residues of Skip involved in the interaction with E7. The mutants 1-179 and 1-219 showed very little or no binding with E7. Strikingly the mutant 1-236 which contains a proline rich sequence (PRS) showed wild type levels of binding, indicating that the PRS sequences with in the SNW domain of Skip as being the region bound by E7 (Figure 15D). This region of Skip is evolutionarily conserved, which suggests that E7 is targeting a functionally important domain of the protein.

3. Skip acts as co-activator of many diverse promoters

I next investigated the biochemical properties of Skip in order to identify the possible modes of regulation mediated by E7 on Skip function. The *Drosophila melanogaster* homologue of Skip has been found to be in complex with the transcriptionally active puffs of salivary glands suggesting that Skip might have a role in the regulation of transcription. In support of this, recent studies have also shown that Skip has a role in the EBNA2 and notchIC activation of target genes (Zhou et al. 2000a; Zhou et al. 2000b). Since Skip plays a role in the transcription of a number of different genes, I thought that this could well represent a more general transcriptional effect of Skip. Indeed, using a variety of different promoters I have been able to show that Skip acts as a general transcriptional co-activator.

I first analyzed the ability of Skip to activate expression from a series of viral and cellular promoters. To do this I analyzed the p53-responsive p21 promoter, the Adenovirus E2 promoter and the HSV TK promoter. In all cases strong transcriptional activation was obtained in the presence of Skip. In contrast, no transcriptional activation was obtained using the inactive GAL4 and the basal pBLCAT3 plasmids. Taken together with the observation that Skip failed to activate expression from the p21 promoter in p53 null cells, suggests that Skip could only stimulate the transcription of already active promoters, implying that it was functioning as a transcriptional co-activator. In order to address this further, transactivation assays were performed with the HPV-16 E2 transactivator on a promoter containing six E2 binding sites upstream of the HSV TK promoter. As expected, the ability of E2 to activate this promoter was greatly increased in

the presence of Skip, providing additional evidence that Skip can be classed as a general transcriptional co-activator. In support of our findings, Skip has also been recently reported to stimulate TGF-β dependent transcription by binding to the MH2 domain of Smad proteins (Leong et al. 2001). It is interesting to note that the amino-terminal domain of E2 functions as a transactivation domain and is believed to recruit transcription factors both to the viral origin of replication and to the promoter, thereby modulating the viral promoter activity. Such proteins include the cellular transcription factors Sp1 (Li et al. 1991), TBP (Steger et al. 1995), AMF1 (Breiding et al. 1997; Peng et al. 2000) and CREB-binding protein (CBP) (Lee et al. 2000a). CBP/p300 is a multi-functional transcriptional co-activator and Skip and p300 seem to share some characteristics, since both are involved in the regulation of several cellular and viral transcription factors. Therefore, it is plausible to argue that E2 might recruit Skip in a coactivator complex to the promoter for augmenting viral transcription. The coactivators CBP/p300 contain an intrinsic histone acetyltransferase activity (HAT) activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996) and have been shown to modify chromatin structures by acetylation of histones (Cho et al. 1998). It has been postulated that recruitment of coactivators bearing HAT activity by promoter-bound transcription factors results in histone acetylation of nearby nucleosomes, thus enhancing access of the transcriptional or replication machinery to DNA (Grunstein 1997; Mizzen and Allis 1998; Struhl 1998). It will therefore be extremely interesting to investigate whether Skip also associates with p300 or any of the histone acetylases required for transcriptional activation.

4. Skip and Ski cooperate in transcriptional activation

I next proceeded to investigate the mechanisms underlying the transcriptional activation function of Skip. An obvious candidate for a role in this activity of Skip was Skip's known interacting cellular partner, c-Ski which is known to be a transcription factor. Indeed, addition of c-Ski to Skip transactivation assays produced a dramatic synergistic activation of diverse target promoters, indicating that Skip and c-Ski cooperate in promoter activation. It appears that the exogenous expression of Ski alone on transcription is promoter dependent, since it only activated the AdE2 promoter, but no significant effect on HSV TK promoter. However, when both Ski and Skip were included together, clear synergistic activation was observed on both HSV TK and AdE2 promoters.

5. HPV-16 E7 silences the co-activation function of Skip

Having characterized Skip as a potent transcriptional coactivator of Ski, I next wanted to determine the effects of E7 on the function of this co-activator complex. Therefore I first investigated the impact of E7 on the HSV TK promoter in U2OS cells in the presence of Skip+Ski. Expression of E7 *in vivo* in U2OS cells inhibited the transcriptional activities of the Skip+Ski complex in a dose-dependent fashion (Figure 22). However, previous studies have shown that E7 is capable of inducing apoptosis (Jones et al. 1997a; Stoppler et al. 1998) and one possible explanation for this reduction in Skip-Ski transactivation activity could be due to E7 induced apoptosis. There are two lines of evidence to suggest that this is not the case. Firstly, I was able to see the dose dependent activation of the AdE2 promoter with E7 in the parallel set of transient transfection experiments (Figure

22), demonstrating that the E7 protein was functional and that no apoptosis was occurring under these experimental conditions. Secondly FACS analysis was also performed following co-transfections of Skip, Ski and E7 in an effort to analyse any potential changes in the cell cycle. Although all these studies were negative, at no time did I observe any signs of apoptosis (data not shown), further supporting the notion that inhibition of Skip-Ski co-activator function is specific and not due to the induction of apoptosis by E7.

Because of the possibility that, E7 might be doing this in co-operation with Rb, I carried out a similar set of experiments in an Rb-/- background. As can be seen from Figure 23, E7 also abrogated the transcriptional activities of Skip-Ski in Saos2 cells, where pRb is inactive, suggesting that E7's inactivation of Skip might be independent of pRb. This is further evident from the analysis of E7 mutant p24Gly, which is impaired for binding pRb (Dyson et al. 1992; Phelps et al. 1992). As shown in Figure 12, p24Gly was able to bind Skip with the same affinity as wt E7, further suggesting that the effects of E7 on Skip are not mediated via pRb. These results demonstrate that one consequence of the E7-Skip interaction is inhibition of Skip-Ski transcriptional activation of diverse promoters.

Next, I analyzed the behaviour of E7 mutants p31Arg/p32Pro and Δ52-56, both of which are defective in binding Skip, in the transcriptional reporter assay containing Skip and Ski. Interestingly, the ability of E7 to inhibit Skip-Ski mediated transactivation is impaired by the same deletion mutants. Since these two mutants have been previously shown to express similar levels of protein as the wild type E7 (Barbosa et al. 1990; Massimi 1999), this suggests that suppression of Skip transcriptional activity by E7 is a

consequence of E7's ability to bind Skip, and this raises the possibility that the ability of E7 to abrogate the function of Skip may contribute to its transforming activity. The observation that v-Ski and EBNA2 also interact with Skip for their transforming activities (Dahl et al. 1998; Zhou et al. 2000a), in addition to high risk E7, suggests a role for Skip in protecting cells from transformation. Taken together, these data further support the hypothesis that many distinct regulatory pathways must be subverted by E7 in order to transform mammalian cells.

6. Why does E7 inhibit Skip function?

How might binding of Skip contribute to the growth-promoting effect of HPVs? A recent study suggested that Skip activates TGF-β dependent transcription through an interaction with the Smad proteins (Leong et al. 2001). TGF-βs are multifunctional cytokines which are involved in differentiation, block cell cycle progression of epithelial cells and inhibit the immune response (Massague et al. 1992). TGF-βs activate a binary system of membrane receptors that signal via serine/threonine kinases, and stimulate inhibitory Smad proteins (Miyazono 2000, review). Loss or altered expression of TGF-β1 has been described as an early event in cervical carcinogenesis and is frequently observed in CIN (Comerci et al. 1996). Frequent loss or decreased expression of TGF-β2 has also been described in cervical dysplasia (Xu et al. 1999). The latter observations are consistent with the idea that TGF-βs function as tumour suppressors in cervical epithelia and also confirm that because loss of TGF-β expression is observed in HPV-infected cells, it is not restricted to observations in cell culture (Glick et al. 1994). Further, it is known that TGF-β1 downregulates proliferation inducing molecules and induces differentiation in HPV-

transformed cells (Ozbun and Meyers 1996; Shier et al. 1999a; Shier et al. 1999b). Interestingly, it has also been recently demonstrated that HPV-16 E7 inhibits differentiation dependent expression of TGF-β2 in cervical keratinocytes (Nees et al. 2000). Taking all these observations into consideration, one may speculate that E7 may be inhibiting TGF-β expression via its association with Skip. The consequence therefore being inhibition of cell differentiation and possibly evading the host immune response. In addition, it is also tempting to suggest that E7 may downregulate the transcription of other genes that may be regulated by Skip which could conceivably be involved in either differentiation, apoptosis or host defense mechanisms. Skip association with E7 presumably also enables E7 to inactivate cellular genes incompatible with the outgrowth of premalignant cells during the development of cervical cancer. Therefore, the interaction between Skip and HPV-16 E7 offers a novel target for designing drugs to inhibit high risk HPVs.

E6, with which E7 cooperates in the immortalization of human keratinocytes, is also been shown to be capable of abrogating the co-stimulatory function of CBP and p300, resulting in the decreased ability of these factors to trans-activate p53-, NF-κB and c-jun responsive promoters (Patel et al. 1999). E6 also represses the transactivation function of IRF-3 (Ronco et al. 1998) and more recently E6 was also found to suppress transactivation activities of Gps2 which is suggested to be involved in transcriptional regulation (Degenhardt and Silverstein 2001). Taken together, these reports suggest that both E6 and E7 collectively inhibit the transcriptional activities of these key elements which play a protective role in the HPV replication and immortalization process.

7. Mechanism of E7 inactivation of Skip

In order to elucidate the possible mechanisms of E7 mediated inactivation of Skip, I first, analyzed the levels of Skip and pRb in HPV positive and negative human keratinocytes. It is interesting to note that Caski cells, which contain about 600 copies of HPV-16 showed low steady state pRb levels when compared to SiHa cells, which have only 1-2 copies of HPV-16. It is suggested that degradation of pRb by HPV-16 E7 is important for the functional inactivation of this tumour suppressor (Boyer et al. 1996; Berezutskaya and Bagchi 1997; Jones and Munger 1997). In contrast, expression levels of Skip were found to be similar both in HPV positive and negative cell lines (Figure 26), this gave the first indication that inactivation of Skip might not involve proteolytic degradation induced by E7. This was also confirmed from a series of transient transfection experiments (Figure 27). An alternative explanation might be inhibition of the Skip-Ski interaction, since this seemed to be required for transcriptional activation. However, competition binding experiments including increasing concentrations of E7 in Skip-Ski interaction assay suggested that, E7 does not disrupt complex formation between Skip and Ski. Therefore, at present it is unclear how E7 is blocking Skip-Ski transcriptional activities, and this is compounded by the fact that, we don't know the precise mechanism by which Skip+Ski cooperate to activate gene expression. However, one possible mechanism one could envisage at this point is that, the direct interaction of E7 and Skip may involve E7 recruitment of HDACs to certain promoter elements, thereby silencing the transactivation functions of Skip-Ski, in a manner similar to the recent finding of E7 recruitment of HDAC1 to inhibit the transcriptional activities of IRF-1 (Park et al. 2000).

The amino acid sequence of Skip contains a potential cyclin A/E-Cdk2 phosphorylation motif SP-X-R at the amino acid residues 233-236, and the known consensus sequence motif for cyclin A/E-Cdk2 phosphorylation is S/T-P-X-K/R (Kitagawa et al. 1996), which makes Skip a good candidate to be regulated by phosphorylation during the cell cycle, similar to the situation with pRb (Buchkovich et al. 1989; Chen et al. 1989). This suggests that potential phosphorylation of Skip might affect E7 binding. It is known that E7 upregulates the expression levels of cyclin A and E, thus modulating the cyclin regulation (Tommasino et al. 1993; Zerfass et al. 1995b; McIntyre et al. 1996; Martin et al. 1998). This also raises the intriguing possibility that E7 might possibly be inactivating Skip function through its association with the cyclin-Cdk complexes.

8. Ski interacts with the evolutionarily conserved SNW domain of Skip

Since one consequence of the interaction between E7 and Skip is abrogation of Skip's transcriptional activity, this also suggests that the PRS/SNW domain of Skip may be required for its transcriptional activity. In the third part of the thesis, I show evidence that indeed SNW is the transcriptional activation domain of Skip. The known binding partner of Skip, Ski, is involved in inducing either transformation or muscle differentiation of quail embryo fibroblasts, depending on the growth conditions (Colmenares and Stavnezer 1989; Colmenares et al. 1991). Ski overexpression also causes postnatal hypertrophy of type II fast muscle fibers in transgenic mice (Sutrave et al. 1990). The capacity of Ski to induce both transformation (growth) and differentiation, which is usually associated with the cessation of growth, is an intriguing paradox. Because of its nuclear localization and its ability to induce expression of muscle-specific genes in quail cells (Colmenares and

Stavnezer 1989), Ski has been assumed to be a transcription factor. Indeed, Ski can function either as a transcriptional activator (Engert et al. 1995; Tarapore et al. 1997) or as a repressor (Nicol and Stavnezer 1998) depending on the specific promoters involved. Recombinant c-Ski protein purified from *E. coli* fails to bind DNA, whereas c-Ski in mammalian cell nuclear extracts retains DNA binding activity, suggesting that c-Ski binds to DNA only when it is associated with other proteins (Nagase et al. 1990). Ski activates transcription of muscle specific promoters and certain viral promoters (Kelder et al. 1997), and has also been found to enhance transcriptional activation by binding nuclear factor I (NF1) sequences (Tarapore et al. 1997). In addition, c-Ski has been found to be a component of the histone deacetylase (HDAC1) complex (Nomura et al. 1999; Tokitou et al. 1999) and also interacts with Smad proteins to regulate TGF-β signaling (Sun et al. 1999a; Sun et al. 1999b), which might partly explain the role of Ski in differentiation. Despite these observations, the precise molecular mechanisms by which Ski transforms cells and regulates differentiation remains unknown, as does the role of Skip in these processes, although, Skip was found to interact with the amino terminal region of Ski essential for its transforming activity. I investigated the specificity and biochemical significance of the Ski-Skip interaction and show that the amino acids from 179-292, which lie within the evolutionarily conserved SNW domain of Skip are essential and sufficient for binding Ski. I first assessed the ability of a series of Skip mutants to interact with c-Ski in a series of GST pull down assays and found that although a weak interaction could be mediated by amino acid residues 1-179, the principal region of Skip responsible for binding to c-Ski was mapped to the central portion of the protein spanning amino acid residues 179-292, which is a part of the SNW

domain (aa 176-333). No binding was detected within the carboxy terminal half of the protein between residues 350-536. Strikingly, this c-Ski binding region of Skip is very highly conserved amongst all the Skip proteins that have so far been characterised from different species (Figure 14), including *D. melanogaster* (Wieland et al. 1992), *Dictyostelium discoideum* (Folk et al. 1996) and *Saccharomyces cerevisiae* (Diehl and Pringle 1991; Harris et al. 1992) (Figure 14), and also suggesting a central role for the SNW region in the function of the two proteins. This further indicates that the Ski-Skip interaction is essential and evolutionarily conserved in all these organisms, from yeast to humans. Since the potential phosphorylation site is also located in the SNW domain, this raises the possibility that Skip-Ski interaction is cell cycle regulated.

9. SNW is the key functional domain of Skip

I then wanted to determine whether the transcriptional activities of Skip and c-Ski were related to the physical interaction of the two proteins. Indeed, when the mutants of Skip were assessed for their ability to activate gene expression, only those which retained the ability to interact with c-Ski could also upregulate promoter activity (Figure 30). In addition, the synergistic transcriptional activity was only obtained when both Skip and c-Ski were present, and with Skip mutant proteins which could still interact with c-Ski. These results therefore provide a function for this highly conserved SNW region of Skip, and further demonstrate that the ability of Skip to interact with Ski is required both for its intrinsic transcriptional activity and for its ability to act synergistically with Ski. This could also mean that, the intrinsic transcriptional activity of Skip is via its interaction with endogenous Ski. The fact that the transforming activity of Ski requires its ability to

interact with Skip suggests that one of the ways in which Ski brings about transformation is through its co-operation with Skip. These results are summarized in Figure 32, and taken together, demonstrate that the transcriptional activation activity of Skip and its ability to synergise with c-Ski are both dependent upon the ability of Skip to interact with c-Ski. The role of Skip in the conversion of CBF1 from a transcriptional repressor to an activator by switching the interaction between the corepressor SMRT and NotchIC (Zhou et al. 2000a), raises the intriguing possibility that Skip may be playing a similar role in case of Ski, thus converting Ski from a transcriptional repressor to an activator under certain conditions. This may possibly take place via Skip sequestration of corepressors such as NcoR, SMRT or Rb from the Ski repression complex, a mechanism similar to that suggested for Hoxc-8, a homeodomain transcription factor, and for Smad1 (Shi et al. 1999). Taken together, these results define a function of the Skip/Ski complex and provide a possible explanation for the transforming effects of Ski.

In summary, we have identified Skip as a new cellular target of HPV-16 E7. E7 functionally inhibits the transcriptional activation activity of Skip by directly binding to the PRS domain of Skip. Mutants of E7, which fail to interact with Skip, are greatly reduced in their ability to transform cells, suggesting that this interaction is important for its transforming activity.

10. Future prospects

The presence of Skip in the Ski DNA complexes (Nagase et al. 1990) and the Smad3 and Smad4 DNA complexes (Leong et al. 2001) suggest that Skip binds to DNA. However, it

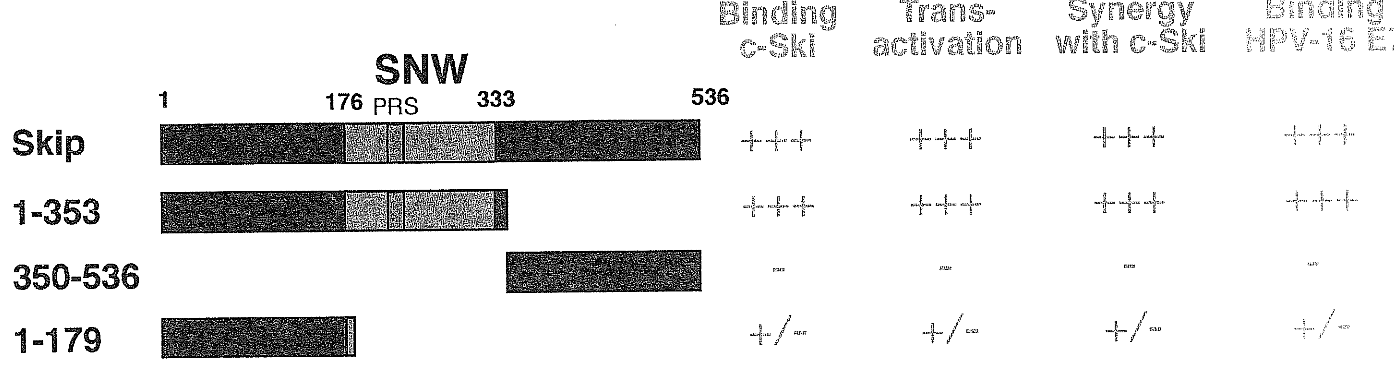

Figure 32. Schematic diagram showing the domains of Skip required for binding c-Ski, activating transcription and synergising with c-Ski. Also shown is the summary of E7-Skip interactions. Numbers refer to the amino acid residues and the central red boxed region corresponds to the evolutionarily conserved SNW region. The green region within the SNW domain represents the conserved Proline rich sequence (PRS).

remains to be investigated whether it binds directly or in association with other proteins. It will therefore be of interest to see whether or not E7 modulates the DNA binding activity of Skip. It is interesting to note that Skip also interacted with TBP in an *in vitro* interaction assay (unpublished observations), which suggest that Skip may also directly interact with the basal transcriptional machinery. Since E7 also interacts with TBP, further experiments could be designed to address this aspect. For example, assays can be done to analyze whether E7 competes with Skip for the binding sites on TBP, or alternatively whether the three proteins can form part of a multiprotein complex.

Since Skip also interacts with NotchIC, the intracellular active domain of Notch, this suggests that Skip could act as a link for exploring the possible links between HPV and the notch signaling pathway, since Notch signaling has been suggested to play a role in the evolution from CINIII lesions to tumours (Daniel et al. 1997). More recently, Adenovirus 13SE1a, which shares many properties with E7, has also been reported to bind the CBF1 growth suppressor (Fortini and Artavanis-Tsakonas 1994), a downstream transcription factor from Notch signaling pathway, and as a consequence was found to dissociate corepressor complexes from the CBF1 complex (Ansieau et al. 2001). This would suggest that E7 might also target CBF1 through its association with Skip. In addition, future experiments should obviously be addressed at investigating the possible mechanisms of E7 inhibition of Skip, such as whether E7 recruits HDACs to inhibit Skip+Ski transactivation.

Finally, it is also interesting to speculate how phosphorylation may potentially regulate the E7-Skip interaction. There is now growing evidence of differential regulation of E6+E7 by differential phosphorylation. However, in this case, the E7 binding site on Skip

is very close to the potential Cdk2 phosphorylation site. This raises the intriguing possibility that the E7-Skip interaction may also be cell cycle regulated.

MATERIALS AND METHODS

1. Yeast two-hybrid Screen

The yeast two-hybrid system was used to screen a human lymphoid cell library, which was kindly provided by Stephen Elledge (Durfee et al. 1993). This human cDNA library was constructed in λ ACT using mRNA prepared from Epstein-Barr virus (EBV)-transformed human peripheral lymphocytes. The library was reported to contain 1.1 X 10^8 total recombinants with more than 95% inserts. This library was made to contain only inserts of more than 600 bp inserts. It was amplified and plasmid DNA was prepared in bulk for yeast transformation. Full-length HPV-16 E7 was cloned in frame with pAS1-GAL4 and was used as bait. Deletion mutants of E7 were also constructed in frame with pAS-GAL4 and were used in the crosses. The yeast strain used are, Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112+URA3::GAL= lacZ, LYS2::GAL=HIS3 cyh^r) and Y187 (MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL=lacZ).

Yeast 190 cells with the plasmid pAS-16 E7 was grown in Synthetic Complete (SC)-Trp till saturation in overnight in culture. This was used to inoculate 1 litre of SC-Trp at 1:250 dilution, grown until the OD reached 0.7 to 0.8 at 27°C/150 rpm (approximate time taken was 12-14 hrs). Cells were harvested at 2000 rpm for 5 minutes, pooled and washed once with 50 ml LiCl/TE. The pellet was resuspended in 1.6 ml of LiCl/TE in a falcon tube and kept on a shaker for 15 minutes at room temperature. Salmon sperm DNA was used as a career for transforming the Y190 cells harbouring the pAS-E7 plasmid. 100 μl of DNA mix (containing 200 μl of shared salmon sperm DNA heated at

94°C for 7 minutes + 800 μl of LiCl/TE+150 μl of library DNA: 0.3 μg/ μl) was mixed with 100 μl of cells and incubated at room temperature for 15-20 minutes. 900 μl of 40% PEG 3350 (Sigma) in LiCl/TE was added to the cells containing the library DNA and incubated for 30 minutes at room temperature, mixed with 140 μl of DMSO, heat shocked for 5 minutes at 42°C. Cells were centrifuged at 3000 rpm for 4 minutes, washed once with 1X YPD (containing Yeast Extract: 5 g, Peptone: 10 g, 10ml 50X Adenine (50X stock: 2.75 g/l), 5 ml of 100 X Ura (100X Stock: 5.5g/l). All the transformants were pooled into 400 ml SC-Trp and recovery of the cells was done for 3 hrs at 27°C/150 rpm. Aliquote of this culture (20/100/200 μl) was plated separately on SC-Trp-Leu plates to determine the transformation efficiency. The recovered cells were harvested in a 50 ml falcon tube, washed once with SC-His-Leu-Trp, resuspended in 4.5 ml of SC-His-Leu-Trp and plated 300 μl each on fifteen 15 cm petridishes containing SC- His-Leu-Trp, but containing 3-AT. The 3-AT concentration for the pAS-E7 bait was pre-titrated and 50 mM concentration was found to have no background. Approximately 2.5 X 10^6 transformants were plated for 3 to 5 days at 30°C. His+ colonies were streaked onto fresh SC-His-Leu-Trp plates and then analyzed in a semiquantitative ß-galactosidase assay using a filter lift assay (Kühne and Banks 1998). The colonies were restreaked on to X-gal containing plates (containing 50ml/litre 1M KH_2PO_4 pH 7.0, to a final concentration of 0.05 M adjusted with KOH + X-Gal 40 mg/l dissolved in Dimethylformamide) overlaid with Sch&Sch BA85 nitrocellulose filter and incubated at 30°C and analyzed for the development of blue colour which indicates the expression of lacZ reporter from the clones. The colour development was ranged from 1 hr to overnight. His+ and 3-AT

resistant blue colonies were scored. Colonies corresponding to positives in this screen were then patched onto a master plate and analyzed further.

1.1. Backscreening of the positive clones

For confirmation of the screen, the total DNA from the 3-amino-triazole-resistant and ß-galactosidase positive colonies were purified and rescued in *Escherichia coli* DH5α and retransformed into *S. cerevisiae* Y187, which were then crossed with *S. cerevisiae* Y190 containing pAS-GAL4 fused deletion mutants of E7. These crosses were analyzed further by ß-galactosidase assay.

The DNA from positive clones from the crosses were purified by CsCl gradient and were analyzed by restriction digestion with XhoI enzyme to detect the sizes of inserts. A set of 50 clones were sequenced by Sanger's dideoxy chain termination method using a kit from Amersham. The nucleotide sequences were analyzed for the homology search using either blast or fasta analysis from the databases. The DNA from the potential interesting candidates were labeled (Amersham) and were used as probes for the southern/dot-blot hybridization to estimate the redundancy of the screen and to eliminate the repetitive clones. Signals were detected using the ECL detection system (Amersham). I could read about 200-300 bp from the manual sequencing. The targets that did not show any homology with any genes of function from the first manual sequences were sent for ABL automated sequencing (Primm). From a total of 202 positive clones, I have identified a unique set of 22 complementation groups for E7 (Figure 8 and 31).

2. Plasmids

To construct yeast expression plasmids containing GAL4-E7 fusion genes E7 specific primers were used in a PCR reaction to amplify E7 sequences from pJ4Ω16E7 and pJ4Ω11E7 (Storey et al. 1988). PCR products were digested with BamHI and SalI and ligated in frame with the GAL4 DNA-binding domain of the yeast expression vector pAS1 (Durfee et al. 1993). Vectors expressing GST-fusion proteins of E7 were constructed by ligating the PCR products into the vector pGEX 2.0. GST-Skip was constructed by subcloning the Skip from the original library plasmid pACT-Skip into the vector pGEX 3X at the BamHI site. GST-Rb has been described previously (Quin et al. 1992). The different Skip expression plasmids used in this study were cloned into the BamHI/EcoRI sites of pcDNA 3.1, by PCR amplification from pCGSP-Skip, kindly provided by M.J.Hayman (Dahl et al. 1998). These constructs were verified by DNA sequencing (Amersham) using the dideoxy chain termination method (Sanger et al. 1977) and were used for *in vitro* translations. The cellular Ski expression plasmid, pMT2-Ski, was kindly provided by M.J. Hayman (Dahl et al. 1998) and the GST-Ski expression plasmid was a kind gift from Shunsuke Ishii (Nomura et al. 1999). pJ4Ω.16 E2 has been described previously (Bouvard et al. 1994). The pTKM.32 CAT reporter was kindly provided by F. Thierrry (Thierry et al. 1990). GAL4 CAT (pG5CAT), and contains five consensus GAL4 binding sites upstream of the E1B minimal promoter, was obtained from commercial sources (Clontech.). The other CAT reporter constructs, p21 CAT (pWWP-CAT), pBLCAT2, pBLCAT3 and AdE2CAT have all been described previously (Murthy et al. 1985; Luckow and Schutz 1987; El-Deiry et al. 1993). For making His_6-16 E7, the gene was cloned in pET7His6 at BamHI-EcoRI sites. pJ4Ω16E7 was used for

expression of E7 in mammalian cells (Storey et al. 1988). The HPV-16 E7 mutants used in this study have all been described previously (Barbosa et al. 1990).

3. Cells, Transfections and CAT assays

The following cells were used in the study.

U20S: derived from human osteosarcoma

Saos-2: human osteogenic fibrosarcoma,

HaCat: Immortalized epidermal keratinocytes

Caski: human cervical epidermoid tumours. These cells contain an integrated HPV 16 genome, about 600 copies per cell

SiHa: squamous cell carcinoma of cervix, containes 1 to 2 copies of an integrated HPV-16 genome

All these cells were grown in DMEM supplemented with 10% fetal calf serum, 1% (v/v) Glutamine and 200 units/ml Penicillin plus 100 μg/ml Streptomycin in an incubator containing 10% CO_2 at 37°C. Transfections were performed using standard calcium phosphate precipitation with 1 μg of reporter plasmid (unless otherwise stated) along with indicated amounts of expression plasmids. The DNA amounts were balanced with empty vector DNA. After 48h the cells were harvested in 100 μl CAT buffer (40mM Tris-HCl pH 7.5, 150mM Nacl; 1mM EDTA) and subjected to three cycles of freeze-thawing, followed by incubation at 65°C for 10 min. Samples were clarified by centrifugation at 14,000 r.p.m. for 2min and the protein concentration of the supernatant was measured by the Bio-Rad protein assay. CAT assays were routinely performed with

5-10 μg of protein incubated with 2.5 μl acetyl-CoA (33.3 mg/ml) and 1.5 μl [^{14}C] chloramphenicol (50 mCi/mol; Amersham) in a final volume of 50 μl at 37°C for 1h. Following extraction with ethyl acetate, samples were analyzed by thin layer chromatography and quantified with a Packard Instant Imager (Packard, Meriden, CT).

4. Western blotting

Expression of Skip was verified by Western blotting from total cell extracts. Protein concentrations were determined using the Bradford method (Biorad). Cells were washed 2-3 times with PBS on ice, added 200 μl of a lysis solution (250 mM NaCl, 0.15% NP40, 50 mM HEPES ph 7, 1% Aprotinin, 100 μM TLCK, 200 μ TPCK). 10μg samples of protein were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), then probed with the required anti-serum. Western transfer was done in Tris-Glycine buffer (Tris-HCl 12.12 g/l and Glycine 57.04 g/l), supplemented with 20% methanol. Antibody incubation was done in PBS containing 0.5% Tween prepared in 10% milk powder and membrane washes were performed with PBS-Tween. Skip antibodies were raised in rabbit against GST fused N-terminal Skip fragment comprising amino acids 1-219, was used at 1: 20,000 dilution. HA monoclonal antibody (dilution 1:10,000) is from Babco (16B12). C15 (pRb) antibody (dilution 1:10,000) is from Santacruz Biotech. His_6-16 E7 was detected by using a polyclonal antibody raised against the His-tagged HPV-16 E7. Complexes of primary and horse radish peroxidase-linked secondary antibodies (Dako) were detected by ECL (Amersham) according to the manufacturer's instructions.

5. *In vitro* translations and GST-pull down assays

^{35}[S]-labeled proteins were produced in vitro by using a coupled transcription-translation system (Promega TNT) according to the manufacturer's instructions. In brief, 1 μg of circular plasmid DNA encoding the protein of interest under the control of either T7 or Sp6 promoter were added to the TNT reaction mix in the presence of 5U of enzyme and 10Ci of S-Met. The reaction was incubated at 30°C for 90 minutes for T7 RNA Polymerase and 2 h for SP6. A small aliquot of translations were loaded on SDS-PAGE gel and the signals were quantitated with a Packard Instant Imager. Equal amounts of translated products were used in the interaction assays.

For fusion protein production and purification, 100ml of an overnight culture of *E. coli* strain BL-21 containing the GST-Ski expression plasmid were inoculated into 1 litre of Luria Broth containing Ampicillin and incubated at 37°C for further 1h. Recombinant protein expression was induced for 3h with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication and cell debris was removed by centrifugation. The levels of protein induction were then determined by SDS-PAGE and Commassie Brilliant Blue R staining.

Equal amounts GST and GST-Ski fusion protein bound to Glutathione-linked agarose (Sigma) were incubated with the *in vitro* translated proteins for 1h at room temperature in a buffer containing 50 mM Tris HCl pH 7.5, 100 mM NaCl and 2.5 mM EDTA. Bound proteins were washed extensively in PBS containing 0.5% NP40 before analysis by SDS-PAGE and autoradiography.

6. Purification of His$_6$-HPV-16 E7

The BL-21 cells containing the E7 plasmid were induced with 1mM IPTG for 3 hrs. The cells were harvested by resuspending them in 6M Guanidinium HCl (pH 8.0 in 10 mM phosphate buffer), disrupted by sonication and cell debris was removed by centrifugation. The supernatant was incubated with Ni-NTA for 1 hr and then loaded onto columns prewashed with buffer containing 6M Guanidinium HCl. The bound protein was eluted with increasing molar concentrations of Imidazole. Fractions were collected and protein concentrations were checked. Purified protein was then used both for raising antibodies against E7 and also for the direct *in vitro* interaction assay with GST-Skip.

7. Production of Polyclonal antibodies against His$_6$-HPV-16 E7

For the first immunization 200 μg of purified protein was injected into two New Zeland rabbits in incomplete Freunds's adjuvant. In the following injections (7) 100 μg of protein were used every 21 days. The specificity and the dilutions of the antiserum was tested with immunoprecipitations performed on HPV-16 E7 translated *in vitro*, in comparison with the pre-immune antibody. Purified antibody was aliquoted and scored at –80°C.

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